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New proteins with inhibitory effects on adipocyte differentiation: effects of TWEAK (TNFSF12) and LIGHT (TNFSF14) on the differentiation, metabolism and secretory function of human primary preadipocytes and adipocytes

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1. Introduction

1.1 Obesity

Obesity is defined as an increase in body fat mass above normal levels. It is associated with a number of adverse changes in overall health and with increased mortality (1-3). It is the most crucial modifiable risk factor for the development of type-2-diabetes and causes disturbances in lipid metabolism. It is believed to be the most important determinant for the metabolic syndrome, a cluster of cardiovascular risk factors including elevated fasting serum triglycerides, low HDL-cholesterol, hypertension and impaired glucose metabolism. The metabolic syndrome is linked with an increased risk for cardiovascular complications. Other undesirable effects of obesity include an elevated risk for certain cancers, joint diseases and hormonal dysregulations among others. Today, obesity is regarded as a status of mild but chronic inflammation due to the increased secretion of pro-inflammatory cytokines which further rise with a gain in adipose tissue mass (4-6). Some of the above described comorbidites of obesity are thought to be caused by or linked to this chronic low-grade inflammatory state.

Besides these physical complications obese patients more frequently suffer from a reduced quality of life and from psychological and social problems than normal weight individuals (7;8).

Obesity can be measured as increased body-mass-index (BMI). The BMI is defined as body weight in kilograms divided by the square of the height in meters. It is categorized into four classes (Table 1) as suggested by the World Health Organization (WHO) and the Deutsche Adipositas Gesellschaft (DAG) (3;9).

Table 1: Classification of body weight depending on the BMI following the WHO and DAG definition.

BMI	Classification
≤18.5 kg/m^2	Underweight
18.5 – 24.9 kg/m^2	Normal
> 25.0 kg/m^2	Overweight
≥30.0 kg/m^2	Obesity
• 30.0 - 34.9 kg/m^2	• Grade I
• 35.0 - 39.9 kg/m^2	• Grade II
• ≥ 40.0 kg/m^2	• Grade III

Over the last decades an alarming increase in the prevalence of obesity has occurred in the western world (1). In the USA, the proportion of the adult population (age 20 - 74 years) with

a BMI equal or above 30 kg/m^2 was stable at approximately 15 % between 1960 and 1980. Since then it increased dramatically to more than 30 % in the year 2000 (10). According to the International Obesity Task Force (IOTF), in 2002/2003 more than 70 % of the male and more than 55 % of the female adult population in Germany were overweight with a BMI ≥ 25 kg/m^2 (11). These data include almost one quarter of the adult German population which is obese, i.e. has a BMI of 30 kg/m^2 or greater.

Obesity is nowadays regarded as an epidemic taking the huge number of patients and the tremendous increase in case number into account. The financial consequences of the disease and its comorbidities are enormous. In Germany, the costs are estimated to be more than 10 % of total health expenditures (12). In the USA, almost a quarter of the health care costs are spent on physical inactivity, overweight and obesity, and health care costs rise with increasing BMI (13;14).

The growth in body fat mass may cause two types of obesity: 1) the gluteal-femoral type with increased adipose tissue at the buttocks and thighs and 2) the abdominal type with increased abdominal adipose tissue (15). The pattern of body fat distribution is an important determinant for the development of adverse complications. The abdominal type of obesity is linked to a high risk of cardiovascular events and to disturbances in lipid and carbohydrate metabolism (1;16;17). Nevertheless, the risk for concomitant diseases is increased independently of the type of body fat distribution at a BMI of 30 kg/m^2 and above (18). Although a direct determination of body fat mass is possible e.g. with Dual-Energy X-Ray Absorptiometry (DEXA), the BMI is the most frequently used measure to evaluate unfavorable changes in body fat mass. Supporting information about body fat distribution can be obtained by assessing the waist circumference (Table 2), which defines the risk for cardiovascular and metabolic complications (19).

Table 2: Risk for cardiovascular and metabolic complications in dependency on the waist circumference.

Sex	Increased risk	Significantly increased risk
Female	≥ 80 cm	≥ 88 cm
Male	≥ 94 cm	≥ 102 cm

Only about 5 % of all obesity cases can be attributed to a rare single genetic cause or are secondary to other events such as glucocorticoid medication or diseases such as hypothyroidism (18). Genetic modifications are considered to be an important factor in the development of obesity amounting for an estimated 50 % of total susceptibility. However, the key events in its development are an increased energy intake and a decreased energy

expenditure, i.e. a chronic positive energy balance (1;2;20). Per capita energy intake in the USA increased by 500 kcal since the early 1970s whereas exercise-dependent energy expenditure in Germany decreased by about 500 kcal per day during the last 50 years (21;22). Several strategies have been employed to prevent further weight gain in the healthy as well as in the overweight population and to induce weight loss in overweight patients.

Basal weight loss strategies include three crucial parameters: reduced energy intake by an adequate diet, increased energy expenditure by intensified exercise and behavioral modification (3;9). A decrease in energy intake is achieved via dietary interventions, whereas an increase in energy expenditure needs to be adjusted to the patient's physical possibilities. Additional pharmacological treatment is an option for patients with a BMI above 30 kg/m^2 and also for patients with a BMI above 27 kg/m^2 and comorbidities. It should only be employed after at least three months of unsuccessful basal therapy. Three drugs are available for the treatment of obesity. 1) The inhibitor of serotonin- and noradrenalin-reuptake sibutramin acts centrally and stimulates satiety, 2) the selective cannabinoid-1-receptor antagonist rimonabant reduces appetite by acting centrally and 3) the lipase inhibitor orlistat reduces fatty acid absorption in the gut (23). For morbidly obese patients with a BMI above 40 kg/m^2 or for patients with a BMI above 35 kg/m^2 and comorbidities, surgical treatment offers another option for sustained weight loss. It should only be considered after at least 6 months of basal therapy (24).

A very important criterion for the treatment of obesity following the described rules is to set a realistic goal. A reduction of body weight by 10 % is already sufficient to achieve a significant improvement of comorbidities (3). In many cases, abnormal glucose and lipid metabolism values return to normal with a weight loss in that range.

Unfortunately, long-term studies demonstrate only a transient or modest loss of body weight. Only 10 to 20 % of the treated patients achieve a long-term weight reduction and maintain the reduced weight (25). Most patients return to their initial body weight within five years or exhibit the undesirable undulatory changes in body weight.

Taking the financial burden for the health-care system and the reduced quality-of-life into account, greater efforts are necessary to tackle the global epidemic of obesity by ensuring a stable weight loss or at least limiting weight gain in obese patients. Aside from new approaches for a broad prevention of obesity, new dietary and medical strategies for already obese patients are urgently needed.

1.2. Adipose tissue and adipocytes

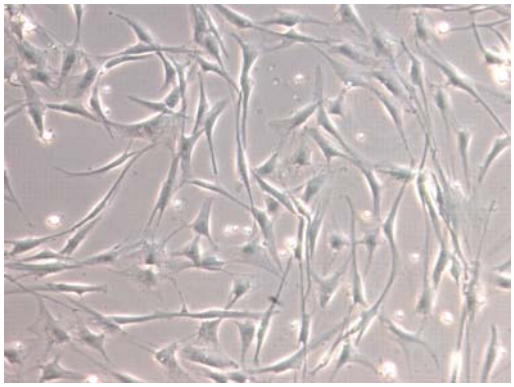
Adipose tissue accounts for 10 to 20 % of total body mass in healthy men and 15 to 30 % in healthy women and is mainly located in subcutaneous (sc) and visceral depots (15). Adipose tissue can be regarded as a discrete organ, since it is comprised of two main tissues, brown adipose tissue (BAT) and white adipose tissue (WAT), that together accomplish the main function, i.e. energy storage (26). However, over the last 10 years a new paradigm in the concept of adipose tissue has emerged. This tissue is no longer regarded as an inert organ for lipid storage, but is also considered an endocrine organ maintaining an intensive crosstalk with many other tissues (27;28). Leptin was the first factor discovered to be secreted by adipose tissue and to regulate bodyweight by a central mechanism of action and in an endocrine manner (26;29). In addition, adipose tissue secretes a variety of other factors including pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) or anti-inflammatory cytokines like IL-10 and the new adipokine adiponectin (30-32). Adipose tissue also produces factors which influence the fibrinolytic system like plasminogen activator-inhibitor-1 (PAI-1) and factors that alter circulatory functions and blood pressure like members of the renin-angiotensin system (33;34).

Human adipose tissue consists of different cell types surrounded by extra-cellular fluid, matrix and capillaries. Mature adipocytes represent about 2/3 of the cell number and (due to their extraordinary cell size) over 95 % of the tissue volume (35). Adipose tissue also contains preadipocytes or fibroblast-like cells which are thought to be precursor cells for mature adipocytes. These cells are present at various stages of the differentiation process in human adipose tissue (36). Other cell types present in adipose tissue are endothelial cells, erythrocytes, and cells from the immune system, such as macrophages (35;37).

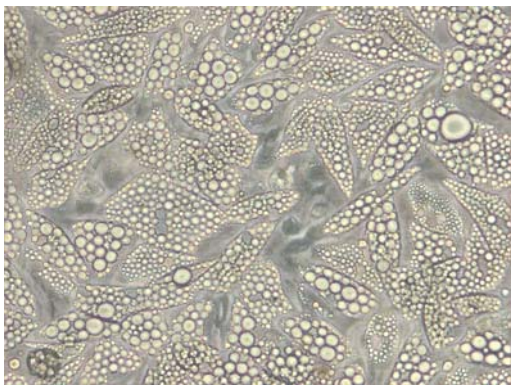
Human WAT can be found at different sites of the body and is classified into two types (36;38;39). Approximately 80 % of the total adipose tissue is located between the skin and the muscle as well as in and around the mammary glands in females as subcutaneous tissue. The remaining 20 % surround organs in the main body cavities (thoracic, abdominal and pelvic) as visceral adipose tissue. Both types of WAT cooperate in their main function of lipid storage and consist of the same main constituents. However, the two types differ in the level of vascularization, the cellular composition and in secretory and metabolic function (30;35-37;40). Vascularization is denser in omental (om) tissue and hence more endothelial cells are present. Macrophages and lymphocytes are also more frequent in the omental adipose tissue. The dense capillary supply points to a higher metabolic activity of omental adipose tissue. While basal lipolysis is comparable in the two types of WAT, norepinephrine-induced

lipolysis is about six-fold higher in omental adipose tissue compared to subcutaneous adipose tissue. Furthermore, omental adipocytes express less insulin receptors and react more sensitive to catecholamines. For this and other reasons, enlarged omental adipose tissue in abdominal obesity is associated with more severe metabolic disturbances than the gluteo-femoral type of obesity mainly found in the female population. Several factors are secreted differently from the two types of WAT: e.g. PAI-1 and angiotensinogen are synthesized to a higher extent by omental WAT while other factors like leptin and TNF-α are produced to a greater extent from subcutaneous adipose tissue. For other factors, e.g. IL-6, no differences in the extent of secretion between the two types of WAT can be found.

Adipocytes develop from preadipocytes, which represent committed cells that descend from multipotent mesenchymal stem cells by largely unknown mechanisms via intermediate adipoblasts (41;42). Differentiation of committed preadipocytes into mature adipocytes, *in vivo* and *in vitro*, requires growth arrest of the preadipocytes with accompanying alterations in the extracellular matrix and the cytoskeleton and distinct alterations in the gene expression profile (43;44). Adipoblasts and preadipocytes have the capacity to proliferate, whereas mature adipocytes irreversibly lose this capacity (41). The process of differentiation can be observed microscopically as termination of proliferation and subsequent change in cell morphology (Fig. 1). During adipogenesis, fibroblast-like preadipocytes become circular and start to accumulate lipid droplets under appropriate culture conditions.



Undifferentiated preadipocytes,
day 0



Differentiated adipocytes,
day 16

Figure 1: Microscopical evaluation of human preadipocyte differentiation

Different factors that alter the transcriptional activity of the preadipocytes are added to the culture medium of preadipocytes to induce their differentiation into lipid-filled mature adipocytes. These factors include the phosphodiesterase-inhibitor isobutylmethylxanthine

(IBMX) which causes an increase in intracellular cAMP levels and, by this mechanism, influences the appearance of transcription factors critical for the early phase of adipogenesis (45). Cortisol is present in the differentiation medium as an important factor affecting transcription factor expression and insulin is essential for a physiological nutrient uptake into the developing adipocyte (46). Thiazolidinediones are added to the culture medium of undifferentiated preadipocytes as high-affinity ligands and activators for the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) (47).

Three families of transcriptional activators induce the morphological changes (42;43;48). These three families appear early during the differentiation process and collaborate in the induction of transcription of adipocyte-specific genes (42;48;49). PPAR-γ is the key transcription factor for adipogenesis in the PPAR-family, a group of nuclear hormone receptors with three known members that form heterodimers with retinoic X receptors (RXR). Secondly, within the CCAAT/enhancer-binding proteins (C/EBP) family, which forms homodimers, the three members C/EBP-α, -β and -δ are important transcription factors for adipocyte differentiation.

The third family of basic-helix-loop-helix leucine zipper proteins (bHLHzip) (which are also active as homodimers) contains the sterol regulatory element-binding protein-1c (SREBP-1c) that is pivotal for adipocyte formation.

C/EBP-β and -δ expression levels are transiently increased during the early phase of differentiation and cause a long-lasting rise in C/EPP-α and PPAR-γ expression and activity. The augmented SREBP-1c expression further stimulates PPAR-γ expression. Together with PPAR-δ, PPAR-γ and C/EBP-α alone and cooperatively stimulate the expression of typical late markers of adipogenesis. These include proteins of the lipid metabolism like adipocyte lipid-binding protein (aP2/ALBP) or fatty acid synthase (FAS) and elements of glucose metabolism like the insulin receptor or the insulin-dependent glucose transporter GLUT-4. In this concerted action of transcriptional activation, PPAR-γ is the key factor for adipogenesis: in the absence of PPAR-γ, cells completely lack the ability to enter the phase of adipogenesis (45). Furthermore, other transcriptional factors were recently shown to be involved in the regulation of adipogenesis (50).

In contrast to the factors described above that enable the process of adipocyte differentiation, several factors are known to have inhibiting effects on adipogenesis. Examples for negative regulators that suppresses adipogenesis by preventing the activation of critical transcription factors like PPAR-γ are Kruppel-like factor-2 (KLF-2) or members of the Wnt signaling pathway (45;51).

The state of adipocyte differentiation and metabolism is also a crucial marker in the development of obesity. Two concepts of obesity are described today: hypertrophic and hyperplastic obesity (Fig. 2). Hypertrophic obesity is marked by intensified lipid storage in existing mature adipocytes and occurs generally in adults with mild overweight. Hyperplastic obesity is characterized by an increase in the number of mature adipocytes and generally occurs early in life (52;53). Yet, hyperplastic changes in adipose tissue can also take place in adult-onset obesity when mature adipose cells reach a critical size (54). During weight loss, a decrease in fat cell size as well as in fat cell number can be observed (55).

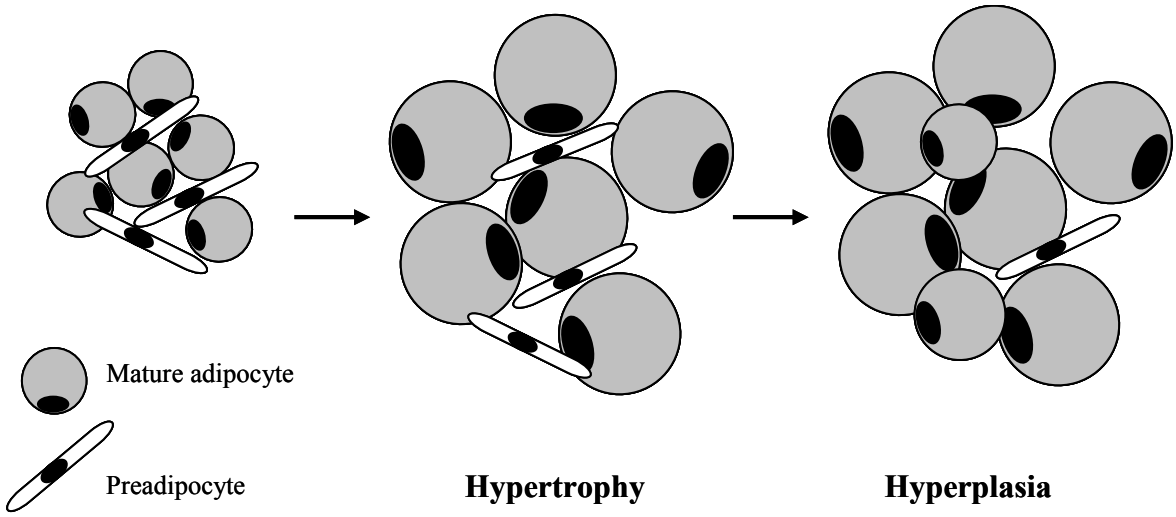


Figure 2: Changes in adipose tissue cellularity with increasing body fat mass. In a first step, existing adipocytes store more energy in the form of lipids. When a critical cell size is achieved, preadipocytes proliferate and new adipocytes develop from these preadipocytes.

Three models are available to study the main aspects of adipose tissue and adipocytes, which are adipose tissue development in physiological and pathophysiological conditions, metabolic and secretory functions and the crosstalk with other tissues. 1) Animal models offer a good opportunity to study the properties of adipose tissue *in vivo*. However, results from these systems may differ from the human system in many aspects. 2) Culture of tissue extracts is a valuable tool to study features of human adipose tissue in its natural composition without the influence of surrounding tissues or systemic parameters. Yet tissue extracts exhibit the weakness of not being supplied with nutrients and oxygen normally provided *in vivo*. Metabolic end products are not removed due to the missing blood circulation. 3) Cell lines offer a convenient and reliable tool to study the effects of different substances on isolated cells under defined and constant conditions. Different murine and rat clonal preadipocyte cell lines have been established for the investigation of adipocyte differentiation and the factors

influencing this process (41;43;55). The most frequently used model is the murine 3T3-L1 swiss mouse embryonic cell line. Unfortunately, no stable and reliable clonal preadipocyte cell lines from white adipose tissue are available for the human system. Recently, a human cell strain that has been derived from the stromal cell fraction of subcutaneous adipose tissue of an infant with Simpson-Golabi-Behmel syndrome (SGBS) was described (56). This cell strain can be proliferated for up to 50 generations and shows a fibroblast-like morphology comparable to isolated primary preadipocytes. The preadipocytes differentiate into mature, lipid droplet-filled adipocytes under defined conditions.

Primary cultures of human preadipocytes and mature adipocytes isolated through collagenase digestion from human subcutaneous and omental adipose tissue provide the possibility to overcome the limitations caused by the rare human models of white preadipocytes (35). Primary culture facilitates the study of precursor and mature cells in their original status under defined conditions. Nevertheless, the variability of the cells depending on the donor and the possible effects of the isolation procedure need to be taken into account when using this model. Furthermore, this model can only be used for a limited life span due to the decrease in differentiation capacity with increasing number of cell divisions (35;41). As described above, differentiation of primary preadipocytes can be induced by supplementation with IBMX and rosiglitazone, a thiazolidinedione. Cortisol and insulin are required in the cell culture medium to further stimulate the expression of adipocyte-specific genes and nutrient uptake. Depending on the donor and the culture conditions, up to 80 % of the cells in the isolated stromal-vascular fraction (SVF) of adipose tissue differentiate into mature adipocytes (35;57). Contaminating endothelial cells can be detected microscopically by their cobblestone-like morphology; however, no specific markers are available to distinguish the preadipocytes from other fibroblast-like cells (35;41;58).

Using murine cell culture models as well as human primary culture and SGBS cells, the effects of a variety of factors, such as hormones and cytokines, on adipocyte differentiation, metabolism, gene expression and secretory function have been investigated. The anabolic hormone insulin stimulates adipogenesis by enhancing glucose uptake and has growth-promoting effects (41;42). Human growth hormone (GH) inhibits differentiation and stimulates lipolysis under serum-free culture conditions in human primary culture and causes a reduction of body fat mass *in vivo* (59;60). IL-6 and transforming growth factor-β (TGF-β) are two examples for pro-inflammatory cytokines with known effects on immature and mature adipocytes (61;62). Both proteins inhibit adipocyte differentiation and IL-6

additionally stimulates lipolysis. TNF-α is a potent and thoroughly investigated component that influences adipocytes and adipose tissue and will be discussed in detail in chapter 1.4.

1.3. Functional genomics screen for modulators of adipogenesis

High-throughput screening of cDNA (deoxyribonucleic acid) libraries for various functional hits in different cellular systems with robotics platforms has been previously described (63-65).

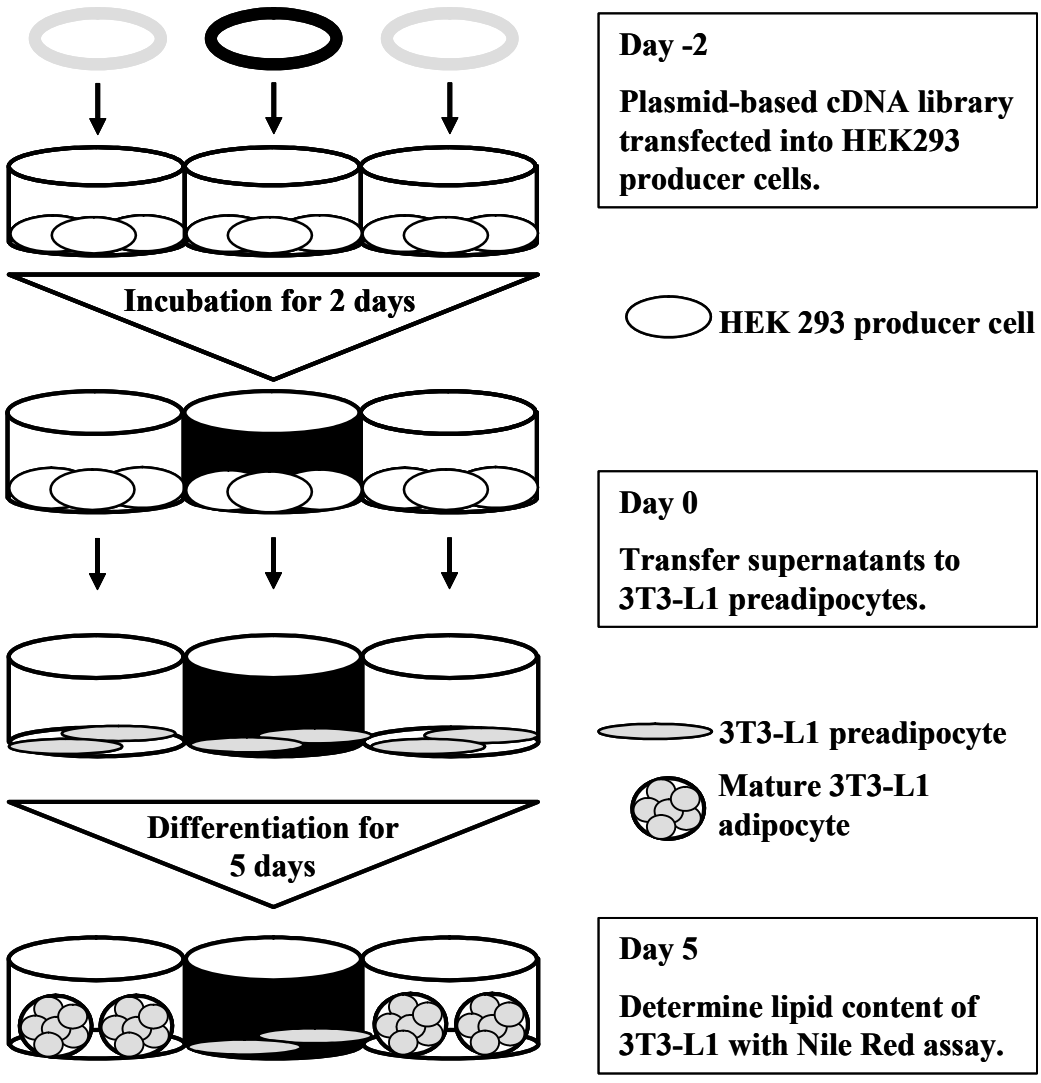


Figure 3: Screening principle. Single cDNAs were transfected into Hek293 (human embryonic kidney) cells on day -2. Supernatants were transferred to 3T3-L1 preadipocytes two days after transfection. 3T3-L1 cells were differentiated under adipogenic conditions for 5 days. The lipid content was determined with the Nile Red assay.

Two libraries of approximately 200 000 clones were screened for factors affecting adipocyte differentiation or lipidation: a full-length clone collection of approximately 35 000 clones and

a so-called “metabolic library” enriched for full-length clones (67 % of the total clones). The metabolic library was generated from the transcriptome of subcutaneous and omental adipose tissue obtained from lean and obese individuals as well as from the transcriptome of hypothalamus, liver, pancreas and skeletal muscle. Proteins discovered in this screen may offer the possibility to block adipose tissue growth via the inhibition of differentiation or to even stimulate weight loss by increased lipolysis. The cDNAs were automatically transfected into HEK293 producer cells (Fig.3) so that cDNAs encoding for secreted proteins would lead to the accumulation of the corresponding proteins in the HEK293 supernatants.

Two days after transfection, the respective HEK293 supernatant was transferred to 3T3-L1 preadipocytes, which were kept under adipogenic conditions for the following five days. 3T3-L1 adipocyte maturation was assessed with the lipophilic dye Nile Red five days after induction of differentiation and addition of the HEK293 supernatant. This dye delivers a fluorescence signal at the excitation wave length 485 nm and the emission wave length 590 nm only when bound by unknown mechanisms to hydrophobic cellular components. Disappearance or decrease of the Nile Red signal can either be caused by an inhibition of adipocyte differentiation or by an effect on lipid or glucose metabolism, i.e. alterations in glucose uptake or lipolysis. The critical value for the determination of hits was set as two standard deviations below the whole plate mean. Hits were traced back to the respective cDNA which disclosed amino acid sequence and led to the identification of the protein. Results were confirmed in 3T3-L1 cells with commercially available proteins where possible.

The described Nile Red assay on 3T3-L1 cells is a reliable tool for detecting a decrease in adipocyte lipid content as judged by the Z-factor, a statistical characteristic for the quality of high-throughput assays (66). The Z-factor is calculated from the mean and the standard deviations of the negative and the positive controls of the high-throughput screening assay. A value between 0.5 and 1.0 (the maximum achievable value) determines an excellent assay, values below 0 an unemployable assay. The high-throughput screening applying the Nile Red assay on 3T3-L1 cells differentiated in the presence of conditioned media from the HEK293 producer cells delivered a Z-factor of around 0.8. It can therefore be judged as an excellent assay for the identification of possibly inhibitory proteins on adipogenesis.

Another proof of the validity of this assay is given by the recovery of certain established inhibitors of adipogenesis. Among the many known and unknown proteins found in the screen to influence adipocyte differentiation and metabolism, TNF-α and lymphotoxin (LT) as members of the TNF superfamily (TNFSF) have already been previously described as inhibitors of adipocyte differentiation (67;68). Two members of the interleukin family, IL-6

and IL-11, are also known to negatively influence preadipocyte differentiation (61;69). The protein endothelin-1, with depicted effects on the cardiovascular system, and platelet-derived growth factor (PDGF) as member of the growth factor family have also been described to alter adipocyte differentiation (70;71). Among others, the screen revealed two members of the TNFSF that were previously not known to influence adipocyte differentiation: TWEAK (TNF-like weak inducer of apoptosis, TNFSF12) and LIGHT (Lymphotoxin-like, exhibits inducible expression and competes with HSV glycoprotein D for HVEM, a receptor expressed by T-lymphocytes, TNFSF14).

To examine the potential of the detected proteins in the treatment of obesity, the effects of TWEAK and LIGHT on adipocyte metabolism, secretion, and differentiation and their potential cytotoxic and apoptotic effects need to be thoroughly investigated. The established properties of TNF-α (as a well known member of the TNFSF) and of the two new proteins will be described in detail in the following chapters.

1.4. TNF-α and adipose tissue

TNF-α was the first member of the TNF superfamily to be described about 30 years ago as a factor causing necrosis of tumors transplanted into mice (72). Nowadays, almost 20 members of the TNFSF are known to interact with members of the TNF receptor superfamily (TNFRSF) (73). The TNFSF members are type-II-transmembrane proteins with an intracellular N-terminal domain that form homotrimers in the active state. The exception is LT-β, which heterotrimerizes with LT-α. A TNF-homology domain is present in the extracellular C-terminal domain of TNFSF members. Most of the TNFSF members can be cleaved from the cell surface at extracellular cleavage sites into soluble cytokines, whereas members such as LT-α are only found as secreted molecules (73;74). Members of the TNFRSF are typically type-I-transmembrane proteins, except for a few type-III-proteins and two soluble members without a transmembrane domain. TNF receptors contain one or more cysteine-rich domains (CRD) in their N-terminal extracellular domain; most TNF-receptors contain death domains (DD) or TNF-receptor associated factor (TRAF)-interaction motifs (TIM) as functional characteristics in their intracellular domain (75;76). The interactions of TNFSF and TNFRSF members play a crucial role in the development, homeostasis, and function of the immune system. This role is important for the physiological operating of the immune system in host defense against infections and tumors. At the cellular level, the interactions between members of the TNFSF and the TNFRSF influence cell survival, proliferation, and differentiation. Additional, but contrary to this role are the host-damaging

effects of TNFSF members, e.g. in sepsis or autoimmune diseases (74;77). Effects of TNFSF members are mediated by binding to TNFRSF members in a distinct but partially overlapping way; so far, more than 40 receptor-ligand pairs have been discovered (74;75).

Monomeric membrane-bound TNF-α has a molecular weight of 26 kilo Dalton (kDa) and its cleavage results in a 17 kDa soluble protein. Both, membrane-bound and soluble forms of the protein form homotrimers and bind to two members of the TNFRSF which show different tissue distribution: the DD-containing TNFR1 (p60) is expressed in most tissues, the TRAF-binding TNFR2 (p80) is mainly expressed in immune system cells (78). Both receptors can be cleaved to form soluble receptors. TNF-α shares receptor binding with the LT-α homotrimer and the LT-αβ heterotrimers (74;79). TNFR1 activation on the one hand protects cells against apoptosis and causes the transcription of inflammatory proteins. These effects are mediated by TRAF-1 and -2 binding to TNF-associated DD (TRADD) and the subsequent activation of nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK). TNFR1 activation may on the other hand mediate apoptosis via the recruitment of TRADD and the activation of caspases under certain conditions (74;80). TNFR2 activation leads to the binding of TRAF-2 to TIM and to the subsequent activation of the pathways described above for TNFR1/TRAF-2 interaction. Enhancement of TNFR1-induced apoptosis has been described for TNFR2 activation (78).

TNF-α acts as important pro-inflammatory cytokine and is expressed in a variety of human tissues and cells with macrophages being the main source (78).

On the one hand, several antibodies against TNF-α are available for humans for the treatment of chronic inflammatory diseases such as rheumatoid arthritis (RA) and Crohn's disease. They significantly reduce disease symptoms and severity, but may display side effects like increased risk of infections and lymphoproliferative diseases (81). On the other hand, using the stimulatory effect of TNF-α on the immune system, anti-tumor therapy has been successful when severe complications occurring under systemic TNF-α administration were avoided by application of TNF-α closely to the tumor (82).

TNF-α expression and secretion in adipose tissue has been described; the expression is low in mature adipocytes and high in the stromal-vascular fraction. TNF-α expression and secretion correlates positively with BMI, and accordingly TNF-α mRNA (ribonucleic acid) expression decreases with weight loss (83;84). Circulating plasma TNF-α levels are low and contribution of adipose tissue derived TNF-α to plasma levels is discussed controversially, but does not seem to be crucial (83;85-87). Both TNF receptors (TNFRI and TNFRII) are expressed in

adipose tissue and both, the receptor mRNA expression and the serum levels of the soluble TNF receptors are up-regulated in obesity (85;88).

Since all three members of the TNF-α signaling complex are expressed in adipose tissue while TNF-α plasma levels are not substantially increased in obesity, an important autocrine or paracrine role for TNF-α in adipose tissue has been suggested (85;89;90). The protein inhibits the differentiation of preadipocytes to mature adipocytes and causes a recurrence of the preadipocyte phenotype when added to differentiated adipocytes (68;91;92). Adipocyte differentiation is altered by decreasing PPAR-γ and C/EBP-α mRNA expression and protein content (93-96). Multiple effects of TNF-α on the metabolic and secretory function of adipose tissue and adipocytes have been investigated. Interference of TNF-α with insulin signaling was observed at the level of both, the insulin receptor (IR) and the IR substrate-1 (IRS-1) phosphorylation in rat and murine cells and subsequently in human adipocytes (97-99). Consequently, phosphatidylinositol-3 kinase (PI3K) activity, insulin-stimulated glucose uptake, and GLUT-4 mRNA expression are also decreased in human adipocytes following TNF-α incubation (96;97;100). In human skeletal muscle cells, a major site for insulin-stimulated glucose uptake, TNF-α also inhibits IRS-1 as well as protein kinase B (AKT) phosphorylation (101). Aside from its effects on glucose uptake, TNF-α stimulates lipolysis in human primary adipocytes and decreases lipoproteinlipase (LPL) mRNA expression and activity (100;102;103). In human differentiated adipocytes, TNF-α treatment increases the mRNA expression and protein secretion of pro-inflammatory cytokines such as IL-6 and monocyte chemoattractant protein-1 (MCP-1) (104;105). A decrease in adiponectin mRNA and protein expression was observed in differentiated adipocytes and in adipose tissue extracts upon TNF-α exposition (104;106). Taken together, this information indicates a role of TNF-α in the control of body weight and adipose tissue mass (27;79;89); the increase in local TNF-α concentration parallels an increase in adipose tissue mass and causes an inhibition of preadipocyte differentiation and a decrease in lipogenesis as well as an increase in lipid mobilization. Yet, a further pathophysiological increase in adipose tissue mass as observed in obesity may lead to the emergence of unwanted insulin resistance and the production of typical pro-inflammatory proteins.

1.5. TWEAK (TNFSF12)

1.5.1. Ligand and receptor properties

TWEAK was described for the first time in 1997 as a protein that induces cell death in the human adenocarcinoma cell line HT-29 in the presence of interferon-γ (IFN-γ) (107). TWEAK is a type-II-transmembrane protein with a length of 249 amino acids (aa) and structural similarities with other members of the TNFSF. The 35 kDa full-length protein can be shed into a functionally active soluble 18 kDa protein (Fig. 4) of 156 aa length that contains the receptor binding domain. In the active state, the protein is a N-glycosylated homotrimer as are most of the other members of the TNFSF (107-109).

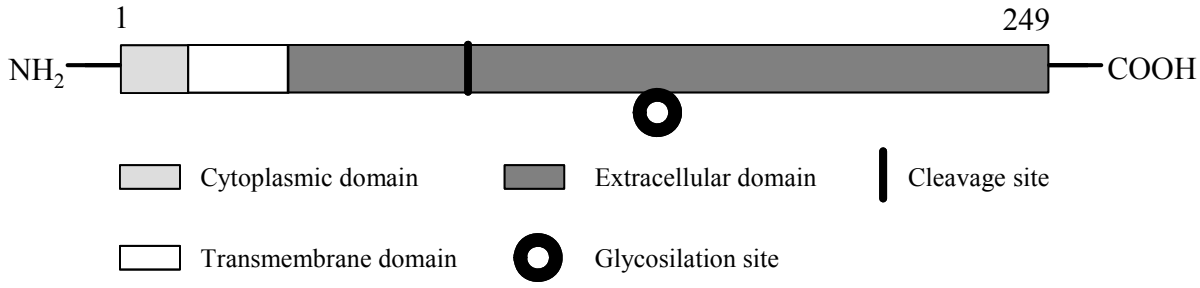


Figure 4: TWEAK protein structure. The 249 aa protein contains a short cytoplasmic domain (18 aa) and a transmembrane domain (27 aa). The extracellular domain can be cleaved to release a soluble protein with a length of 156 aa.

The TWEAK receptor protein, fibroblast growth factor-inducible-14 (Fn14, TNFRSF12A) was described four years after TWEAK (110). Fn14 is a type-I-transmembrane protein with a length of 129 aa and a molecular weight of 14 kDa. Cleavage of a 27 N-terminal signal peptide delivers the mature 102 aa receptor (Fig. 5). Fn14 contains one extracellular cysteine-rich domain, which is typical for members of the TNFRSF. Fn14 is the smallest member of this receptor family. As for the other TNFRSF members, homotrimerization upon ligand binding is required for the activation of Fn14. Only TRAF-binding sites, but no death domain can be found in the short intracellular domain (109-111). Until recently, Fn14 was considered to interact with TWEAK alone and vice versa, although this binding pattern is unusual for members of the TNFSF and TNFRSF. In many cases, blocking of Fn14 with antagonistic antibodies completely abolished TWEAK's function, and a binding study for members of the TNFSF and the TNFRSF revealed no further interaction partners for TWEAK and Fn14 (73;112;113). This binding concept was challenged during the last years.

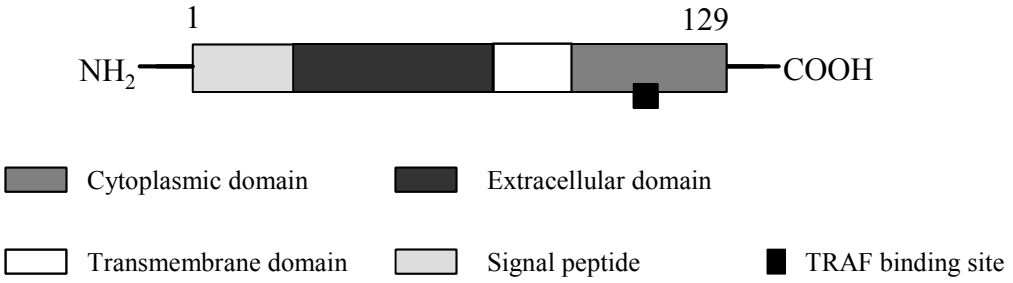


Figure 5: Protein structure of Fn14. The signal peptide is cleaved to deliver the mature receptor. The type-I-transmembrane protein contains a 53 aa extracellular domain and a transmembrane domain with 21 aa. The short intracellular C-terminus (28 aa) carries one TRAF-binding site.

A second ligand for Fn14 was proposed since effects mediated via Fn14 could not be completely blocked by anti-TWEAK antibodies (114). Furthermore, a second TWEAK receptor has been proposed because TWEAK provokes effects on cells that do not express Fn14 on their cell surface and its effects can not be completely blocked by antagonistic anti-Fn14 antibodies in all cases (115;116).

1.5.2. Ligand and receptor expression

TWEAK mRNA is expressed in many tissues including heart, lung and skeletal muscle as well as in lymphoid organs like spleen and lymph nodes. The expression patterns differ between humans and mice (107). There is evidence that TWEAK mRNA expression does not correlate with protein expression (109). Protein expression has been found in macrophages and in monocytes from peripheral blood mononuclear cells (PBMC) upon IFN-γ stimulation (117;118).

Fn14 mRNA expression has been observed in many adult and embryonic tissues with high levels in heart and lung (109;111). mRNA expression is upregulated in regenerating nerve cells as well as in proliferating endothelial cells, smooth muscle cells and fibroblasts (110;111;119;120). Fn14 cell surface expression has also been found in human tumor cell lines (121;122).

Combined receptor and ligand protein expression have been detected in cells of the human innate immune system with an upregulation upon IFN-γ stimulation, but not on B- or T-cells (123;124).

TWEAK and Fn14 protein expression have been observed in several human diseases and in cellular and murine models of these diseases. These include lupus erythematosus (LE) and lupus nephritis as well as atherosclerosis and rheumatoid arthritis (124-129). In mouse

models, ligand and receptor mRNA and protein levels are upregulated upon cerebral ischemia and encephalitis (130-132). TWEAK and Fn14 mRNA and protein expression have been found in a variety of human tumors and tumor cell lines (133-135). In human subcutaneous adipose tissue TWEAK mRNA correlates negatively with body weight and Fn14 mRNA is expressed only in extremely obese but not in normal-weight patients (136).

Only little information is available on the signaling pathways that regulate TWEAK expression; TWEAK mRNA expression in mammary epithelial cells appears to be regulated via the inhibitor of κB-kinase (IKK)-2/NF-κB pathway through NF-κB and Drosophila forkhead transcription factor (FOXO) consensus sites in the TWEAK promoter (137). TWEAK internalization and nuclear localization has been found in several cellular models and nuclear localization sites are present in the protein (137;138). Fn14 expression was suggested to be regulated by the Rho/ROCK pathway (139).

1.5.3. Animal models

Two mouse models over-expressing liver-specific TWEAK show no gross alterations but increased liver progenitor cell proliferation, and the mice are viable for many generations (140;141). Yet mice over-expressing TWEAK in the muscle suffer from a decrease in body and muscle mass and die within two weeks after birth (142). TWEAK-/- mice are also viable with no visible disturbances except for an increased peripheral natural killer cell (NK) pool (123).

Fn14 deficient mice are also viable with no visible abnormalities and have an increased resistance to cerebral ischemia (141;143;144).

1.5.4. Ligand and receptor effects

As expected from the first description of TWEAK's functions and from the subsequent investigations, the main functions of the TWEAK/Fn14 interaction are the stimulation of apoptosis and cell growth as well as the promotion of pro-inflammatory cytokine secretion. TWEAK is involved in the following major pathological processes: autoimmune diseases, rheumatoid arthritis, atherosclerosis, tumor growth and central nervous system diseases. Furthermore, TWEAK is involved in the physiological process of wound healing and tissue repair.

The stimulation of pro-inflammatory responses upon TWEAK incubation has been mainly described *in vitro*; TWEAK stimulates the secretion of a variety of cytokines from a variety of cell lines and primary cells, e.g. human umbilical vein endothelial cells (HUVEC) and

primary human astrocytes, a human bronchial epithelial and a murine osteoblast cell line (115;122;145;146). These cytokines include IL-8, MCP-1, granulocyte-monocyte colony-stimulating factor (GM-CSF), receptor activator of NF-κB-ligand (RANKL), and IL-6. *In vivo*, the injection of TWEAK increases kidney MCP-1 levels in mice, whereas the injection of anti-TWEAK antibody in mice with RA significantly decreases MCP-1 serum levels and disease severity (129;147). TWEAK has also been implied in the development of human chronic arthritic diseases because of its pro-inflammatory effects on human synoviocytes and primary chondrocytes (128;148).

Contrary to these results, in TWEAK-/- knock-out mice significantly more IFN-γ is secreted from the innate immune cells after lipopolysaccharide (LPS) injection, suggesting a limiting role for TWEAK in the pro-inflammatory response of the innate immune system (123).

Since it stimulates the release of pro-inflammatory cytokines, which is an important precondition for the onset of LE, TWEAK has been linked to this disease but no proof has been found for the involvement in the initiation of the disease (149).

The pro-inflammatory effects of TWEAK and Fn14 have been presumed to be involved in central nervous system diseases. In addition to the described stimulatory effect on cytokine secretion in murine and human astrocytes, anti-TWEAK antibodies decrease the severity of experimental autoimmune encephalitis (EAE) and infarct size in a model of cerebral ischemia in mice (131;140;143;145;150). Besides these effects TWEAK/Fn14 interaction also increases the permeability of the blood-brain-barrier (151). Thus, TWEAK seems to be involved in brain inflammation, yet its expression has also been found in the normal brain and an inverse correlation has been found between TWEAK mRNA expression and the onset of acute and chronic autoimmune diseases in mice (117;145).

The apoptotic effects of TWEAK probably also contribute to its impact on central nervous system interferences. While TWEAK causes apoptosis in primary murine neurons, inhibition of the protein with antagonistic TWEAK antibodies decreases apoptosis in a primary mouse neuronal cell model for ischemia (130).

Apoptotic properties of TWEAK have been investigated in many cellular systems *in vitro*. Induction of apoptosis has been described mainly in the presence of sensitizing agents like IFN-γ, e.g. for the HT-29 cell line and the gastric adenocarcinoma cell line KATO-III (107;118). However, in other human tumor cell lines apoptotic or cytotoxic effects have not been observed; TWEAK even delivers anti-apoptotic effects in HUVEC cells (122;152). TWEAK secreted from IFN-γ stimulated monocytes and macrophages is responsible for their cytotoxic effects on tumor cells (114;118). Concerning the increased expression of TWEAK

in human tumors, interesting results were obtained from a murine model. TWEAK seems to be involved in tumor rejection by attracting cytotoxic macrophages to the tumor site (153). However, TWEAK-over-expressing HEK293 cells cause an increased tumor growth when injected into mice; in a mammalian breast tumor cell line (Eph4), TWEAK causes proliferation and branching (133;135).

The increased tumor growth following injection of TWEAK-over-expressing HEK293 cells in mice is also caused by TWEAK's stimulatory effect on cell proliferation, because the tumors are vascularised to a greater extent in comparison to tumors without TWEAK over-expression (133). Proliferative effects of TWEAK on HUVEC and *in vivo* stimulation of angiogenesis has been already described earlier (112;154). In two tumor cell lines, contradictory results were obtained; while TWEAK increased proliferation of the HCC human hepatocellular carcinoma cell line, it inhibited proliferation of a melanoma cell line (A375) (107;122).

The stimulatory effects of TWEAK on cell proliferation also affect wound healing and tissue repair. TWEAK leads to the proliferation and upregulation of adhesion molecules in HUVEC and synergizes with basic fibroblast growth factor (bFGF) in a wound healing assay and in the induction of morphogenetic changes of HUVEC (112;152). However, the proliferative effects of TWEAK on HUVEC and smooth muscle cells were accompanied with the secretion of pro-inflammatory cytokines and the expression of adhesion molecules, also considered to be involved in the pathogenesis of atherosclerosis (139).

During nerve regeneration in mice, Fn14 is upregulated and Fn14 over-expression in a rat neuron cell line (PC12) stimulates nerve outgrowth (119). TWEAK/Fn14 signaling is probably also involved in liver and in muscle regeneration (141;144;155).

The stimulatory effects of TWEAK on murine primary myoblast proliferation are accompanied by an inhibition of differentiation (144). These inhibitory effects on cellular differentiation have also been described for primary human chondrocytes and osteoblast precursors (128). TWEAK stimulates differentiation of the human macrophage/monocyte cell line RAW264.7 to functionally active osteclasts and induces morphological changes in rat neurons (116;119).

1.5.5. TWEAK and Fn14 signaling

TWEAK/Fn14 signaling is mainly mediated through TRAF binding to the receptor complex (109;112;156). This leads to activation of the canonical and the alternative NF-κB pathways in cellular systems and *in vivo* (122;127;142;151;157). These pathways are mainly responsible for the pro-inflammatory and pro-survival effects of TWEAK, but NF-κB

stimulation is also involved in the pro-apoptotic effects of TWEAK (115;130;147;158). The ligand and receptor actions are also mediated through several other signaling pathways: AKT, glycogen synthase kinase-3β (GSK-3β), the Rho GTPase Rac-1 and RhoA as well as the mitogen-activated protein kinase (MAPK) extracellular regulated kinase (Erk)-1/-2, p38-MAPK and JNK are involved in the effects in different environments (116;119;120;138;142;145;146;159). The activation of caspases in the apoptosis as well as of cathepsin B in necrosis has been described for TWEAK mediated cytotoxic effects (160).

1.5.6. Summary

TWEAK exerts a variety of effects *in vitro* and *in vivo* which include pro-inflammatory and proliferative as well as cytotoxic effects. The properties of receptor and protein are largely dependent on the cellular context and the pathophysiological alterations. Even in the *in vivo* models the effects of TWEAK and Fn14 are dependent on the type of model and the status of the animals.

It has to be taken into account that most of the results described above were obtained in different disease models and may not reflect the effects of TWEAK under healthy, physiological conditions. Most of the pro-inflammatory effects were described *in vitro* in different cell systems. Thus, no information is available so far regarding the main sources and the physiological role of TWEAK in humans.

1.6. LIGHT (TNFSF14)

1.6.1. Ligand and receptor properties

LIGHT was identified in an extensive gene search in 1995 and was described four years later as ligand for herpesvirus entry mediator (HVEM) (161;162).

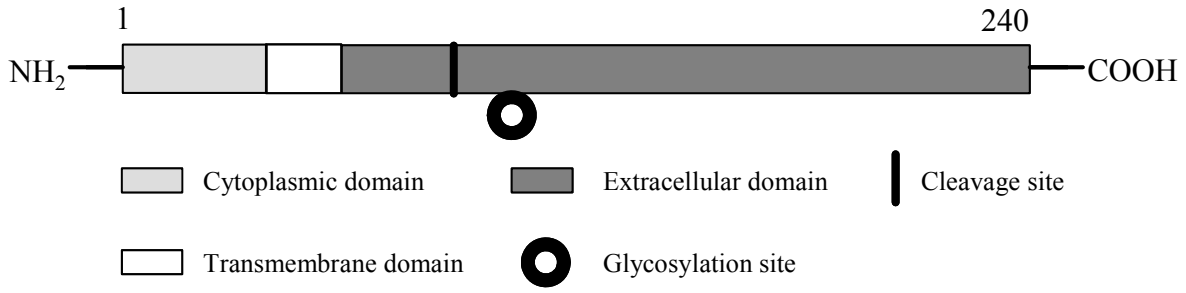


Figure 6: LIGHT protein structure. The short hydrophobic transmembrane domain (20 aa) links the intracellular N-terminus (38 aa) to the cleavable extracellular C-terminus (182 aa). The extracellular domain contains one N-glykosylation site.

LIGHT, as a typical member of the TNFSF, is a type-II-transmembrane protein. A 20 aa transmembrane domain links the short cytoplasmic N-terminus to the extracellular domain that contains the receptor-interacting domain (Fig. 6). A soluble protein with a predicted molecular mass of 21.7 kDa can be shed by metalloproteinase cleavage from the extracellular domain. The protein can be N-glycosylated at one site within the soluble domain, the molecular weight of 24 kDa in the monomeric form corresponds to this glykosylation. LIGHT forms a homotrimer with a molecular mass of 65 kDa (161;163-166). Its sequence is approximately 30 % similar to TNF-α, LT-α and LT-β (161).

LIGHT binds to three known members of the TNFRSF: the HVEM, the Lymphotoxin-β receptor (LTβR) and the soluble decoy receptor DcR3 (161;163;167). LIGHT shares HVEM binding with LT-α, LTβR binding with LT-$\alpha_1\beta_2$ and Dc3 binding with Fas ligand (FasL). All these members of the TNFSF, together with TNF-α, are important in the development and maintenance of the immune system (161;168-170).
HVEM is a type-I-transmembrane protein; the N-terminal extracellular sequence contains two glykosylation sites and the ligand binding domain (Fig. 7). The molecular weight of the mature receptor is approximately 32 kDa after signal peptide cleavage (171;172).

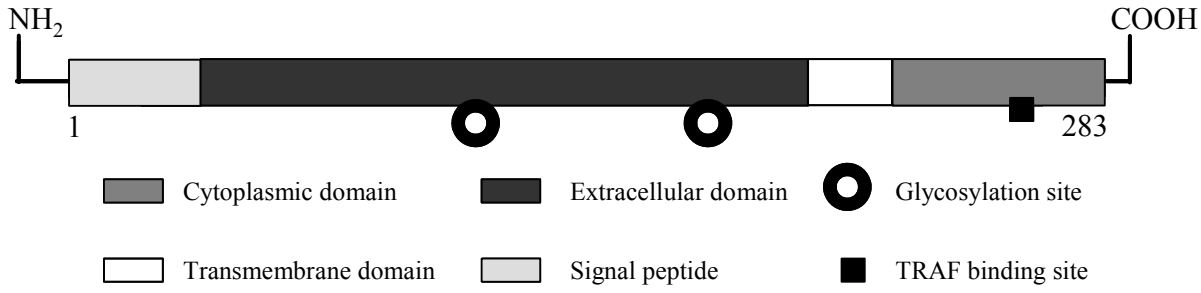


Figure 7: HVEM protein structure. The short transmembrane domain (23 aa) connects the intracellular C-terminus (58 aa) to the extracellular N-terminal ligand-binding domain (166 aa).

LTβR is similar to the HVEM; it also is a type-I-transmembrane protein with a signal peptide and a short transmembrane domain (Fig. 8). The immature precursor has a molecular weight of approximately 47 kDa and contains two glykosylation sites in the extracellular domain (173). Both, HVEM and LTβR, have cysteine-rich repeats in their extracellular domain that characterize the TNFRSF. They do not contain death-domains, but TRAF-binding sites in their intracellular C-terminus. In its active form, HVEM trimerizes upon LIGHT binding, whereas LTβR dimerizes upon LIGHT binding (169-172;174;175).

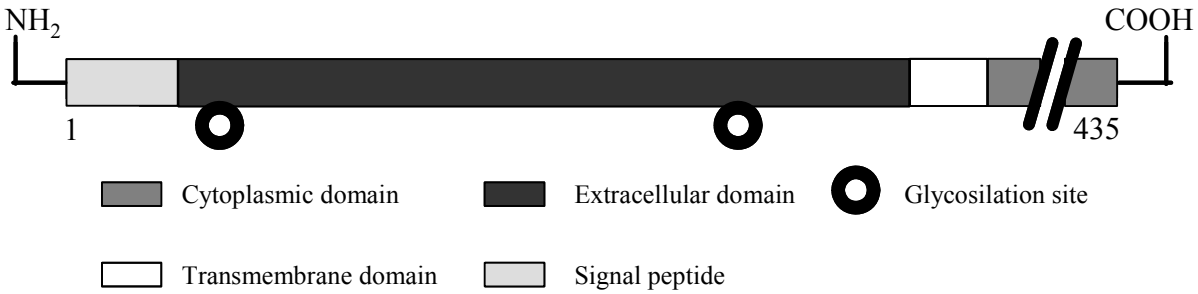


Figure 8: LTβR protein structure. The short transmembrane domain (21 aa) connects the intracellular C-terminus (187 aa, not displayed in full length) to the extracellular N-terminal ligand-binding domain (197 aa) that contains two glykosylation sites.

DcR3 is a soluble receptor; the mature protein consists of 271 aa and has a molecular weight of 40 kDa. It contains one N-glykosylation site and four cysteine-rich domains. The receptor is probably secreted and competes with HVEM and LTβR for LIGHT binding (167).

1.6.2. Ligand and receptor expression

LIGHT mRNA is weakly expressed in the peripheral lymphoid tissues, the heart, the placenta, the liver, the lung and the kidney; a stronger signal is found in the spleen and the brain (161). LIGHT mRNA and cell surface protein expression has been found in activated T-lymphocytes and in immature dendritic cells (DC) (161;163;176;177).

HVEM mRNA is expressed at high levels in the thymus, spleen, lung and kidney and is also detectable in cells of the immune system, such as monocytes and B and T-cells (171;172). HVEM protein expression is detectable on T- and B-lymphocytes and on dendritic cells isolated from PBMC (171;178-180). HVEM and LIGHT co-expression has been described for T-lymphocytes from PBMC and the gut with a reciprocal regulation upon stimulation (upregulation of LIGHT and down-regulation of HVEM) (178;181). LTβR expression is not found on cells of lymphoid origin, but on cells of stromal origin like fibroblasts, on epithelial cells, and on cells of myeloid origin such as monocytes and dendritic cells (170;182).

HVEM and LTβR cell surface co-expression has been detected on several human carcinoma cell lines, on human primary hepatocytes, on HUVEC, and on synovial fibroblasts (163;183-185).

LIGHT protein expression and increased serum levels have been observed in several human diseases; these include human LE and RA as well as scleroderma patients (186-190).

The intracellular signals mediating LIGHT basal expression include activation of the transcription factors Sp1 and Ets in the human Jurkat T-lymphocyte cell line. Stimulated

transcription is mediated via an increase in intracellular calcium levels, the phosphatase calcineurin and the activation and binding of nuclear factor of activated T-cells (NFAT) transcription factors to the appropriate sites in the LIGHT promoter (191).

1.6.3. Animal models

Murine models with LIGHT knock-out mutations display no gross abnormalities and are viable and fertile. CD8+ T-cell proliferation, but not effectiveness, is impaired; however, the mice show normal lymphoid architecture (192-195). LTβR knock out mice display disrupted spleen architecture and overall disturbances in the lymphoid system (182). HVEM-/- mice are healthy and fertile comparable to LIGHT-/- mice and show no alterations in the lymph system, although they show an increased reactivity of isolated T-cells (196).

Contrary to the results from knock-out mice over-expression of LIGHT in T-lymphocytes leads to severe alterations. The animals have an increased T-cell population in the peripheral and central pool and spontaneously develop severe autoimmune diseases. They are not viable and fertile and display a decreased size (197-200). Yet LIGHT has the ability to partly restore the lymphoid deficiencies when over-expressed in LTα-/- mice (201).

1.6.4. Ligand and receptor effects

In primary human cell culture as well as in human and murine cell lines, the main actions of LIGHT encompass stimulation of apoptosis as well as proliferation, the induction or inhibition of morphological changes and the stimulation of pro-inflammatory cytokine secretion. From these *in vitro* models and from *in vivo* studies in animals, the involvement of LIGHT in a variety of medical conditions like autoimmune diseases, tumor growth and rheumatoid arthritis has been suggested. LIGHT is also presumed to be involved in the physiological processes of lymphoid system development and normal immune system function.

The stimulatory effect on the secretion of pro-inflammatory cytokines, e.g. IL-6 or IL-8, and the expression of adhesion molecules, e.g. intracellular adhesion molecule-1 (ICAM-1), has been shown *in vitro*, e.g. in a human rhabdomyosarcoma cell line (RD), in HUVEC, and in synovial fibroblasts (184;190;202). Yet the most important targets of LIGHT seem to be cells of the immune system such as T-cells, dendritic cells, and monocytes, from which LIGHT stimulates release of a variety of cytokines such as IFN-γ, TNF-α, and IL-8 (176;179;203). *In vivo* inhibition of LIGHT decreases IL-6 and IFN-γ mRNA expression in smooth muscle cells in a model of graft arterial diseases, while T-lymphocytes from LIGHT transgenic mice

display an increased IFN-γ and GM-CSF secretion (199;204). Stimulatory effects of LIGHT on cell proliferation have been found *in vitro* and *in vivo* for cells of the lymphoid system (176;199). A stimulatory effect of LIGHT on apoptosis has been mainly described for tumor cells, such as LIGHT-over-expressing human breast cancer cell line or upon co-stimulation with IFN-γ in HT-29 cells (163;164). In RD cells, LIGHT inhibits proliferation without causing cytotoxic effects but stimulates morphological changes which are part of the differentiation process (202). LIGHT is also involved in morphological changes that are part of DC maturation (179).

The roles of LIGHT in pathophysiological conditions include a combination of the discrete effects described above. As judged from LIGHT transgenic animals, the protein is involved in the development of auto-immune diseases and of chronic inflammation (199;200). Inhibition of LIGHT ameliorates graft versus host disease and inhibits the development of type-I-diabetes and RA in murine models (165;205-208). LIGHT stimulated secretion of pro-inflammatory cytokines and T-cell stimulation may be involved in the development of the inflammatory bowel disease in rats (209).

The physiological roles of LIGHT in the development of the immune system are not clarified. As described above, LIGHT-/- animals show no severe alterations, yet LIGHT can partly mediate the restoration of lymphoid architecture in LT-α deficient mice. LIGHT also seems to play a role in negative selection in the thymus (198). LIGHT also participates in the physiological role of the immune system in the defense against bacteria and tumors (203). LIGHT expression in murine tumors leads to rejection of established tumors which is mediated via increased DC and T-cell immigration and activation and the upregulated chemokine and adhesion molecule production (210;211). In this and other cases, tumor rejection seems not to be achieved via the direct pro-apoptotic effects of LIGHT but via an increased infiltration and activation of immune cells (163;212;213). LIGHT was also suggested to be involved in the development of RA and hepatitis (206;214). Concerning its role in metabolism, LIGHT was found to cause unfavorable disturbances in lipid metabolism when over-expressed in T-lymphocytes besides the already described initiating effects on type-I-diabetes (215).

1.6.5. LIGHT, LTβR and HVEM signaling

The two membrane-bound receptors for LIGHT have been observed to interact with TRAF-1, -2, -3 and -5 (164;216). Yet under physiological conditions, only LTβR seems to signal via TRAF-3, which seems to be important for LIGHT induced apoptosis of human tumor cell

lines (164). While the effects mediated through HVEM are mainly important for T-cell proliferation, maturation and cytokine secretion, LTβR is responsible for pro-inflammatory effects like cytokine secretion and adhesion molecule expression in non-lymphoid cells and may cause apoptosis in certain tumor cell lines (170;174).

As for most members of the TNFRSF, LIGHT has been found to activate the classical and the alternative NF-κB pathway in different environments (166;183;202;217). This mediates effects like cytokine secretion, adhesion molecule expression and DC maturation (185;218;219). LIGHT mediates cytotoxic effects in HT-29 via activation of JAK/STAT-1 and p38-MAPK (220). JNK and Erk-1/-2 are activated by LIGHT in the human epithelial carcinoma cell line HeLa and in dendritic cells, respectively, and LIGHT stimulates vascular endothelial cell adhesion molecule-1 (VCAM-1) expression via the activation of PI3K in human fibroblast-like synoviocytes (185;217;218).

1.6.6. Summary

Although cell specific, LIGHT affects mainly the immune system; effects are mediated via the LIGHT receptors HVEM and LTβR. LIGHT seems to be involved in lymphoid system development, though its exact role is not clarified. The protein has potent pro-inflammatory effects when over-expressed in T-cells and is involved in the development of auto-immune diseases and inflammatory processes. It seems to also play a role in tumor rejection. LIGHT protein effects were mostly investigated in different disease models or by protein over-expression and with membrane bound LIGHT. Therefore, more studies need to be conducted to draw conclusions about the physiological role of the protein.

2. Objective

Considering the huge number of affected patients and its tremendous increase during the last decades, obesity is referred to as an epidemic. Taking the enormous burden for the health-care system as well as the pathophysiological and psychological consequences for the affected individual into account, more effective strategies for the prevention and therapy of the disease are needed. This is the more important since the established therapeutical concept of dietary intervention, increase of physical activity and behavioral modification turned out to be rather of limited effectiveness. Therefore, new concepts including pharmaceutical therapies should be developed. Inhibition of adipocyte differentiation may offer a novel therapeutic strategy to prevent further weight gain in obese patients.

Two members of the TNF superfamily, TWEAK and LIGHT, which have previously not been described to affect adipose tissue development, were discovered in a high-throughput screening approach to inhibit adipocyte development in murine 3T3-L1 adipocytes.

TWEAK and LIGHT were applied in their shed form as soluble recombinant human proteins for a thorough investigation in human primary preadipocytes and adipocytes. Besides the confirmation of their inhibitory activity on the differentiation of human preadipocytes, they were investigated for possible cytotoxic, apoptotic and proliferative effects. A potential use as therapeutic also implies that the proteins do not exert stimulatory effects on the secretion of pro-inflammatory cytokines or deteriorate metabolic parameters in human adipocytes. Therefore, the central objective of the work was to carefully characterize the effect of these members of the TNF superfamily in human adipose tissue.

3. Methods

The precise composition of all media and buffers is supplied in the appendix section as are all materials and equipments including information on the respective suppliers.

3.1. Primary culture

Experiments were either performed in freshly isolated human primary preadipocytes before or after differentiation into adipocytes or in freshly isolated mature adipocytes. Human primary preadipocytes were isolated as described previously (221). Adipose tissue was obtained from patients undergoing elective surgery or abdominal surgery for herniectomy. All patients gave written informed consent. Patients with severe diseases like cancer were excluded, and, unless otherwise stated, no selection was made for BMI, age or gender. Tissue was obtained under sterile conditions and was immediately transported in basal medium to the laboratory. Isolation of human primary preadipocytes was either performed at the same or the next day after an overnight storage at 4 °C. Small differences in yield, but no differences in differentiation capacity were observed after overnight storage (data not shown). Connective tissue and blood vessels were excised, and tissue was minced into small pieces; tissue obtained from liposuction was washed with phosphate buffered saline (PBS). Collagenase digestion was performed for 2 hours in PBS with 2 % bovine serum albumin (BSA) and 200 U/ml collagenase in a shaking water bath (80 rpm) at 37 °C. After centrifugation at 200 x g for 10 minutes, the supernatant containing mature adipocytes in the digestion solution was removed, and the pellet containing the stromal-vascular fraction was incubated for 10 min in erythrocyte lysis buffer and subsequently filtered through a 150 µm polypropylene membrane. After a second centrifugation step, the pellet was resuspended in basal medium and filtered through a 70 µm filter. Cells were stained with Trypan Blue and counted in duplicate in a Neubauer chamber. Seeding density for differentiation was 40 000 cells/cm^2 for 12-well and 55 000 cells/cm^2 for 6-well plate. In 96-well plates, cells were inoculated at 90 000 cells/cm^2 for differentiation and 6500 cells/cm^2 for proliferation experiments. The cells were kept in basal medium supplemented with 10 % fetal bovine serum (FBS) for 24 to 48 hours to ensure attachment of preadipocytes to culture dishes and were then (day 0) washed twice with PBS to remove cell debris and contaminating erythrocytes. Differentiation was induced on day 0 by adding induction medium for four days. Medium was afterwards changed to fresh differentiation medium every four days. Cells started producing visible lipid droplets on day 7 and were used for experiments only if more than 50 % were differentiated as judged by

microscopical counting in five randomly selected areas. Upon induction of differentiation, undifferentiated control cells of the same patient were harvested. For Nile Red experiments, undifferentiated control cells were measured at the same day as differentiated cells and until then kept in differentiation medium lacking hydrocortisone and triiodothyronine (T_3).

Mature adipocytes were isolated according to the protocol of Rodbell (222) at the day of surgery with the following modifications: Minced tissue was prepared as described for preadipocytes and collagenase digestion was performed in Krebs-Ringer-Phosphate Buffer (KRP) containing 4 % BSA and 100 U/ml collagenase in a shaking water bath (37 °C, 80 rpm) for 30 to 60 minutes. Floating cells were aspirated and filtered through a 2000 µm and subsequently through a 250 µm filter. Cells in the flow-through were washed three times with KRP containing 0.1 % BSA and were finally collected by aspiration of the wash solution. 1.5 ml of cells were inoculated in 4.5 ml basal medium.

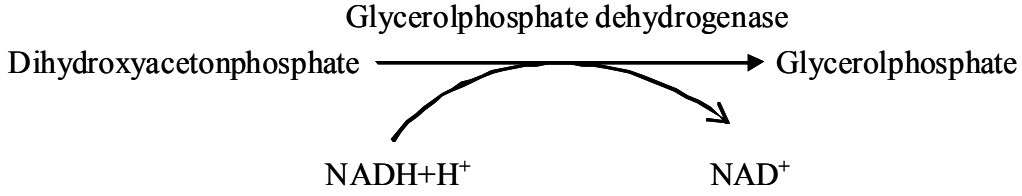
The protocol for human tissue collection was approved by the ethical committee of the Faculty of Medicine of the Technische Universität München.

3.2. SGBS preadipocytes

SGBS cells are derived from the stromal cell fraction of subcutaneous adipose tissue of an infant with Simpson-Golabi-Behmel syndrome (SGBS). SGBS preadipocytes were cultured as described elsewhere (56). In brief, the cells were proliferated in T75 flasks in basal medium supplemented with 10 % FBS until they reached confluence. Following two washing steps with PBS, the cells were detached with trypsin solution for 5 minutes at 37 °C. Digestion was terminated by adding basal medium supplemented with 10 % FBS, and the cells were collected by centrifugation for 10 minutes at 200 x g. A cell aliquot was incubated with Trypan blue and counted in a Neubauer chamber. The SGBS cells were seeded at 150 000 cells per T75 flask for sub-cultivation. Seeding density was 2000 cells/cm^2 for 96 well plates in basal medium supplemented with 10 % FBS for differentiation. Upon confluence (5 to 7 days after seeding), the cells were washed twice and differentiation was induced (day 0) with induction medium. The medium was changed to differentiation medium after four days. One doubling cycle of SGBS cells, as judged by cell counting after trypsination, is referred to as one generation. The cells were used until generation 50 at the latest.

3.3. Glycerolphosphate dehydrogenase (GPDH) assay

GPDH activity in the cell lysate is frequently used as an established marker of adipocyte differentiation (223;224) and was determined as described elsewhere (225). The assay applies the principle of photometric determination of nicotinamide adenin dinucleotide (NADH) consumption (see reaction).



The cell lysates were mixed with GPDH reaction buffer and the reaction was started by the addition of dihydroxyacetone phosphate (DHAP). GPDH present in the cell lysates at a concentration that correlates with the degree of adipocyte differentiation catalyzes the conversion of DHAP into glycerol-1-phosphate with a parallel consumption of NADH.

Cells cultured for 4, 8, 12, 16 or 20 days after induction of differentiation were detached with cell scrapers in GPDH harvest buffer on ice and were subsequently sonicated on ice for 7 seconds. After centrifugation at 10 000 x g and + 4 °C for 10 minutes, GPDH activity in the supernatant was determined photometrically. 80 µl of cell extract were incubated with 415 µl reaction solution and the reaction was started by the addition of 5 µl DHAP solution. NADH consumption was measured as decrease in absorption at 340 nm for 10 minutes at 25 °C. GPDH in mU/ml was calculated from linear NADH consumption and put in relation to the protein content (mg/ml) determined by the Bradford assay (see below).

3.4. Determination of protein content

The protein content for GPDH measurement was determined with the Bradford assay (226). This assay is based on a shift in absorption of Coomassie Brilliant Blue G-250 upon protein binding. Briefly, 50 µl cell lysate were incubated with 350 µl water and 100 µl Bradford reagent for 10 minutes at room temperature. The protein content was measured photometrically at 595 nm with different BSA concentrations being used for the standard curve.

For all other purposes, protein content was determined with the "BCA Protein Assay Kit" following the manufacturer's instruction. This kit applies the principle of the Biuret assay, a method which utilizes the reduction of copper through proteins and a subsequent colorimetric detection of the cuprous cation. In brief, cell lysates were incubated with a cupric sulphate

solution and a reaction partner for the cuprous cation (bicinchoninic acid). The formation of the colored end-product was determined at 562 nm after 30 minutes of incubation at 37 °C.

3.5. Nile Red assay

The lipophilic, fluorogenic dye Nile Red was utilized to determine the lipid content of cells. According to the manufacturer, the intracellular lipophilic binding partners of Nile Red have not been identified. Fluorescence was determined at an excitation wave length of 485 nm and an emission wave length of 590 nm. Undifferentiated control cells were measured in parallel to determine the degree of unspecific binding of Nile Red to membranes and other hydrophobic cell compounds. The results for differentiated and treated cells were corrected for the results obtained from undifferentiated control cells. The experiments were only used in the analysis if the signal from the differentiated cells was at least twice the signal of the undifferentiated control cells.

The cells were cautiously washed with 200 µl PBS after removal of 150 µl of the applied 200 µl of culture medium. 50 µl Nile Red solution was added to 110 µl medium/wash solution remaining per well after the washing step. The cells were incubated for 3 hours at 37°C and 5 % CO_2. Each single assay was performed in 3 to 6 wells for each condition and in at least three independent replicates. The results obtained with the Nile Red assay paralleled those for the established GPDH assay as shown in chapter 4.1.1.

3.6. Cytotoxicity

Lactate dehydrogenase (LDH) secretion into the medium as an indicator of cytotoxicity-induced membrane leakage was determined according to a method described by Vassault (227). LDH concentration in the cell culture supernatant was calculated in duplicate from the enzymatic conversion of supplied pyruvate into lactate measured photometrically as NADH consumption.

The cells were incubated with the indicated proteins or with dimethylsulfoxide (DMSO) for 48 hours. 50 µl supernatant was collected and mixed with 625 µl assay buffer for 5 minutes at 30 °C. The reaction was started by adding 125 µl pyruvate buffer, and the linear decrease in absorption at 339 nm was assayed photometrically for 10 minutes at 30 °C.

Additionally, the number of viable cells was determined with the "CellTiter 96® AQ_{UEOUS} One Solution Cell Proliferation Assay". Absorption of the soluble, bioreduced formazan product that is only generated in viable cells was measured at 490 nm. According to the

manufacturer's instruction, the cells were incubated with the assay solution for 2 hours at 37 °C after the 48 hour incubation with the proteins.

3.7. Apoptosis

Apoptosis was determined with the "Caspase-Glo® 3/7 Assay" according to the manufacturer's instruction. In this assay, the activity of caspase-3 and caspase-7 is measured. This is achieved by addition of a substrate to the lysed cells. If caspases are activated in the cells, this substrate is cleaved by caspases 3 and 7 to a product that delivers a luminescent signal.

Briefly, differentiated and undifferentiated cells were incubated with the respective protein alone or in combination with 10 µg/ml cycloheximide (CHX) for 24 hours. Subsequently, assay substrate was added to the cells. By shaking the cells for 1 minute at 500 rpm, the assay solution causes cell lysis. The lysates were incubated for 3 hours at room temperature and caspase 3 and caspase 7 activity was measured luminometrically.

3.8. Proliferation

Preadipocyte proliferation was assayed using the "Cell Proliferation Enzyme-linked Immunosorbent Assay (ELISA)" according to the manufacturer's instruction. For this assay, proliferating cells are incubated with the pyrimidine analogue 5-bromo-2`-deoxyuridine (BrdU). Since proliferating cells replicate their DNA, BrdU is incorporated into the DNA instead of thymidine and can be detected photometrically with labeled anti-BrdU-antibodies.

Briefly, cells were seeded in 96-well plates at a density of 6 500 cells/cm^2 in basal medium supplemented with 10 % FBS for 24 hours. After a washing step with PBS, the medium was changed to basal medium containing 0 %, 5 % or 10 % FBS with or without the indicated protein or epidermal growth factor (EGF). The cells were kept under these conditions for 5 days. Thereafter, the cells were incubated with substrate solution containing BrdU for 24 hours at 37 °C and 5 % CO_2. The cells were fixed and subsequently incubated with a horseradish peroxidase (HRP) coupled anti-BrdU antibody. Photometric detection was performed at 450 nm using a colorimetric substrate for the HRP.

3.9. Glucose uptake

Glucose uptake was performed as described previously (100). For this assay, differentiated adipocytes are incubated with the glucose derivative 2-deoxy-D-[1-^{3}H]-glucose, which can be

incorporated into, but not metabolized in the cells. The radioactive labeling of the glucose derivative allows the detection of the glucose uptake characteristics.

At day 13 after induction of differentiation the cells were washed three times with PBS and incubated in glucose-reduced basal medium (5 mM glucose) for 3 hours. The proteins were added for the last 60 minutes. Glucose uptake was initiated by stimulating the cells with 10^{-7} M insulin for 15 minutes and 2-deoxy-D-[1-^{3}H]-glucose (1µCi/ml) was added for additional 20 minutes. The reaction was terminated by placing the cells on ice, and cell lysis was performed with IGEPAL buffer for 20 minutes on ice. The uptake of radioactively labeled glucose was assessed in a scintillation counter. The results were corrected for unspecific glucose uptake.

The cells were counted to determine variations in cell number and degree of differentiation. No significant differences were found within the experiments (data not presented).

3.10. Western blot

Determination of AKT serine phosphorylation, a key step in the insulin signaling cascade, was determined by Western blots. For Western blotting, cell lysate proteins were separated by size in a gel electrophoresis chamber and were subsequently blotted onto a membrane. Antibodies against AKT and against phosphor-serine-Akt were applied on the membrane and detection was performed with a secondary HRP-labeled antibody.

Mature adipocytes were incubated in basal medium for 24 hours at 37 °C and 5 % CO_2. The proteins or the positive control (100 nM Wortmannin) were added for 15 minutes. Stimulation with 10^{-7} M insulin was performed for additional 5 minutes. The cells were harvested in Ripa buffer and lysed by two freeze-thaw cycles in liquid nitrogen and on ice. After centrifugation for 5 minutes at 5000 x g at + 4 °C, the cell lysates were used for protein determination, and Ripa buffer was added to achieve equal protein concentrations in all samples. The samples were boiled for 5 minutes at 95 °C in Laemmli buffer and were separated by 10 % acrylamide-sodium dodecyl sulphate (SDS) gels in running buffer. Semi-dry transfer was carried out onto polyvenyl difluoride (PVDF) membranes in transfer buffer. Blocking and antibody incubation were performed according to the manufacturer's instruction using the "ECL (enhanced chemiluminescence) Advance Western Blotting Detection Kit" and Tris-buffered saline Tween-20 (TBST) buffer. The films were exposed for the indicated times and developed manually.

Subsequently, the membranes were treated with stripping buffer at 60 °C for 30 minutes and, after extensive washing in TBST, were retreated as described above to determine total AKT.

3.11. Lipolysis

Glycerol content in the medium as marker of lipolysis was determined according to an established procedure (228;229). This assay makes use of the secretion of glycerol, the adipocyte end-product of triglyceride degradation, into the medium of adipocytes. Added glycerokinase catalyzes the conversion of glycerol into glycerol-1-phosphate with a measurable parallel consumption of ATP.

At day 12 after induction of differentiation the cells were washed with PBS. The cells were then incubated for 48 hours at 37 °C and 5 % CO_2 in colorless basal medium with the respective proteins or 1 µM isoproterenol as positive control. The "ATP kit SL 144-042" was used to determine the glycerol content luminometrically. This kit applies a luciferase reaction that stably emits light. ATP consumption through the glycerokinase reaction causes a linear decrease in light emission dependent on the amount of enzyme. Glycerol dissolved in medium was used for the standard curve.

The results of the glycerol assay were corrected for the number of viable cells by using the "CellTiter 96® AQ_{UEOUS} One Cell Proliferation Assay" as described in chapter 3.6.

3.12. Real-time polymerase chain reaction (PCR)

For this assay, RNA was extracted and subscribed to cDNA. A real-time PCR with 40 cycles allows for online monitoring of DNA replication and gives information about the expression level of the individual mRNAs.

The cells were differentiated in the presence of the respective proteins for 4 or 12 days. Total RNA was obtained with the "Total RNA Isolation Kit" following the manufacturer's instructions. The RNA content was determined photometrically. 0.35 µg of RNA were transcribed to cDNA using the "iScript™cDNA Synthesis Kit". TWEAK, LIGHT, glycerol aldehyde phosphate dehydrogenase (GAPDH), PPAR-γ, hormone-sensitive lipase (HSL) and adiponectin were determined with respective "Taqman Probes" and "Taqman Universal PCR Mastermix". Receptor (Fn14, HVEM, LTβR) mRNA was determined with primers designed with Vector NTI software and with "Absolute™ QPCR SYBR® Green ROX Mix". PCR was performed in the "Mastercycler Realplex ep" system with the following setup: 95 ° for 10 min, 40 cycles (50 cycles for HSL) with 95 °C for 15 seconds and 60 °C for 1 minute. All signals were standardized to the housekeeper gene GAPDH (230). Primer sequences and Taqman Primer/probe order numbers are described in detail in the appendix section.

3.13. Fluorescence-activated cell sorting (FACS)

Proteins on the cell surface were incubated with fluorescence-labeled specific antibodies and cells were measured individually in a FACS device to determine the level of protein expression on the cell surface.

Undifferentiated cells at day 0 and differentiated cells at day 12 were washed with PBS and tentatively detached with a cell scraper in ethylenediamintetraacetate (EDTA) solution after 5 minutes incubation at 37 °C and 5 % CO_2. The cells were counted with Trypan blue in the Neubauer chamber, and at least 50 000 cells were incubated in antibody solution with the respective receptor antibody for 30 minutes on ice. The human Fn14 was detected with ITEM-4 antibody and the human HVEM with MAB356 antibody. Isotype control was mouse IgG2b and IgG1, respectively. The human LTβR was detected with AF629, and normal goat IgG1 was used as isotype control. Following washing steps with antibody solution, the cells were incubated with secondary Alexa Fluor®-coupled antibody in antibody solution for 30 minutes on ice. The results from FACS were standardized to the appropriate isotype control. Overlays were accomplished with the FACS express software.

3.14. Enzyme-linked immunosorbent assay

For this assay, secretory products of the cells in the supernatant or serum levels of cytokines were detected by incubation with labeled antibodies and colorimetric substrate and a subsequent photometric measurement.

Undifferentiated cells at day 0 and differentiated cells at day 12 were incubated with the respective protein for 48 hours. Supernatants were collected and centrifuged for 5 minutes at 5000 x g and 4 °C in order to remove cell debris. The medium from mature adipocytes was obtained after a 48 hour incubation with the respective proteins immediately after cell isolation. Enzyme-linked immunosorbent assays were performed for IL-6, leptin and adiponectin following the instruction of the manufacturer. This includes several incubation steps with the respective antibodies or substrate solution as well as several washing steps with supplied washing solution.

The serum levels of TWEAK and LIGHT were determined after serum centrifugation for 10 minutes at 16 000 x g and 4 °C. The assays were performed as described by the manufacturer's instruction.

3.15. Bio-Plex cytokine assay

The Bio-Plex system is based on the Luminex technology and applies the principle of a sandwich ELISA: cytokines are coupled to an antibody-coated bead. Fluorescence labeled beads are specific for each cytokine and allow for multiplex measurements. Detection of cytokine-bound beads is achieved with phycoerythrin-labelled (PE) streptavidin bound to biotinylated secondary antibodies. Multiplex assays were performed for IFN-γ, regulated upon activation, normal T-cell expressed, and secreted (RANTES) and GM-CSF with a "Bio-Plex Multiplex Cytokine Assay" and for IL-8 and TNF-α with a "Human Adipocyte Lincoplex Kit".

Briefly, the supernatants were incubated with antibody-coupled beads as described by the manufacturer's instruction. Supernatants for the Bio-Plex cytokine assay were generated as described in chapter 3.14. The washing steps were carried out by adding washing solution and by vacuum suction through the 96-well plate filter-bottom. The secondary antibody was added for one hour at room temperature. Immediately after streptavidin-PE incubation, the cytokine-coupled beads were measured in the Bio-Plex system. Standard curves for each cytokine were included in the kits. Measurements were performed with supernatants from two wells for each condition.

3.16. Bio-Plex phosphoprotein assay

The principle of the "Bio-Plex Phosphoprotein Assay" and the "Bio-Plex Total Target Assay" equals that of the cytokine assay. The supernatants are incubated with antibody coupled beads for phospho- and total-protein of p38-MAPK, Erk-2, JNK and inhibitor of κ-Bα (Iκ-Bα) and detected with PE-streptavidin bound to biotinylated secondary antibodies.

At day 12 after induction of differentiation the cells were incubated with the respective protein for 30 minutes. The cells were placed on ice, and lysis was performed with the "Bio-Plex Cell Lysis Kit". The cells were detached with cell scrapers and, following two freeze-thaw cycles in liquid nitrogen and on ice, were centrifuged for 5 minutes at 5000 g and +4 °C. The protein content was determined as described in chapter 3.4., and an assay/lysis buffer mixture was applied to equalize the protein content in all samples to 10 µg/50 µl.

Following washing steps, the incubation with primary antibody was carried out overnight and with secondary antibody for 30 minutes and the Bio-Plex assay was performed immediately after the last incubation with streptavidin-PE. The results for phospho-proteins were corrected for total-protein content.

3.17. Activated NF-κB ELISA

This assay applies the principle of a sandwich ELISA for the detection of NF-κB subunits. 96-well plates are coated with oligonucleotides containing NF-κB consensus binding sites. After binding of the NF-κB subunits from cell lysates, detection is performed photometrically with labeled secondary antibodies.

On day 12 after induction of differentiation, the cells were incubated with the respective protein for 30 minutes. The cells were placed on ice, and cell extract together with nuclear extract was obtained through lysis in Digman C buffer. The cells were detached with cell scrapers, and lysis was completed by two freeze-thaw cycles in liquid nitrogen and on ice. The extracts were obtained by centrifugation for 10 minutes at 16 000 rpm, 4 °C and protein content was determined as described in chapter 3.4. The protein content was equalized with Digman C buffer to 2 µg/µl. NF-κB binding activity was determined with an "TransAm™ NFκB Family Kit". Following coating of the 96-well plate with oligonucleotides containing NF-κB consensus binding sites, the cell extract was added. The plates were incubated for 1 hour at room temperature and the HRP-coupled secondary antibody was added for 1 hour at room temperature after a washing step. The extent of NF-κB binding to the respective oliognucleotide was determined photometrically at 450 nm and a reference wave length of 655 nm). The internal positive control Raji nuclear extract was supplied by the manufacturer.

4. Results

4.1. Inhibition of differentiation

In a high-throughput screening approach, HEK293 were transfected to produce soluble proteins with a potential inhibitory effect on differentiation of 3T3-L1 murine preadipocytes. Subsequent addition of the HEK293 supernatants to differentiating 3T3-L1 preadipocytes caused a marked inhibition of differentiation for 21 different cDNAs (screening data not shown). Among the 21 hits with inhibitory effects on adipocyte differentiation, six proteins have been described previously to influence adipocyte differentiation: TNF-α and LT as members of the TNFSF, IL-6 and IL-11 as members of the interleukin family as well as endothelin-1, and PDGF (61;67-71). Two members of the TNFSF previously not described to alter adipocyte differentiation were also found to reduce adipocyte lipid content in this screening approach: TWEAK (TNFSF12) and LIGHT (TNFSF14). The other proteins found in this approach are currently under investigation and therefore can not be displayed. The screening results for TWEAK and LIGHT were confirmed by addition of soluble recombinant protein to differentiating 3T3-L1 cells on the day of induction to differentiation (Fig. 9). TWEAK or LIGHT were present for five days under adipogenic conditions at the indicated concentrations. Adipocyte lipid content was thereafter measured with the Nile Red assay.

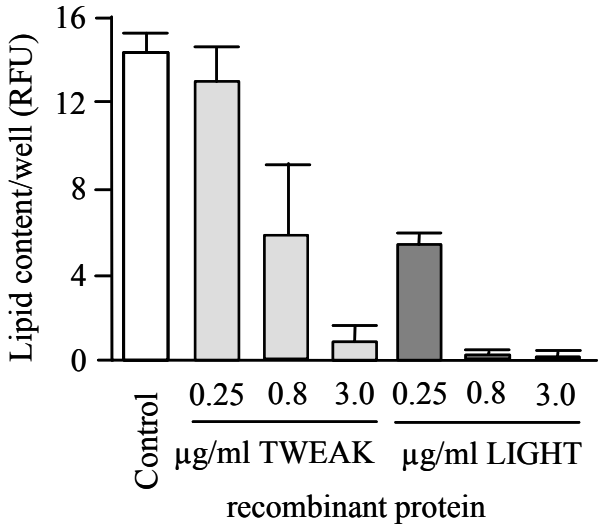


Figure 9: Inhibition of differentiation in 3T3-L1 preadipocytes. Cells were incubated with 250 ng/ml, 800 ng/ml or 3 000 ng/ml TWEAK or LIGHT from the day of induction until day 5. Adipocyte lipid content was determined with the Nile Red assay. n = 3 for triplicate measurement in each single experiment.

Both proteins displayed a concentration-dependent inhibitory effect on murine 3T3-L1 preadipocyte differentiation. To confirm these results for a possible application of TWEAK and LIGHT in the human system, human primary preadipocytes and human SGBS preadipocytes were incubated with rising TWEAK or LIGHT concentrations for the whole differentiation period under adipogenic conditions.

4.1.1. Comparison of Nile Red and GPDH assay

As described above, the determination of lipid content in adipocytes was performed with the Nile Red assay. The measurement of GPDH activity in cell lysates from adipocytes is an established parameter for the degree of cell differentiation (223;224). The cytosolic enzyme is crucial for triglyceride biosynthesis in the adipocyte, its product is substrate for the first step of triacylglycerol synthesis, i.e. the formation of diacylglycerol from glycerol-3-phospate and activated fatty acids (231). To confirm the suitability of the Nile Red assay as a marker for adipocyte lipid content and differentiation, results of this assay were compared to results of the established GPDH assay. Cells from six patients seeded in 12-well plates as described in the methods were induced for differentiation on day 0. Cells were harvested for GPDH measurement on day 8, 12, 16 and 20 and from four of the patients additionally on day 4. Cells were in parallel seeded in 96-well plates for the measurement of Nile Red signal following the same time scheme.

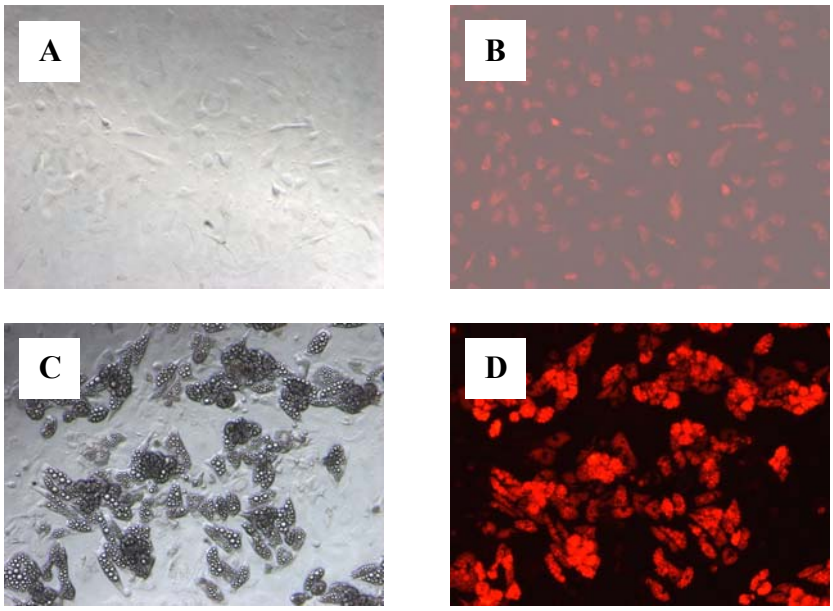


Figure 10: Microscopical evaluation of undifferentiated preadipocytes (A, B) and differentiated adipocytes (C,D). Cells were stained with Nile Red as described in methods and photographs were taken in normal (A,C) and fluorescent light (B, D). Magnification 50 x.

The dye Nile Red interacts with undefined hydrophobic cell compounds and delivers a considerable increase in the fluorescence signal upon differentiation (Fig. 10).

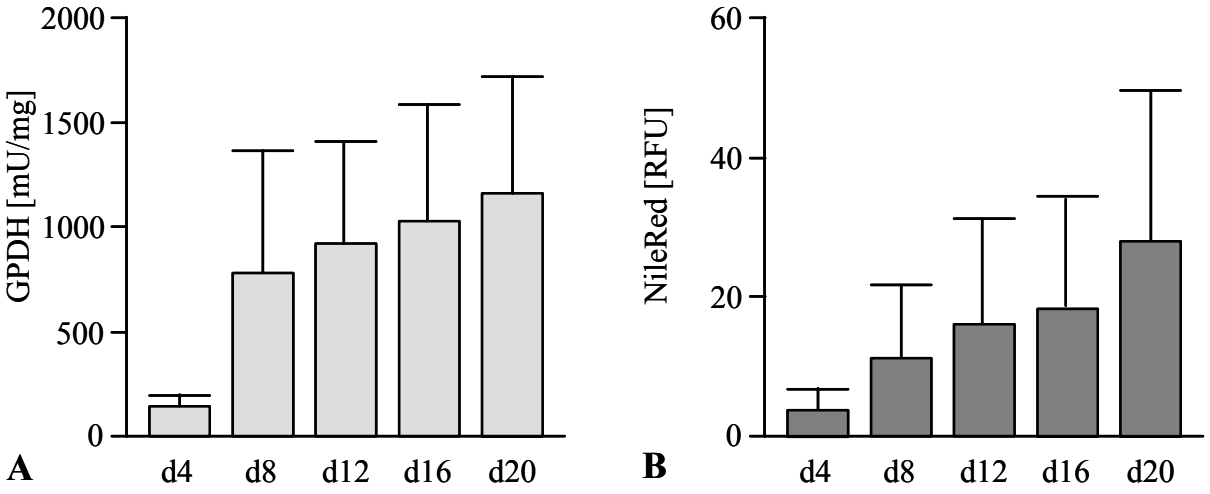


Figure 11: Differentiation-dependent values for GPDH and Nile Red signal. Cells were assayed for GPDH content and Nile Red signal on day (d) 4, day 8, 12, 16 and 20. GPDH is given in mU enzyme per mg of protein content in cell lysates. Nile Red is indicated in relative fluorescence units (RFU). GPDH was measured in duplicate from 2 combined wells for each single donor, Nile Red was determined in six wells for each donor.
n = 4 for day 4, n = 6 for day 8 to 20.

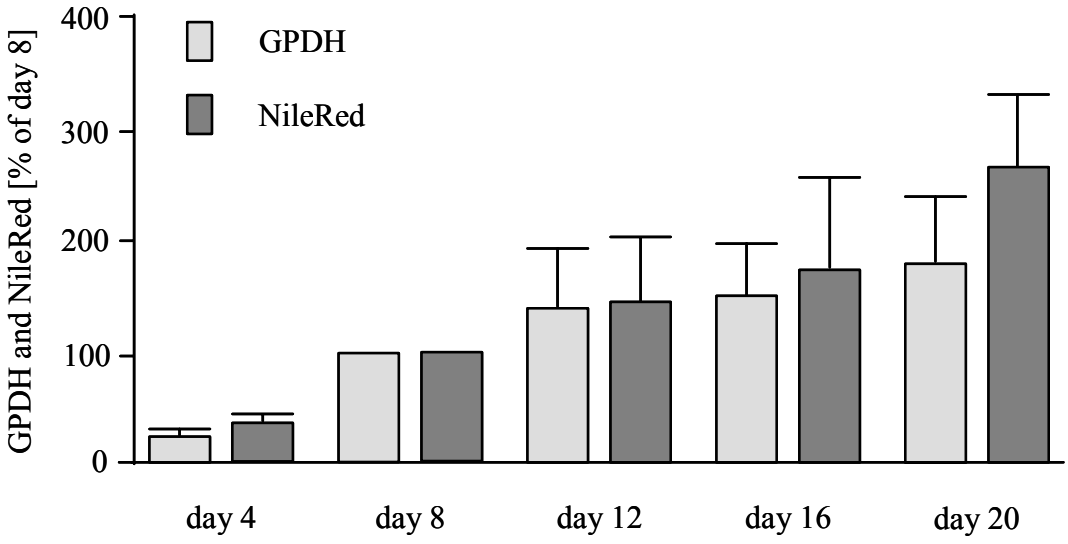


Figure 12: Comparison of GPDH and Nile Red values in primary human adipocytes for increasing periods of differentiation. Results are relative to the results obtained on day 8 for each set of experiments. n = 6, n = 4 for day 4.

GPDH increased from values around the detection level in undifferentiated cells (day 4) to an average of 1163 ± 558 mU/mg on day 20 (Fig. 11 A). Fluorescence data measured for Nile Red increased from values around 2.5 RFU (day 4) to 27.6 ± 20.9 RFU on day 20 (Fig. 11 B). These results from GPDH and Nile Red measurement already reveal a limitation of the human primary culture system. A high inter-individual variation results in high standard deviations in this model. Results were therefore displayed relative to respective control cells to facilitate comparison between different treatments. To compare the results obtained from the two different methods for the determination of adipocyte differentiation, results were within each method standardized to results on day 8 since most of the following results were obtained on day 8. No principal differences were observed between the results of the two assays for the degree of differentiation in adipocytes differentiated for different periods of time (Fig. 12). Thus, the Nile Red assay offers an easy-to-use alternative for the determination of intracellular lipid content and differentiation in comparison to the GPDH assay, which is the most widely used parameter of adipose differentiation.

4.1.2. Concentration-dependent effects of TWEAK and LIGHT in subcutaneous human adipocytes

To determine the effect of TWEAK and LIGHT on the differentiation of human subcutaneous primary preadipocytes, cells were differentiated for 8 days in the presence of the respective protein. The highest investigated concentration was 1000 ng/ml, the minimal concentration was 0.025 ng/ml and protein was increased by factor four to 0.098, 0.39, 1.56, 6.25, 25, 100 and 400 ng/ml.

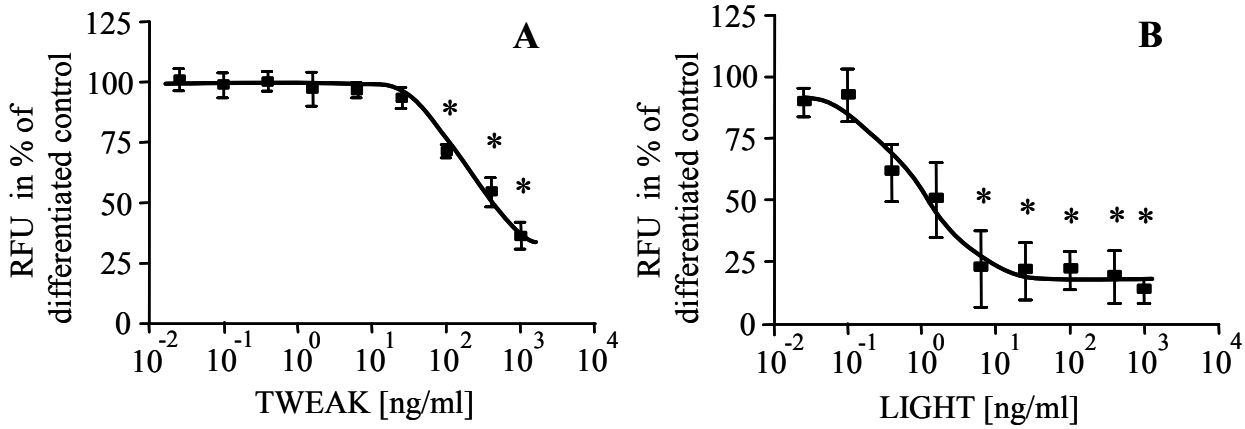


Figure 13: Concentration-dependent effects of TWEAK (A) and LIGHT (B) on the differentiation of human primary preadipocytes cultured in the presence of soluble recombinant protein for 8 days. Sigmoidal dose-response-curves were obtained with GraphPad Prism Software. n = 3, results for each donor were an average of three wells. * $p < 0.05$ vs. differentiated, untreated control.

Results are presented in percent of fully differentiated control cells in the absence of any of the factors under investigation (Fig. 13). Cells cultured in parallel in 2F+C medium to prevent proliferation and differentiation delivered values for the background of Nile Red staining. This is necessary due to the modest interaction of the dye with hydrophobic cell compartments. These values were subtracted from the results of differentiated untreated and treated cells. A concentration-dependent effect on adipocyte differentiation was visible for both proteins. For TWEAK, the maximum effect could not be observed due to technical limitations. For both proteins, microscopical evaluation revealed intact cells with no detachment at all protein concentrations (Fig. 14).

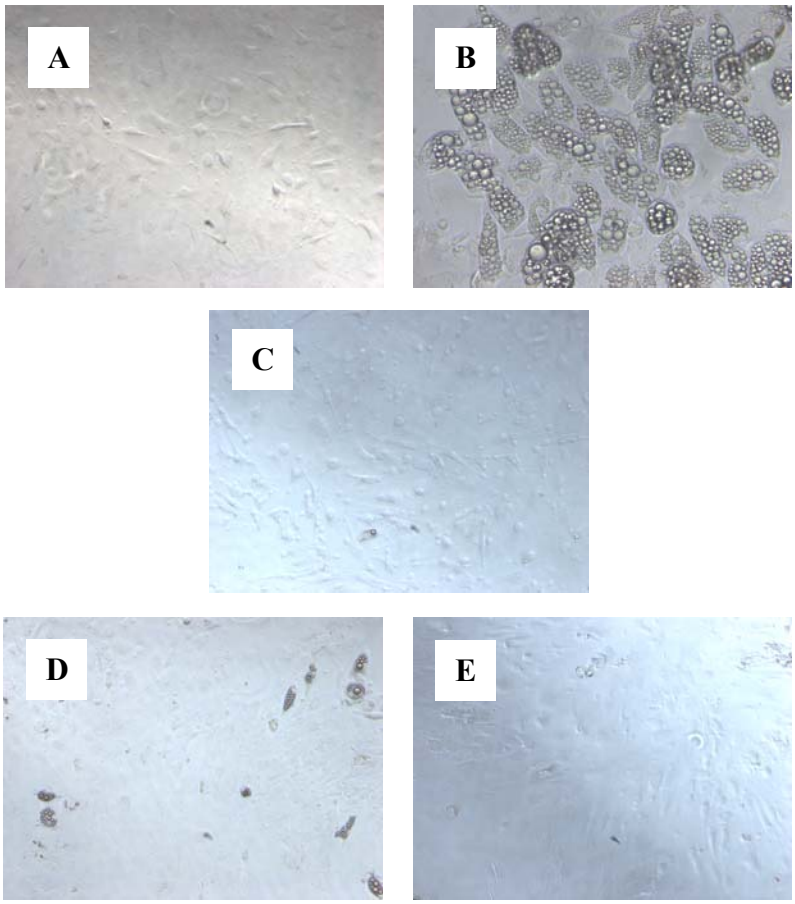


Figure 14: Microscopical evaluation for the effect of TWEAK, LIGHT, and TNF-α, respectively. Undifferentiated preadipocytes (A) and differentiated adipocytes (B) were compared to cells differentiated in the presence of 100 ng/ml TNF-α (C), 1000 ng/ml TWEAK (D) or 100 ng/ml LIGHT (E). Magnification 50 x.

The half maximal inhibitory effect (EC50) of TWEAK was calculated to be ~ 200 ng/ml (~ 10 nM) and of LIGHT to be ~ 10 ng/ml (~ 0.4 nM). TWEAK and LIGHT were human, soluble, recombinant proteins produced in E. coli and HEK293 cells respectively.

4.1.3. Concentration-dependent effects of TWEAK and LIGHT in omental preadipocytes

To further examine the effect of the cytokines for human adipose tissue, the effect of TWEAK and LIGHT was also investigated in human primary omental preadipocytes. Three different concentrations (10, 50 and 100 ng/ml) of TWEAK and LIGHT were applied. TNF-α at 50 ng/ml (2.9 nM) was used as a positive control in omental cells. TNF-α inhibited differentiation almost to the level of undifferentiated control cells (Fig. 15). TWEAK and LIGHT exhibited concentration-dependent effects (Fig. 15) with LIGHT being the more potent inhibitor under the applied conditions as already observed for the subcutaneous adipocytes.

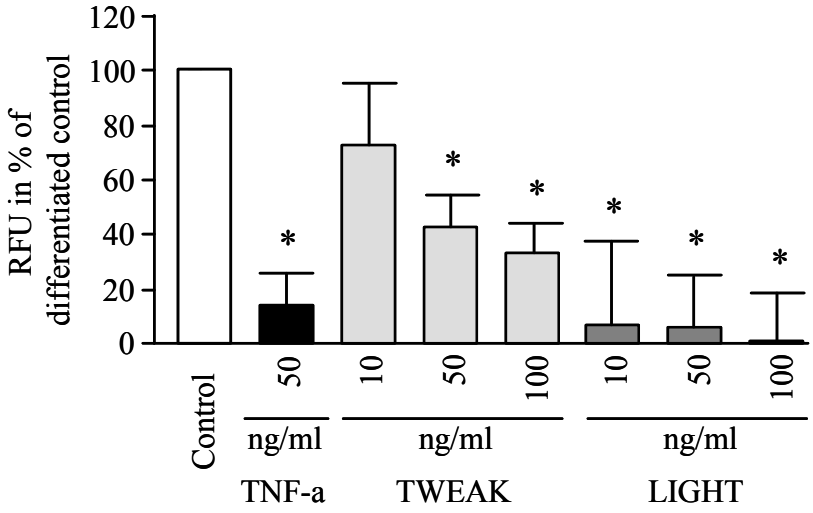


Figure 15: Concentration-dependent effects of TWEAK and LIGHT on human primary omental preadipocytes in comparison to TNF-α. Cells were differentiated for 12 days in the presence of the specified concentrations of the respective protein. n = 3, results for each donor are the means of 6 wells. * $p < 0.05$ in comparison to differentiated, untreated control cells (Control).

LIGHT inhibited differentiation of omental adipocytes to a level comparable to TNF-α and all three LIGHT concentrations were obviously above the EC50 in omental preadipocytes which differs from the results in subcutaneous preadipocytes. This indicated a slightly stronger responsiveness to LIGHT in the omental fat depot. TWEAK displayed a significant effect starting from 50 ng/ml, and inhibited differentiation to more than 50 % with 100 ng/ml, which is a slightly stronger effect than observed in subcutaneous preadipocytes.

4.1.4. Concentration-dependent effects of TWEAK and LIGHT in SGBS cells

SGBS preadipocytes were differentiated in the presence of increasing concentrations (10, 50 and 100 ng/ml) of TWEAK and LIGHT for 8 days. For comparison, cells were in parallel differentiated in the presence of 50 ng/ml TNF-α. TNF-α inhibited differentiation to the level of undifferentiated control cells (Fig. 16). As observed in human omental and subcutaneous preadipocytes, LIGHT was more potent than TWEAK in human SGBS cells. LIGHT inhibited differentiation approximately to the level of undifferentiated cells. TWEAK already reduced lipid content to less than 50 % at the lowest concentration of 10 ng/ml, which indicates a lower EC50 in the SGBS system.

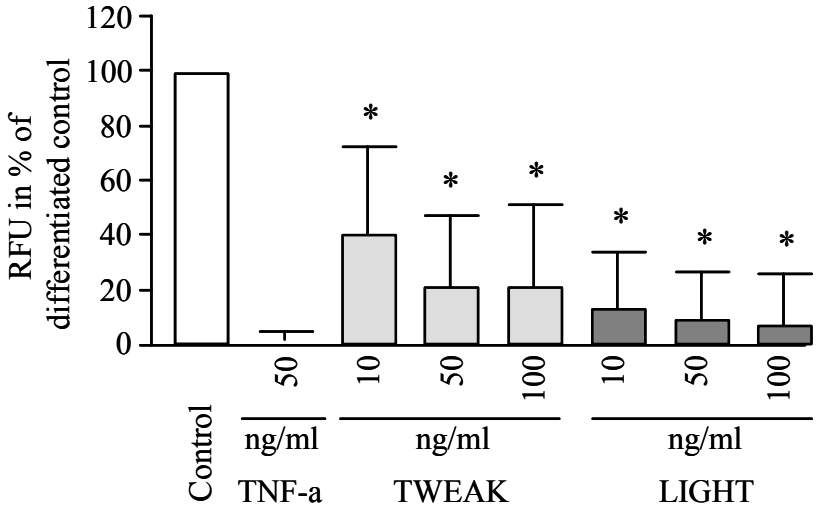


Figure 16: Concentration-dependent effects of TWEAK and LIGHT on SGBS cells differentiated for 8 days in the presence of the specified concentrations in comparison with 50 ng/ml TNF-α. n = 5, results for each donor were the mean of 6 wells. * $p < 0.05$ in comparison to differentiated, untreated control cells (Control).

4.1.5. Time-dependent effects of TWEAK and LIGHT in human subcutaneous preadipocytes

TNF-α, as well-studied member of the TNFSF, exhibits inhibitory effects on adipocyte differentiation as well as stimulatory effects on lipolysis in terminally differentiated adipocytes (68;100). The decrease in NileRed signal should therefore be visible for TNF-α added the whole period of differentiation and for only a few days to differentiated adipocytes. This was tested as follows: Human primary preadipocytes were differentiated for 12 days. TWEAK at 1000 ng/ml and LIGHT at 100 ng/ml as well as TNF-α at 100 ng/ml were added on day 0, i.e. the day of induction, and were present for the whole differentiation period. In parallel, proteins were added on day 4 or on day 8 after induction of differentiation so that they were present for the last 8 or 4 days of differentiation, respectively. Lipid content was

determined with the Nile Red assay on day 12. The effects of TWEAK and LIGHT were weaker than the effect of TNF-α under all conditions. TWEAK and LIGHT inhibited differentiation significantly ($p \leq 0.05$) in comparison to differentiated, untreated control cells (Fig. 17) only when added on the day of differentiation or on day 4 after starting differentiation.

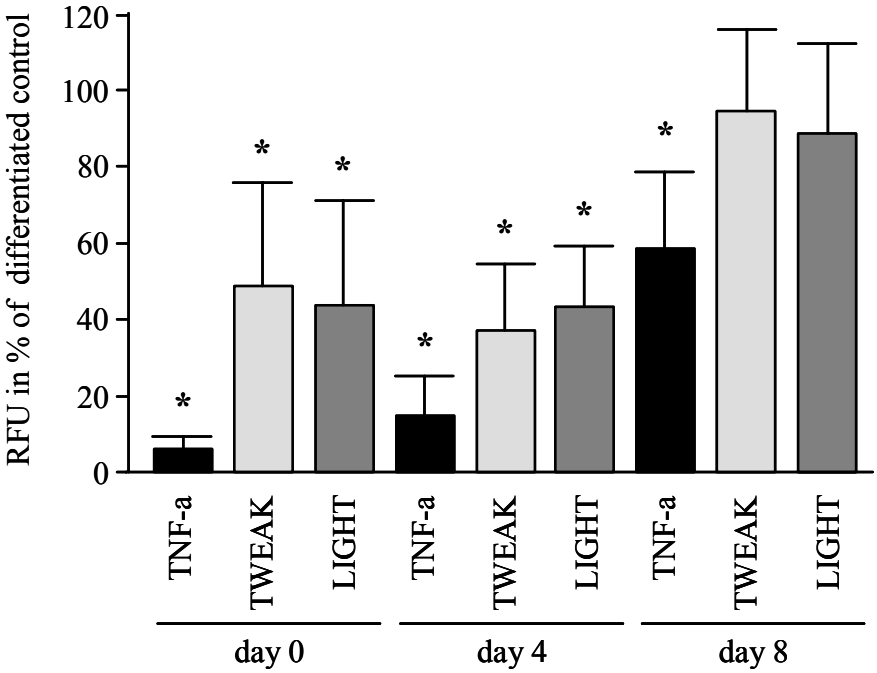


Figure 17: Time-dependent effects of TNF-α, LIGHT and TWEAK on adipocyte differentiation. Human primary subcutaneous preadipocytes were differentiated for 12 days. 100 ng/ml TNF-α, 100 ng/ml LIGHT and 1000 ng/ml TWEAK were either present for the whole differentiation period (added on day 0), or for the last 8 days (added on day 4) or for the last four days (added on day 8). n = 4, results for each donor for an average of 4 wells. * $p < 0.05$ versus differentiated, untreated control.

Contrary to TNF-α no inhibitory effects were visible when the proteins were added to differentiated cell on day 8 and were therefore present for the last 4 days.

4.1.6. Real-time PCR for PPAR-γ

Adipocyte differentiation from preadipocytes is, among other processes, mediated by a tightly regulated alteration in transcription factor activity of C/EBPs, SREBP-1c and PPAR-γ (42;43;48). Activation of the transcription factor PPAR-γ is a critical early step in adipocyte differentiation (45;49). TNF-α is known to interfere with PPAR-γ mRNA expression and this effect is involved in the inhibition of adipocyte differentiation (94-96). To test the effects of TWEAK and LIGHT on this system, cells were incubated for four days with 100 ng/ml TNF-α as positive control and in parallel with 1000 ng/ml TWEAK or 100 ng/ml LIGHT under adipogenic conditions. Cells differentiated for four days displayed a fivefold increase in

PPAR-γ mRNA expression in comparison to undifferentiated control cells (Fig.18 A). This increase was reduced to an only twofold increase when cells were differentiated in the presence of TNF-α. When 1000 ng/ml TWEAK or 100 ng/ml LIGHT were present during the first four days of differentiation, PPAR-γ mRNA expression was significantly decreased to about 60 % and 70 %, respectively, of the mRNA expression in untreated control cells.

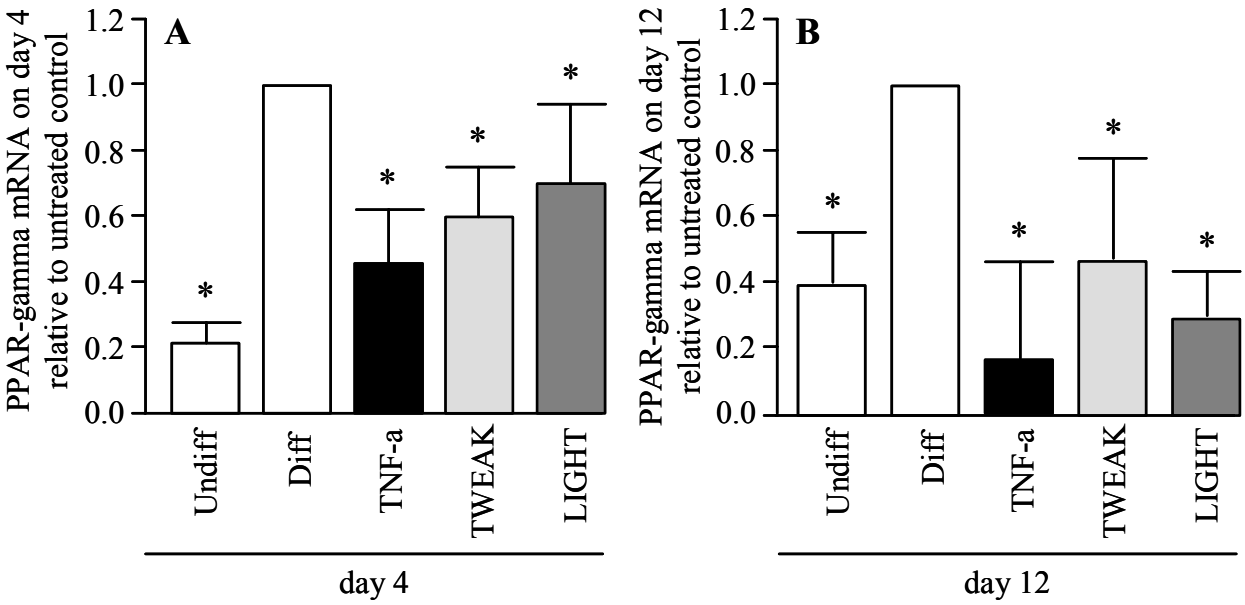


Figure 18: Effects of TWEAK and LIGHT on PPAR-γ mRNA expression. Cells were differentiated for four days in the presence of 100 ng/ml TNF-α, 100 ng/ml LIGHT or 1000 ng/ml TWEAK (A). mRNA was harvested on day 4 and RT-PCR was performed. Results are presented relative to the respective untreated, differentiated control cells. Cells from different donors were differentiated for 12 days following the same protocol (B). n = 8 for day 4 (n = 4 for TWEAK), n = 6 for day 12, results for each donor were obtained in triplicate measures from two combined wells. * $p < 0.05$ vs. differentiated control.

Cells from different donors differentiated for 12 days displayed a decrease in PPAR-γ mRNA expression under the influence of TNF-α, which was comparable to the level of undifferentiated, untreated control cells (Fig. 18 B). Under these conditions, TWEAK at 1000 ng/ml and LIGHT at 100 ng/ml also inhibited PPAR-γ mRNA expression in human primary adipocytes to the level of undifferentiated, untreated control cells. These results indicate that TWEAK and LIGHT also interfere with the differentiation process of human primary preadipocytes via the inhibition of early transcription factor expression.

4.1.7. Real-time PCR for adiponectin and HSL

To gather more information on the mechanisms by which TWEAK and LIGHT mediate the decrease in the Nile Red signal, real-time PCR was performed for adiponectin and HSL as typical markers of fully differentiated adipocytes. The adipokine adiponectin is a typical late marker for adipocyte differentiation. The protein is expressed in and secreted from mature adipocytes, but no expression or secretion is found in preadipocytes (232;233). TNF-α is known to decrease adiponectin expression in mature adipocytes (234). HSL mRNA expression is up-regulated in adipocytes in comparison to preadipocytes and the presence of TNF-α was found to decrease HSL mRNA expression (56;234;235).

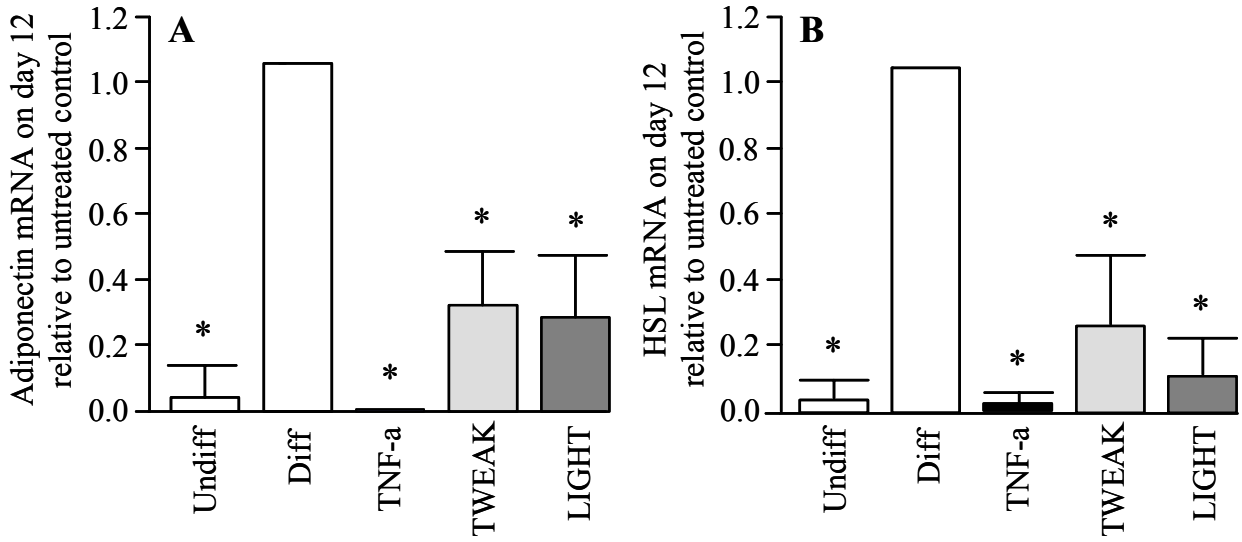


Figure 19: Effects of TWEAK and LIGHT on adiponectin and HSL mRNA expression. Cells were differentiated for twelve days in the absence or presence of 100 ng/ml TNF-α, 100 ng/ml LIGHT or 1000 ng/ml TWEAK. mRNA was harvested on day 12 and RT-PCR was performed for qualification of adiponectin (A) and HSL (B) mRNA. Results are presented relative to the respective untreated, differentiated control cells. n = 6, results for each donor were obtained in duplicates. * $p < 0.05$ vs. differentiated control.

Human primary preadipocytes were differentiated for 12 days in the presence of 100 ng/ml LIGHT or 1000 ng/ml TWEAK. mRNA was isolated on day 12 and RT-PCR was performed for adiponectin and HSL. Undifferentiated control cells were cultured in 2F+C medium in parallel. TNF-α inhibited mRNA expression of adiponectin and HSL to the level of undifferentiated untreated cells (Fig. 19). TWEAK and LIGHT had comparable inhibitory effects on the mRNA expression of adiponectin in cells differentiated in the presence of either protein for 12 days: adiponectin expression was only about 30 % of the values reached in differentiated untreated control cells. For HSL expression, LIGHT seems to be slightly more

potent than TWEAK under the chosen conditions, yet both proteins significantly inhibited mRNA expression.

4.2. Effects of TWEAK and LIGHT on cell viability and proliferation

4.2.1. Cytotoxicity

Negative effects of TWEAK and LIGHT on cell viability have been described (107;118;164). To exclude cytotoxic or pro-apoptotic effects on the course of the decreased Nile Red signal, human primary preadipocytes and adipocytes were incubated with 2000 ng/ml LIGHT or TWEAK for 48 hours. 5 % DMSO was used as positive control for a cytotoxic effect of the two investigated proteins. Cytotoxic effects were evaluated with two different methods in parallel: LDH concentration in the supernatant of treated cells and untreated control cells as a marker of membrane leakage was assayed with a spectrophotometric method determining enzyme activity (227). Additionally, the number of viable cells after TWEAK, LIGHT or DMSO treatment was determined with the MTS assay. This assay measures the optical density of cell lysates originating from a bioreduced product of the applied MTS tetrazolium salt which can only be produced in viable cells. These experiments were simultaneously performed under three different cell conditions for each donor: preadipocytes seeded at normal density were induced for differentiation and the MTS as well as the LDH assay were performed on day 8 in differentiated adipocytes after 48 hours of incubation with protein. Additionally, cells induced for differentiation were immediately incubated with the respective proteins and assays were performed on day 2 after induction. Proliferating cells were seeded at half the density described for differentiated cells and assays were performed after two days of proliferation before cells had reached confluence, which would lead to contact inhibition of cell growth. DMSO caused an increase in LDH activity that was only significant for differentiating cells (Fig. 20). Neither TWEAK nor LIGHT at 2000 ng/ml, which is considerably higher than the EC50 or concentrations applied in the other experiments, displayed a stimulatory effect on LDH activity. The MTS assay revealed a significant decrease in the number of viable cells for 5 % DMSO at all three cell cycle states. Whereas with TWEAK, the number of viable cells was increased under all three conditions, LIGHT did not alter the number of viable cells. This increase was significant in differentiating preadipocytes. In conclusion, neither TWEAK nor LIGHT at the applied high concentrations

caused a cytotoxic effect on proliferating and differentiating human primary subcutaneous preadipocytes or on differentiated adipocytes.

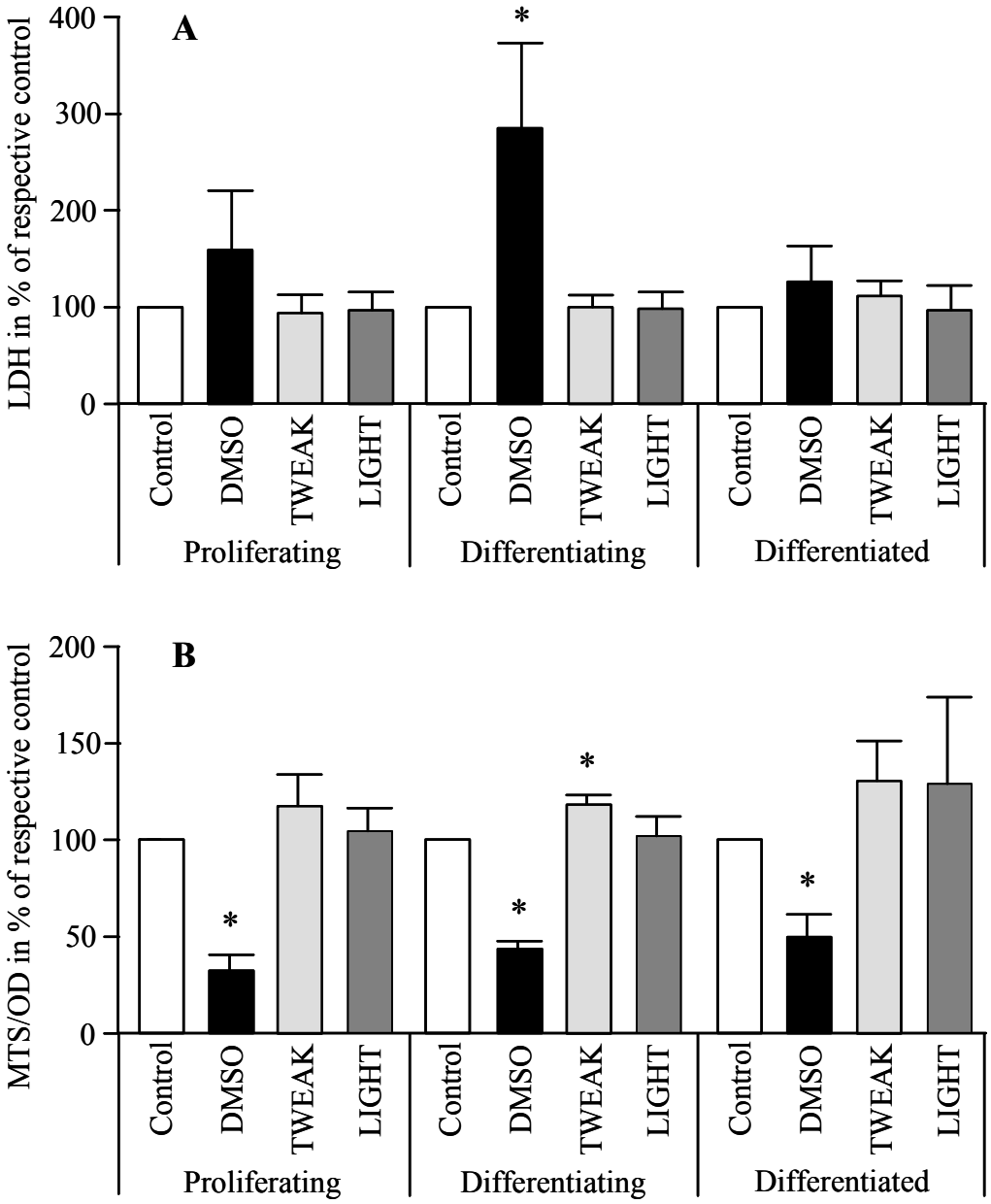


Figure 20: Effects of TWEAK and LIGHT on cell viability. LDH assay (A) and MTS assay (B) were performed in proliferating, differentiating and differentiated cells after 48 hours of incubation with 5 % DMSO or 2000 ng/ml TWEAK or LIGHT. n = 4, results for each donor were obtained from four wells for MTS and in duplicate from four combined wells for LDH. * $p < 0.05$ vs. respective proliferating, differentiating or differentiated control.

4.2.2. Apoptosis

To rule out possible apoptotic effects, human primary subcutaneous cells, differentiating (immediately after the induction to differentiation) as well as differentiated, were used for determination of caspase-3 and caspase-7 activation. Human adipocytes and preadipocytes show an only low sensitivity to apoptosis upon TNF-α, yet sensitivity can be markedly increased by co-incubation with cycloheximide (CHX), an inhibitor of biosynthesis (236). 100 ng/ml TNF-α in combination with 10 µg/ml CHX was used as a positive control and was applied for 24 hours.

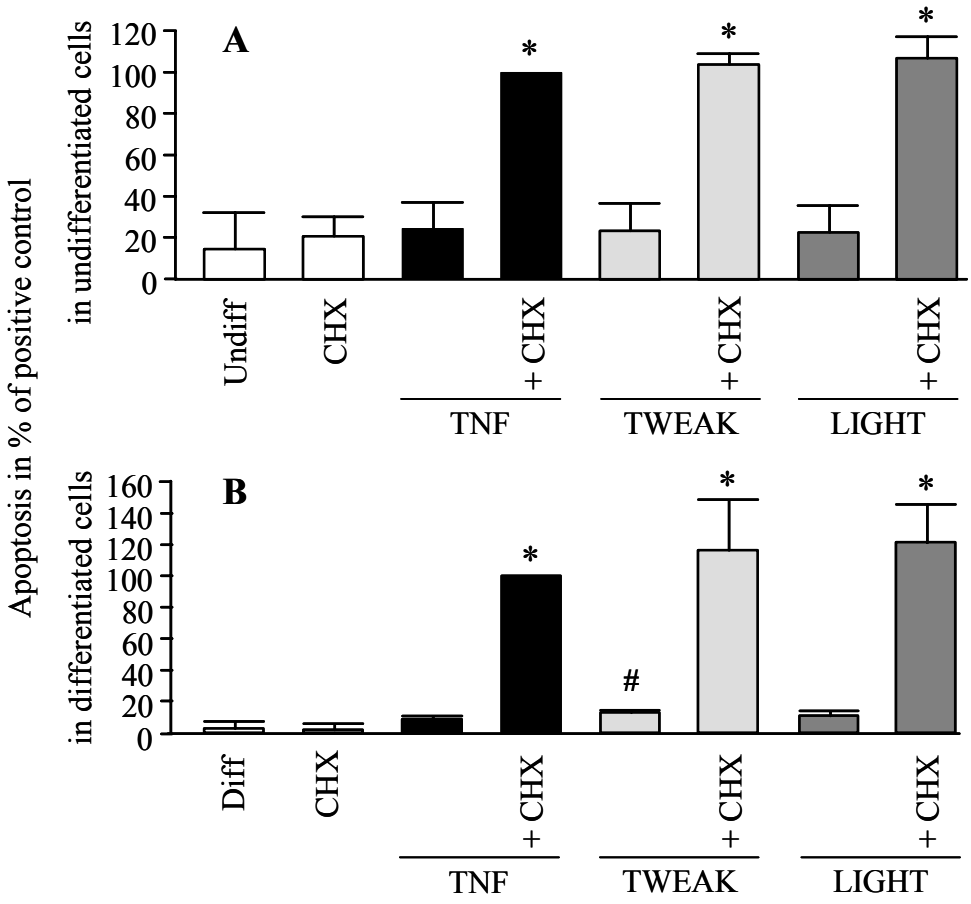


Figure 21: Apoptotic effects of TWEAK and LIGHT. 100 ng/ml TNF-α, 1000 ng/ml TWEAK or 100 ng/ml LIGHT were applied for 24 hours with or without 10 µg/ml CHX in undifferentiated (Undiff) and differentiated (Diff) cells. Caspase-3 and -7 activity was measured via luminescent cleaved substrates. n = 3, results for each donor were obtained in duplicates. * $p < 0.05$ vs. CHX, # $p < 0.05$ vs. Diff.

100 ng/ml LIGHT or 1000 ng/ml TWEAK were applied in parallel for 24 hours without or in combination with CHX. Cells were subsequently lysed and caspase-3 and caspase-7 activity was determined with the Caspase-Glo® 3/7 Assay. This assay measures the luminescence of the cleaved substrates. As described in the literature (236) CHX alone has no effect on

apoptosis (Fig. 21). LIGHT or TNF-α did not affect cell viability when administered alone, yet stimulated apoptosis significantly in comparison to CHX-treated cells when co-incubated with 10 µg/ml CHX. TWEAK showed effects comparable to LIGHT and TNF-α when co-incubated with CHX and had a weak pro-apoptotic effect when administered alone on differentiated adipocytes. However, this pro-apoptotic effect was marginal when compared with the effect of TWEAK co-incubated with CHX.

4.2.3. Proliferation

TWEAK and LIGHT have been described to have stimulatory effects on cell proliferation (122;154;176;237). Effects of TWEAK and LIGHT on the proliferation of human primary preadipocytes were therefore investigated using the BrdU assay. Cells were seeded at a density well below the seeding density for differentiation with 6500 cells/cm^2. Following a

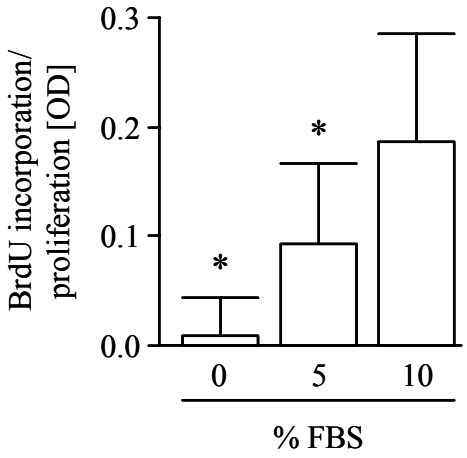


Figure 22: Human primary preadipocytes proliferated in the presence of increasing concentrations of FBS. n = 5, results for each donor were obtained in duplicates. * $p < 0.05$ vs. 10 % FBS.

5 to 6 day proliferation phase in the presence of 1000 ng/ml TWEAK or 100 ng/ml LIGHT with or without 5 % FBS, cells were further proliferated under the same conditions with labeled BrdU for 24 hours. Cells did not reach confluence at any time-point to ensure replication of the cells within the exponential growth phase. Proliferation, i.e. the BrdU incorporation rate, was then determined colorimetrically with a peroxidase-conjugated BrdU-specific antibody. Cells were in parallel incubated with 0 %, 5 % or 10 % FBS. 10 ng/ml EGF was additionally used as positive control. Increasing concentrations of FBS led to a significant increase in BrdU incorporation into proliferating cells (Fig. 22). When cells were proliferated in the presence of the respective protein without FBS, both EGF and TWEAK significantly increased the proliferation rate in preadipocytes (Fig. 23 A). Similar results were obtained

when the assay was performed in the presence of 5 % FBS (Fig. 23 B). LIGHT had no effect on proliferation of human primary preadipocytes under either condition.

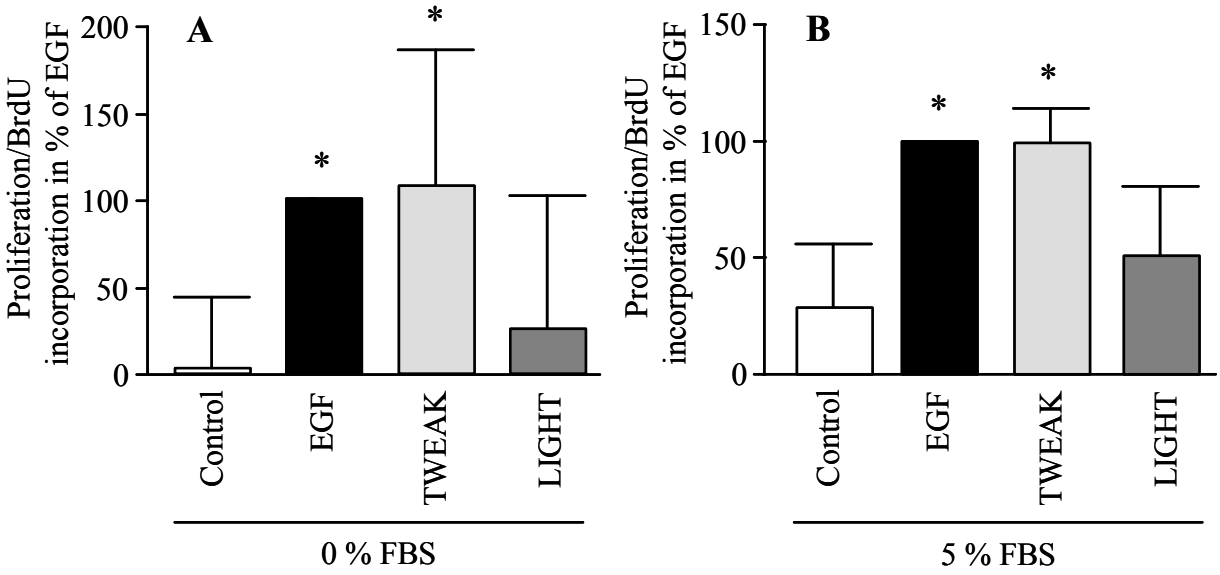


Figure 23: Effects of TWEAK and LIGHT on cell proliferation. Human primary subcutaneous preadipocytes were proliferated for 5 to 6 days in the presence of 10 ng/ml EGF, 1000 ng/ml TWEAK or 100 ng/ml LIGHT and 0 % (A) or 5 % (B) FBS. BrdU incorporation was determined over a period of 24 hours. n = 5, results for each donor were obtained in duplicate. * $p < 0.05$ vs. respective control with 5 % or without FBS.

4.3. Metabolic parameters

Human adipose tissue and in particular adipocytes are the main site of energy storage in the body. Adipocytes react to numerous hormonal and nutritional stimuli. The main functions, lipid storage and release, are based on either glucose and fatty acid uptake and their incorporation into triglycerides or lipolysis, i.e. release of fatty acids and glycerol (238;239).

4.3.1. Glucose uptake

Glucose uptake of adipocytes was determined in human primary subcutaneous adipocytes differentiated for 14 days. Cells were incubated with 100 ng/ml TNF-α, 1000 ng/ml TWEAK or 100 ng/ml LIGHT for 1 hour subsequent to 2 hours of insulin and glucose deprivation. Cells were then stimulated for 15 minutes with 10^{-7} M insulin and glucose uptake was determined for a period of 20 minutes with radioactively labeled deoxy-glucose (a glucose metabolite that is transported into the cell but not metabolized within the cell). Basal glucose uptake was determined in the absence of insulin. Cell number and degree of differentiation

were determined by microscopical counting of three independent and representative areas in each well, no significant differences were observed in cell number or degree of differentiation within each experiment.

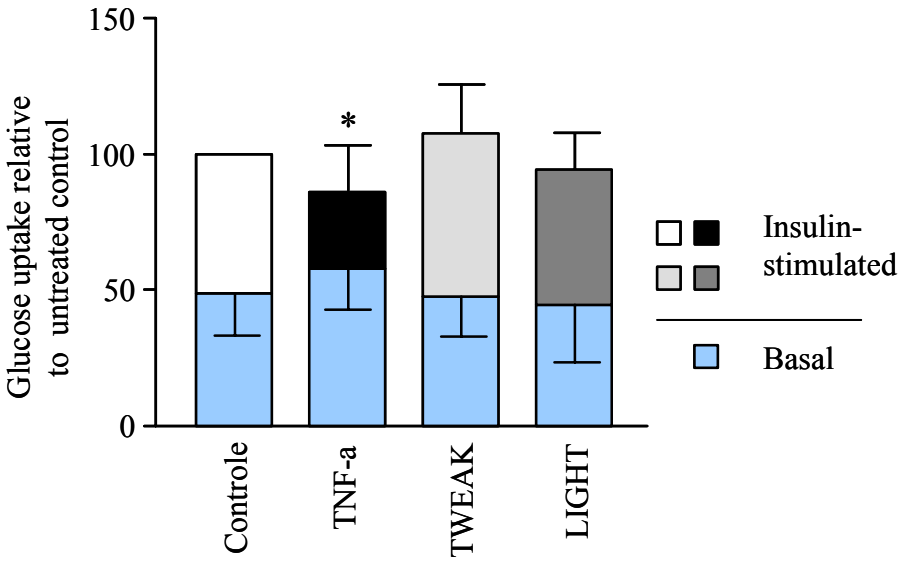


Figure 24: Glucose uptake in human primary subcutaneous adipocytes differentiated for 14 days. Basal glucose uptake without insulin-stimulation and insulin-stimulated glucose uptake was determined after 1 hour of incubation with the respective protein. n = 5. * $p < 0.05$ vs. insulin-stimulated control cells.

Insulin-stimulated glucose uptake was significantly reduced by about 45 % after one hour of incubation with TNF-α (Fig. 24) as described in the literature (96). Neither TWEAK nor LIGHT had a significant influence on basal or insulin-stimulated glucose uptake (Fig. 24).

4.3.2. AKT Western blot

AKT/Proteinkinase B is an important signal mediator for glucose uptake in adipocytes and phosphorylation at the serine residue 473 (Ser473) is a crucial step for AKT activation by insulin (240). Freshly harvested mature adipocytes were incubated with 1000 ng/ml TWEAK or 100 ng/ml LIGHT for 15 minutes. After 5 minutes of incubation with 10^{-7} M insulin, cells were lysed on ice for western blot experiments. Wortmannin at 100 nM was used as a control for the inhibition of insulin-stimulated AKT phosphorylation with an incubation time of 3 hours prior to insulin stimulation (241). Lysates were loaded on SDS polyacrylamide gels after standardization for protein content. Insulin stimulated AKT serine phosphorylation from undetectable levels to a clearly visible signal. Wortmannin inhibited AKT serine phosphorylation (Fig. 25 A). TWEAK and LIGHT did not affect insulin-stimulated AKT

serine phosphorylation when compared to the control. Total AKT protein levels were comparable in all conditions and used for correction (Fig. 25 B).

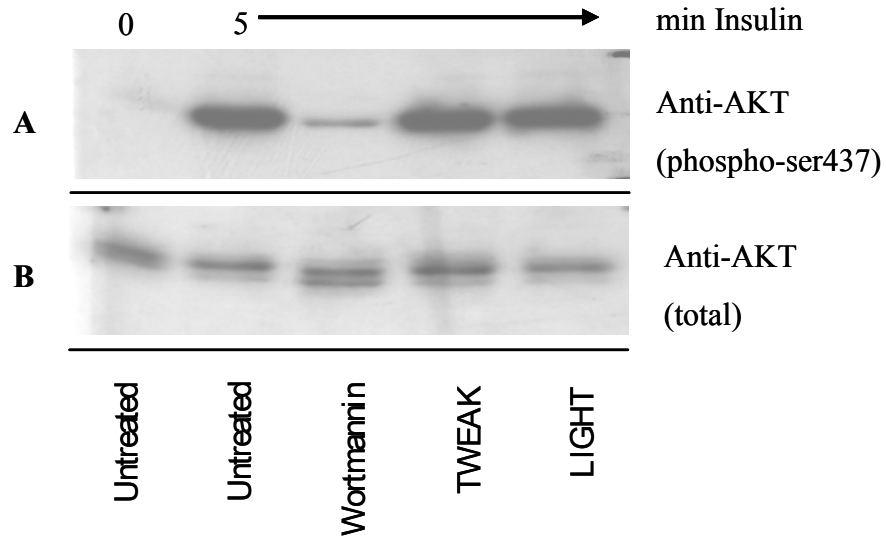


Figure 25: AKT Western blot in human mature adipocytes. Cells were incubated for 3 hours with 100 nM Wortmannin or for 15 minutes with 1000 ng/ml TWEAK or 100 ng/ml LIGHT followed by 5 minutes of stimulation with 10^{-7} M insulin. Results are representative of three independent experiments.

4.3.3. Lipolysis

Lipolysis is a critical step in the energy homeostasis maintained by adipose tissue (242). Fat cells degrade triglycerides via activation of HSL. The resulting monoacylglycerol is further cleaved into glycerol and a free fatty acid moiety by monoacylglyceride lipase. Glycerol can not be reused by the adipocyte for triaclyglycerol synthesis as fat cells do not express glycerol kinase (238). Produced glycerol is therefore transported to the surrounding cell environment and, subsequently, reutilized by the liver for gluconeogenesis. The amount of glycerol in the cell culture supernatant therefore represents the lipolysis rate in adipocytes. TNF-α and the ß-adrenergic agonist isoproterenol are well known stimulators of lipolysis (26;100;243). Adipocytes differentiated for 14 days were incubated with 1000 ng/ml TWEAK or 100 ng/ml LIGHT for 48 hours. 100 ng/ml TNF-α and 1 µM isoproterenol were in parallel incubated as positive controls. The cell culture supernatant was harvested and the number of viable cells was determined with the MTS assay. Glycerol was determined in the cell culture supernatant luminometrically by adenosintriphosphate (ATP) consumption of glycerol kinase. TNF-α and isoproterenol stimulated lipolysis more than twofold after a 48 hour incubation (Fig. 26). Neither TWEAK at 1000 ng/ml nor LIGHT at 100 ng/ml had any effect on lipolysis in differentiated adipocytes under the chosen conditions.

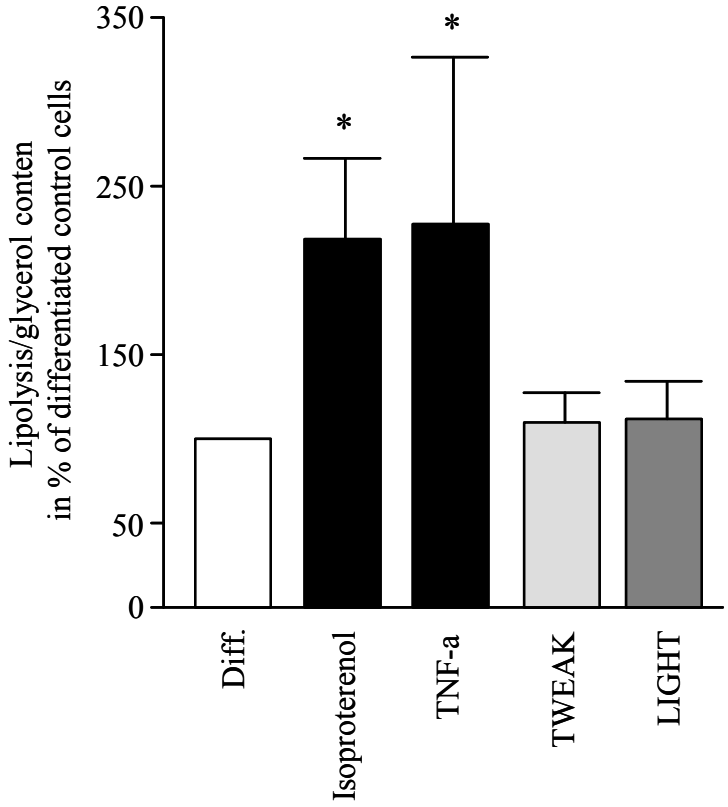


Figure 26: Lipolysis, i.e. glycerol content in the supernatant in human primary sc adipocytes differentiated for 14 days and incubated with the respective protein (100 ng/ml TNF-α or LIGHT or 1000 ng/ml TWEAK) or with 1 µM isoproterenol for 24 hours. n = 7, results for each donor were obtained in duplicate. * $p < 0.05$ vs. untreated, differentiated control (Diff.).

4.4. TWEAK and LIGHT in human serum and adipose tissue

TWEAK and LIGHT mRNA and protein have been found in humans in various tissues and cells, and their roles in numerous disease processes are described in the introduction. Yet only little information is available on the role of TWEAK and LIGHT in physiological processes. The presence of both proteins at mRNA and protein levels was therefore investigated in human adipocytes and preadipocytes and in human serum samples.

4.4.1. Real-time PCR in primary culture

RNA was harvested from undifferentiated preadipocytes on day 4 and from differentiated adipocytes on day 14. RNA was subscribed to cDNA and PCR was performed for 40 cycles with Taqman® gene expression assays for amplification of TWEAK and LIGHT. The primers and probes used in these experiments are shown in the appendix. cDNA results were standardized for GAPDH values measured in all samples. Results (Fig. 27) for TWEAK are

presented relative to the positive control of HepG2 cells, and results for LIGHT relative to the positive control of ionomycin/PMA-stimulated Jurkat cells (122;191).

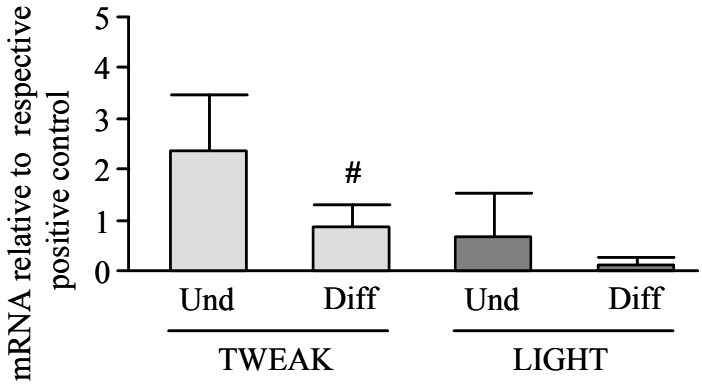


Figure 27: RT-PCR for TWEAK and LIGHT in undifferentiated (Und) and differentiated (Diff) human primary sc preadipocytes. Results are standardized for GAPDH content and are presented relative to the respective positive control (data not shown): HepG2 cells for TWEAK and ionomycin/PMA-stimulated Jurkat cells for LIGHT. n = 4, results were obtained in duplicate for each donor. # $p < 0.06$ vs. undifferentiated cells.

TWEAK mRNA expression decreased during the process of differentiation ($p < 0.06$). No significant differences between undifferentiated preadipocytes and differentiated adipocytes were obtained for LIGHT mRNA expression due to a high standard deviation, but there was a similar trend as for TWEAK.

4.4.2. Western blot for TWEAK and LIGHT in primary culture of human preadipocytes and adipocytes

The soluble, shed forms of TWEAK and LIGHT produce bands of approximately 18 kDa and 25 kDa, respectively, in Western blots (134;244). Human primary subcutaneous preadipocytes were analyzed for the presence of TWEAK and LIGHT in Western blots with respective antibodies. Undifferentiated preadipocytes before and after isolation of macrophages with magnetic beads were available from two donors. TWEAK human recombinant protein was used as positive control and was detectable with two bands (Fig. 28). TWEAK was visible in both donors as a weak band of approximately 20 kDa, i.e. its monomeric soluble form, and was also visible at around 70 kDa with a strong signal in one (Fig. 28) and a weak signal in the second donor (not shown). LIGHT was not visible at the expected size of the monomer in both donors, yet a band at around 65 kDa was detectable in both donors; this band increased in strength after macrophage removal (Fig. 28). In both gels, multiple bands of cleavage

products were observed. In another set of three donors, TWEAK and LIGHT protein expression was investigated with Western blots in undifferentiated and differentiated subcutaneous cells as well as in freshly harvested mature adipocytes.

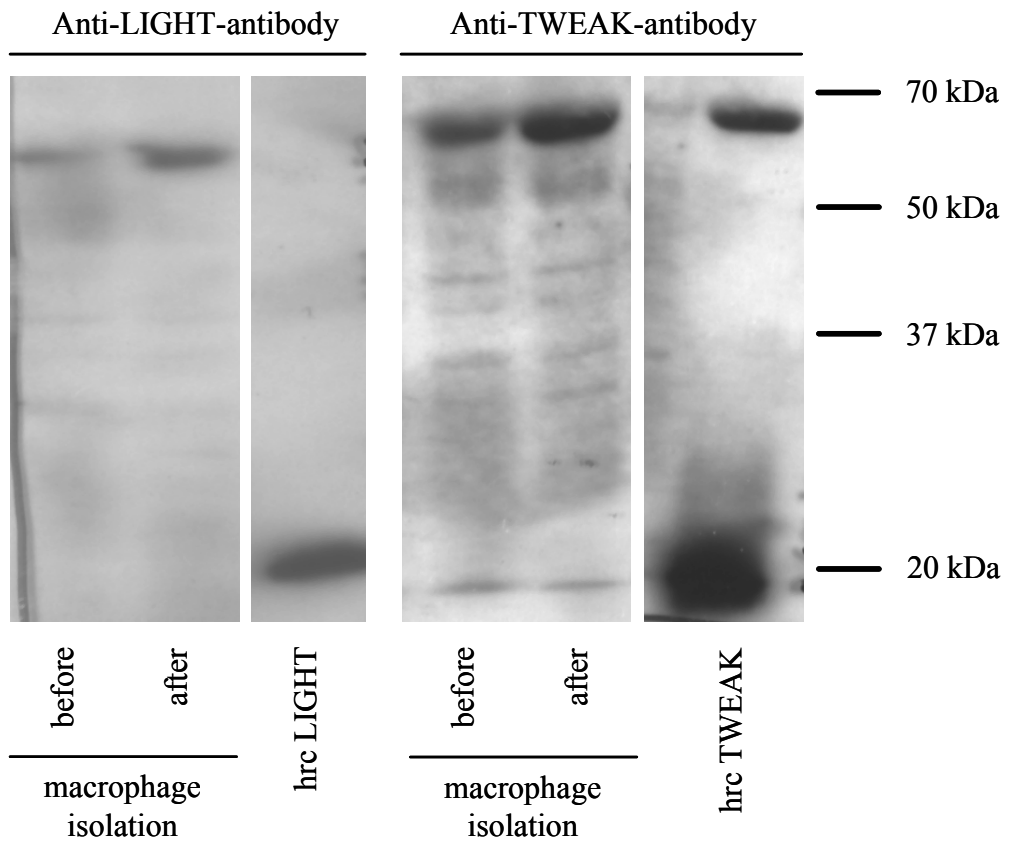


Figure 28: Western blot for TWEAK and LIGHT in human primary subcutaneous preadipocytes before and after isolation of macrophages by antibody-coupled magnetic beads. Human recombinant (hrc) soluble proteins were used as positive control.

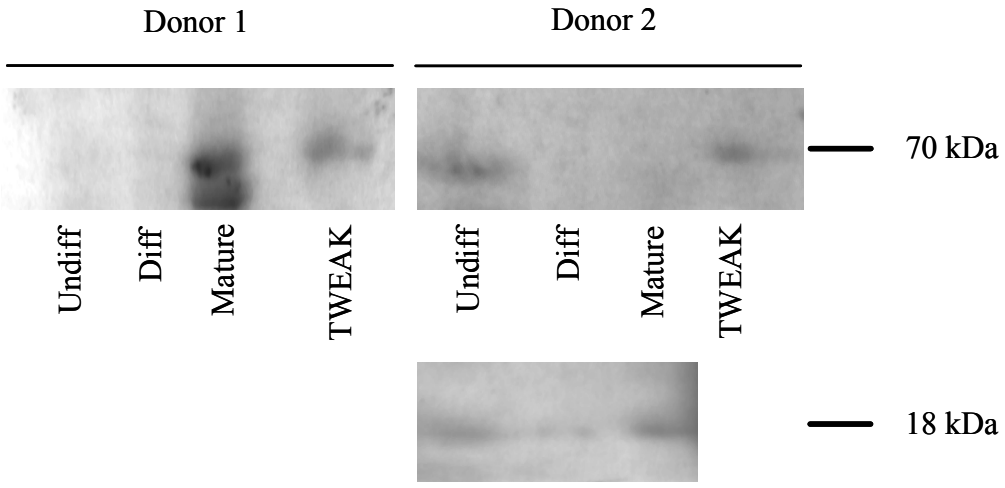


Figure 29: Western blot for TWEAK in human primary preadipocytes (Undiff), differentiated adipocytes (Diff) and freshly harvested mature adipocytes (Mature). Human recombinant soluble TWEAK was used as positive control.

TWEAK was visible as weak band at the expected size of the monomer in all three cell types (Fig. 29). As in figure 28, multiple bands of cleavage products were detectable in all three donors (not shown). A clear signal for the trimeric band in mature adipocytes could be observed in one donor, while in another donor a weak band was detectable in preadipocytes (Fig. 29). As expected from the results in undifferentiated preadipocytes (Fig. 28), LIGHT protein expression was not visible at the expected monomeric size in all three donors (not shown).

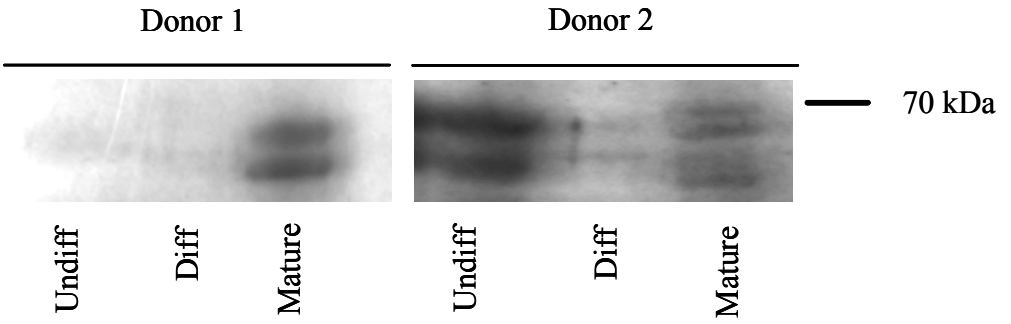


Figure 30: Western blot for LIGHT protein expression in undifferentiated preadipocytes (Undiff), differentiated adipocytes (Diff) and freshly isolated mature adipocytes.

A band at the size of the trimer was clearly visible in mature adipocytes in one donor (Fig. 30), but only weakly visible in the two other donors. Weak bands were visible in undifferentiated preadipocytes in all donors as well as multiple cleavage bands in all three cell types (not shown). To conclude, TWEAK and LIGHT are expressed in preadipocytes and adipocytes at different levels varying between donors and cells differentiation status.

4.4.3. Serum levels

TWEAK and LIGHT fasting serum levels were determined in normal weight and obese patients by ELISA. TWEAK was measurable at values of around 100 pg/ml in the serum of normal-weight (BMI below 25 kg/m^2) donors (Fig. 31). A significant decrease in TWEAK serum levels to around 80 pg/ml in obese, non-diabetic patients could be observed. The decrease from normal-weight to obese patients was about the same for obese patients with diabetes. No difference was noticed between obese patients with and without diabetes. LIGHT was found in the plasma of normal weight, non-diabetic donors with values at the detection limit and was even not detectable in some donors. LIGHT serum levels increased significantly in obese patients to values below 10 pg/ml. This increase was observed in obese patients without diabetes and with type-2-diabetes. No differences were detectable in obese

patients with and without diabetes. Sera from normal weight donors with diabetes were not investigated.

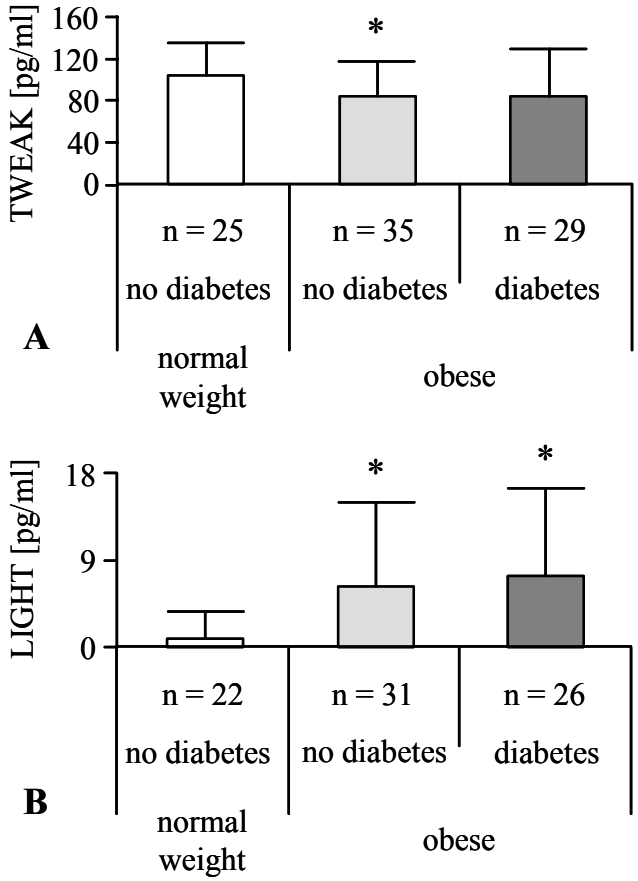


Figure 31: ELISA for TWEAK (A) and LIGHT (B) in fasting sera of three donor groups: normal weight donors and obese patients with and without type-2-diabetes. The group size n is marked in the graph. * $p < 0.05$ vs. respective normal weight control.

4.5. Receptors for TWEAK and LIGHT

TWEAK has been described to signal only via the Fn14 receptor. The presence of a second receptor has been proposed, yet this receptor has not been identified so far (112-116). In a FACS-based interaction study, the exclusive interaction of TWEAK with its receptor Fn14 has been confirmed, a binding pattern which is untypical for members of the TNFSF and the TNFRSF (73).

In contrast to the binding pattern of TWEAK, three different receptors have been reported to interact with LIGHT (161;163;167). HVEM and LTßR are cell surface proteins, whereas DcR3 is a soluble decoy receptor. These interactions and the overlapping receptor binding patterns with other TNFSF family members is typical for TNFSF/TNFRSF members. Binding

of LIGHT to its three proposed receptors was shown in the above mentioned FACS-based interaction study (73).

4.5.1. Real-time PCR in primary culture

First, receptor expression was investigated at the mRNA level. RNA was harvested from differentiated adipocytes on day 14 and from undifferentiated preadipocytes on day 4. After transcription of RNA to cDNA, PCR was performed for 40 cycles with primers for human Fn14, lymphotoxin ß receptor and HVEM as described under methods. Results were standardized for GAPDH values measured in parallel for all samples. The receptor for

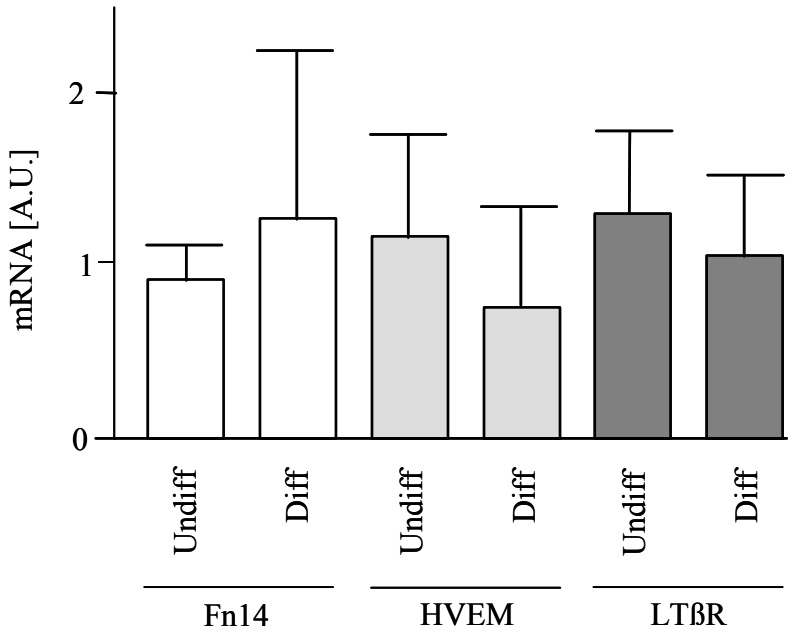


Figure 32: Real time-PCR for the receptors of TWEAK and LIGHT. cDNA of undifferentiated preadipocytes (Undiff) and differentiated adipocytes (Diff) was analyzed with 40 cycles of PCR with the respective primers. Results are corrected for GAPDH values. n = 4.

TWEAK and both receptors for LIGHT were expressed in the stromal-vascular fraction of adipose tissue consisting mainly of undifferentiated preadipocytes and in differentiated adipocytes. No significant differences were observed between undifferentiated and differentiated cells in the expression of all three receptors (Fig. 32).

4.5.2. FACS analysis of receptors

To study receptor expression on the cell surface, FACS experiments were performed in undifferentiated preadipocytes on day 0 and during differentiation on day 10. To detect

surface expression of Fn14, the commercially available, recently developed specific antibody ITEM-4 was applied (121).

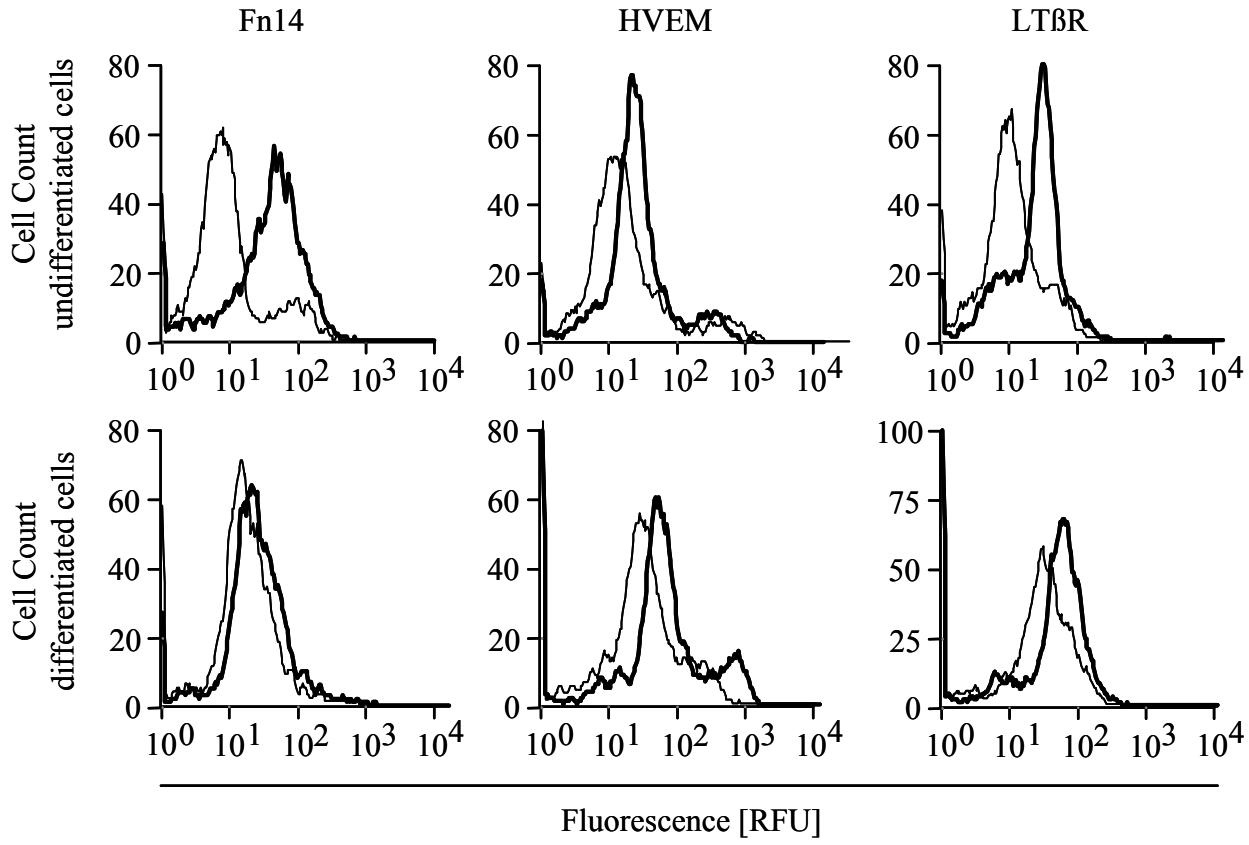


Figure 33: FACS analysis of TWEAK and LIGHT receptors. Antibodies against the Fn14, the LTßR and the HVEM were used for the detection of receptor cell surface expression. n = 3, results are representative of three donors. Isotype controls are presented as thin, specific antibodies as thick line.

Furthermore, binding of commercially available, specific antibodies to the LIGHT receptors LTßR and HVEM was analyzed on undifferentiated and differentiated primary cells by FACS analysis.

Table 3: Median cell count from FACS analysis for three donors presented as quotient from results for specific antibody and the respective isotype control in undifferentiated (Undiff) preadipocytes and differentiated (Diff) adipocytes.

	Fn14		HVEM		LTßR	
	Undiff	Diff	Undiff	Diff	Undiff	Diff
Donor 1	3.4	1.5	2.5	2.4	2.9	1.8
Donor 2	3.7	2.4	1.6	2.0	3.4	1.9
Donor 3	3.8	1.0	2.0	1.8	2.5	1.8

Cells were incubated as described in the methods with the respective first antibody and a fluorescence-labeled second antibody. Results are presented in relation to the adequate isotype control (Fig. 33). As displayed, all three receptors were detected in undifferentiated primary preadipocytes. HVEM showed a trend towards a weaker expression in comparison to Fn14 and LTßR. Fn14 and LTßR expression decreased during cell differentiation, whilst no gross alterations were visible for HVEM. The median cell counts of undifferentiated and differentiated cells are summarized in table 3. Quotients were calculated from the values of relative fluorescence units of the specific antibody. Fn14 expression decreased in all three donors as revealed by the diminished quotients of specific antibody and istoype control. HVEM expression showed less marked alterations: it slightly decreased in two donors, yet slightly increased in one donor. LTßR surface expression was reduced in all three donors. To summarize, all three receptors are present on human primary preadipocytes and on differentiating adipocytes to varying degrees.

4.5.3. Agonistic antibodies

To verify receptor expression and to ensure biological activity of receptors, differentiating preadipocytes were cultured under adipogenic conditions for 8 days in the presence of increasing concentrations of specific agonistic receptor antibodies. An antibody agonistic for the TWEAK receptor has been recently described to cause cytotoxic effects in a mouse T lymphoma cell line over-expressing human Fn14 (121). The antibody agonistic for the lymphotoxin ß receptor is commercially available and is specified to inhibit proliferation of the human coloncarcinoma cell line HT29 in an agonistic manner when immobilized (245). To our knowledge, no agonistic antibody was available for the HVEM while these data were generated. Preadipocytes were incubated with the respective isotype control in parallel to specific antibodies. This isotype control was only investigated in one concentration, i.e. the highest concentration used for the respective agonistic antibody.

As seen in figure 34, TWEAK at 1000 ng/ml and LIGHT at 100 ng/ml inhibited adipose differentiation of human primary preadipocytes when cultured in the presence of either protein for 8 days. The agonistic ITEM-1 antibody against human Fn14 displayed a concentration-dependent inhibitory effect on preadipocyte differentiation (Fig. 34 A). When compared to the respective isotype control at a concentration identical to the highest concentration used for the specific antibody, the antibody was already effective at a concentration of 1 µg/ml. Effects comparable to the action of 1000 ng/ml TWEAK were achieved with an antibody concentration of 5 µg/ml. The agonistic antibody against human

LTßR also displayed a concentration-dependent inhibitory effect on preadipocyte differentiation when applied for the whole period of differentiation (Fig. 34 B).

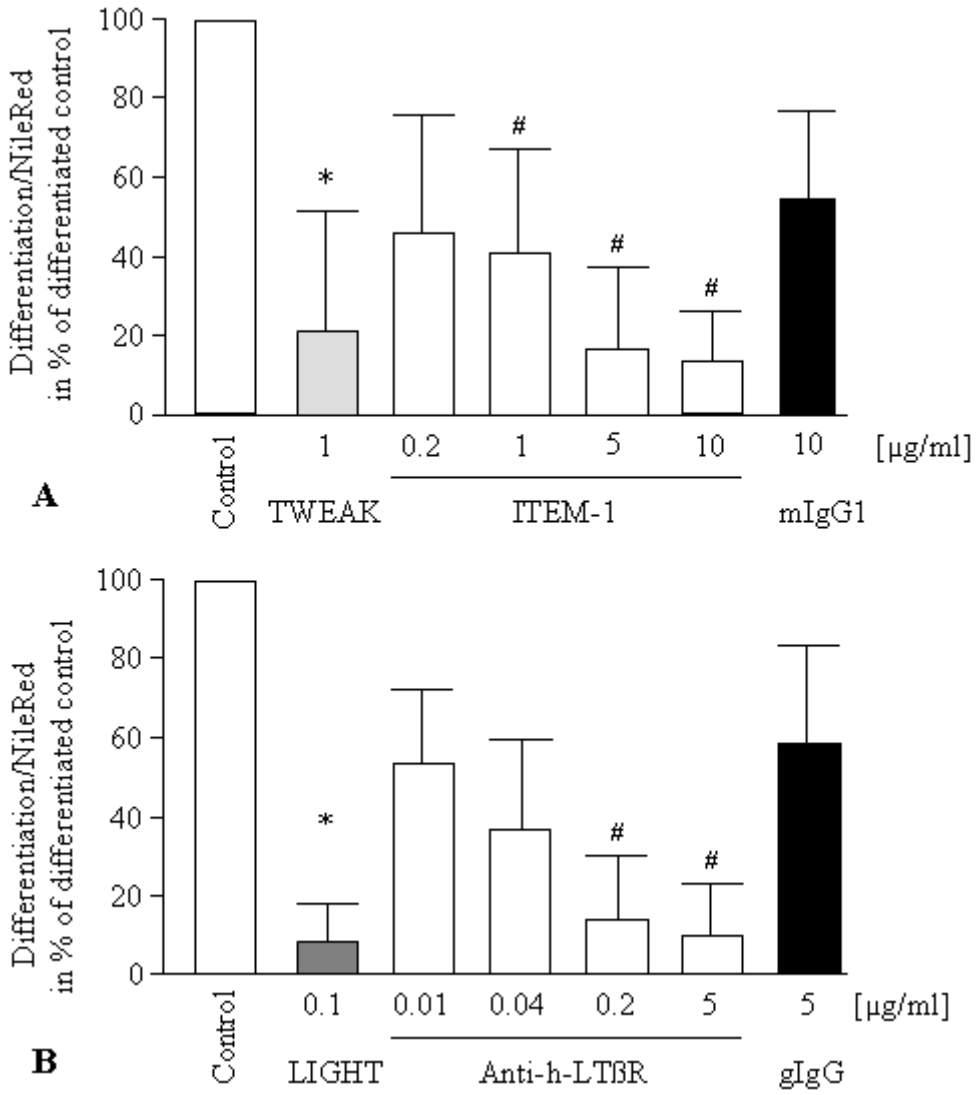


Figure 34: Concentration-dependent effects of agonistic antibodies against the Fn14 (A) or the LTßR (B) obtained with Nile Red assay on day 8. n = 5, results were an average of three wells for each donor for the LTßR and six wells for ITEM-1. * $p < 0.05$ vs. differentiated, untreated control. # $p < 0.05$ vs. isotype control mouse IgG1 (mIgG1) or goat at indicated highest concentration.

This effect was comparable to the effect of 100 ng/ml LIGHT starting from a concentration of 0.2 µg/ml. Significance in comparison to the respective isotype control was achieved at 0.2 and 5 µg/ml. It can be concluded from these data that Fn14 and LTßR are sufficient for the delivery of inhibitory effects of TWEAK and LIGHT, respectively, on differentiation of human primary preadipocytes.

4.5.4. Antagonistic antibodies

To verify that the receptors described above are responsible for the effects mediated by TWEAK and LIGHT, differentiating preadipocytes were co-incubated with blocking, antagonistic receptor antibodies and with the respective protein for 8 days under adipogenic conditions. Due to technical limitations, these experiments were carried out in the SGBS cell strain. ITEM-2 antibody against human Fn14 has been described to have a very weak agonistic effect, but to have a pronounced blocking effect when co-incubated with TWEAK (121). Differentiating cells were co-incubated in parallel with the isotype control mouse IgA and TWEAK to rule out unspecific antibody effects and were incubated with protein or antibody separately. In case of human LIGHT, a blocking antibody for the human HVEM was available, but, to our knowledge, no antagonistic antibody was commercially available against the human LTßR.

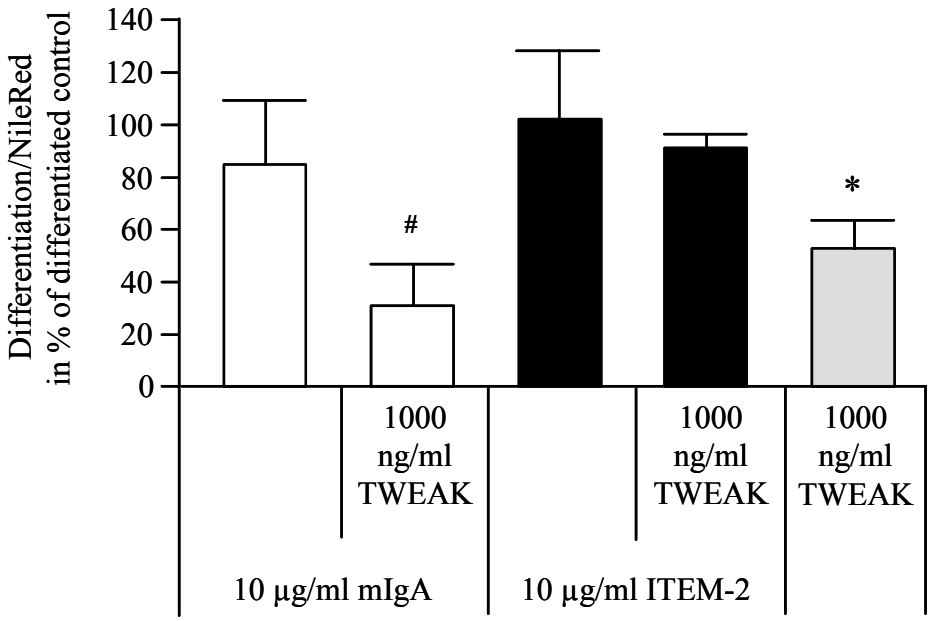


Figure 35: Effect of the blocking, antagonistic antibody ITEM-2 on SGBS preadipocyte differentiation. Cells were differentiated for 8 days in the presence of ITEM-2 in the presence or absence of TWEAK. The same setting was applied in parallel for the isotype control. n = 3, results are the mean of three wells per generation of SGBS. * $p < 0.05$ vs. differentiated, untreated control. # $p < 0.05$ vs. exclusive antibody incubation.

Nevertheless, one polyclonal antibody against the human LTßR was tested for its ability to block LIGHT binding to LTßR, but was shown to be inefficient. As can be seen in figure 35, TWEAK significantly inhibited differentiation of human SGBS preadipocytes at the applied concentration of 1000 ng/ml when present for the whole differentiation period of 8 days. Under equal conditions, the effects of ITEM-2 and the isotype control were small and not

significant. Co-incubation of TWEAK with the isotype control inhibited differentiation even stronger than TWEAK alone. Differentiation was almost unchanged in cells co-incubated with ITEM-2 and TWEAK. It can be concluded from this result that the antagonistic antibody ITEM-2 partly blocked TWEAK binding and the subsequent effects in human SGBS preadipocytes. This allows the conclusion that Fn14 is a biologically active receptor for TWEAK in this human cell system. Unfortunately, no such results could be provided for the LIGHT receptors. Contrary to its proposed antagonistic properties, the antibody against the human HVEM did not block LIGHT-induced inhibition of differentiation in human SGBS preadipocytes kept under adipogenic conditions in the presence of receptor and protein for 8 days (data not shown). Likewise, no blocking effects could be observed with the antibody against the human LTßR, as this receptor antibody even exerted agonistic activity. For this reason, no information could be obtained about the role of the two LIGHT receptors in the human preadipocyte cell system.

4.6. Signaling pathways

TWEAK and LIGHT have been described to activate the MAPK and the NF-κB pathway in different cellular systems besides other signaling molecules (145;156;202;220). Therefore, the effects of TWEAK and LIGHT on these two major pathways were investigated in human primary adipocytes.

4.6.1. Effects of TWEAK and LIGHT on MAPK

TWEAK is known to activate JNK and Erk-1/-2 in RAW cells, and TWEAK-stimulated IL-6 release from astrocytes is mediated via p38-MAPK activation (116;145). LIGHT was found to activate JNK in HeLa cells and Erk-1/-2 in DC, and p38-MAPK activation mediated by LIGHT is in part responsible for the proteins apoptotic effects on HT-29 cells (217;218;220). To gather more information about MAPK activity in human primary cells following incubation with TWEAK or LIGHT, adipocytes differentiated for 14 days were incubated with 1000 ng/ml TWEAK or 100 ng/ml LIGHT for 30 minutes. Cells were incubated in parallel with 100 ng/ml TNF-α as positive control. Cell were lysed as described in methods, and phosphor-JNK, -Erk-2 and -p38-MAPK content was measured with the Bioplex bead-based luminescent technology. Total MAPK content was in parallel determined in all cell lysates to adjust for total protein expression. Content of phospho-protein was related to total protein content. Results are presented in relation to the positive control TNF-α.

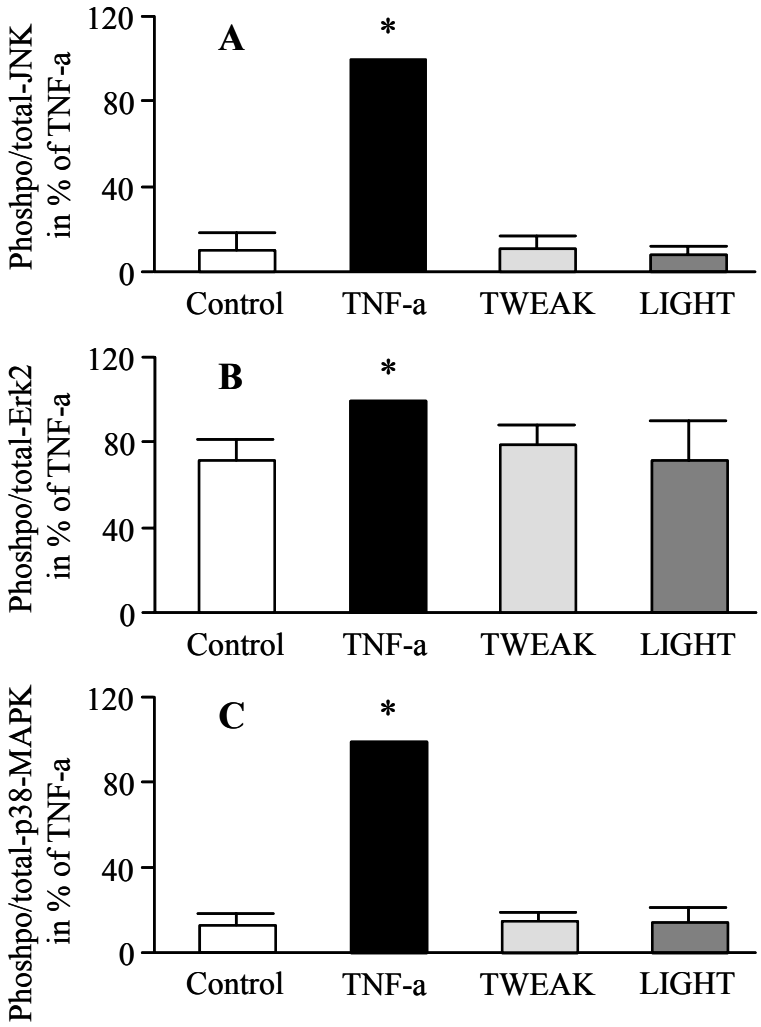


Figure 36: Effects of TWEAK and LIGHT on MAPK activity. Differentiated adipocytes were incubated for 30 minutes with 100 ng/ml TNF-α, 1000 ng/ml TWEAK or 100 ng/ml LIGHT. Phospho- and total protein content of JNK (A), Erk-2 (B) and p38-MAPK (C) was determined. n = 6. * $p < 0.05$ vs. untreated, differentiated control.

As shown (Fig. 36), TNF-α stimulated JNK and p38-MAPK phosphorylation about tenfold, whereas Erk-2 phosphorylation was stimulated only weakly. Neither TWEAK at 1000 ng/ml nor LIGHT at 100 ng/ml altered JNK, Erk or p-38-MAPK phosphorylation and activation. Thus, both proteins did not signal in human primary differentiated adipocytes via the MAPK pathways under the chosen conditions.

4.6.2. Effects of TWEAK and LIGHT on NF-κB

Activation of the classical NF-κB pathway has been described for TWEAK and LIGHT in several systems, e.g. for TWEAK in Kym-1 and HUVEC cells and for LIGHT in T-cells and

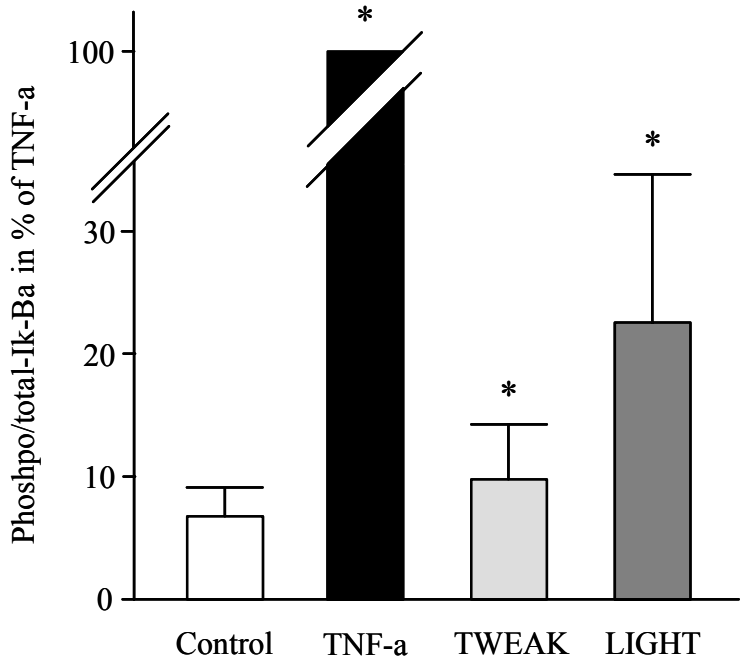


Figure 37: Iκ-Bα phosphorylation in differentiated adipocytes after 30 minutes of stimulation with 1000 ng/ml TWEAK, 100 ng/ml LIGHT or 100 ng/ml TNF-α as positive control. n = 6. * $p < 0.05$ vs. differentiated, untreated control.

in primary hepatocytes (107;112;176;183). Activation of the alternative NF-κB pathway, i.e. IKK-α recruitment or RelB activation, has been depicted for TWEAK in rat fibroblasts and for LIGHT in HeLa cells (157;217). To gather more information about the signaling pathways activated in adipocytes, cells differentiated for 14 days were incubated with 1000 ng/ml TWEAK or 100 ng/ml LIGHT for 30 minutes. Cells were incubated in parallel with 100 ng/ml TNF-α. Activation of the canonical NF-κB pathway through TNF-α has been described, yet activation of the alternative pathway seems to be limited to certain receptors of the TNFRSF (80). Cell lysates were obtained as described in methods. Iκ-Bα phosphorylation was investigated as marker for the activation of the classical NF-κB pathway. TNF-α strongly stimulated Iκ-Bα phosphorylation in human primary adipocytes (Fig. 37). Stimulation by TWEAK (approximately 1.5-fold) and LIGHT (approximately 3-fold) was weak compared to the stimulation seen with TNF-α. To further verify the activation of the NF-κB pathway in differentiated adipocytes, another set of donor cells was incubated with the respective proteins as described for the Iκ-Bα set of experiments. Combined cellular and nuclear lysates were

generated as described in methods. Activation of the different NF-κB subunits was determined by ELISA.

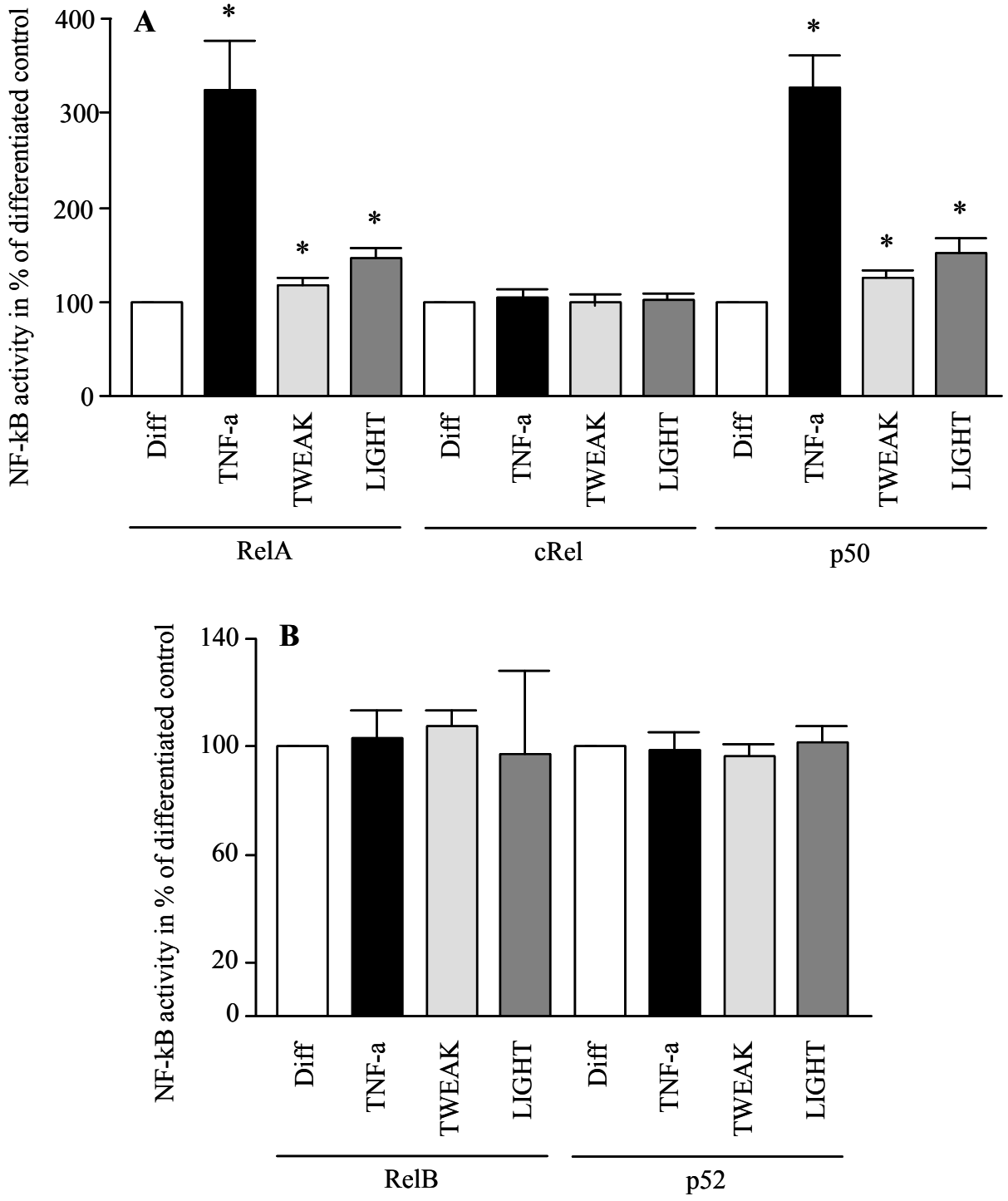


Figure 38: ELISA for NF-κB activity. Differentiated adipocytes on day 14 were incubated for 30 minutes with 1000 ng/ml TWEAK or 100 ng/ml LIGHT or 100 ng/ml of the positive control TNF-α. Combined cellular and nuclear extracts were assayed for NF-κB binding activity for the classical (A) and the alternative (B) pathway. n = 5. * $p < 0.05$ vs. differentiated, untreated control.

The ELISA applied immobilized NF-κB consensus binding sites and labeled antibodies specific for the different subunits. As observed for Iκ-Bα phosphorylation, activation of the classical NF-κB pathway, i.e. RelA and p50, was pronounced with TNF-α (Fig. 38 A), but no activation of cRel or of the alternative NF- κB pathway was observed with TNF-α (Fig. 38 B). As expected from the Iκ-Bα results, TWEAK and LIGHT also stimulated the classical NF-κB pathway (Fig. 38 A). In the presence of TWEAK, RelA and p50 activities increased by around 20 % and 25 %, respectively. LIGHT was more potent than TWEKA, RelA and p50 activities increased about 45 % and 50 %, respectively. However, for both NF-κB subunits, TWEAK and LIGHT exerted much weaker effects than TNF-α, a result in accordance with the findings described above (Fig. 37). No activation of the canonical NF-κB pathway, i.e. RelB or p52 activation, was observed with TWEAK or LIGHT. In summary both, TWEAK and LIGHT, activate the classical NF-κB pathway in human primary differentiated adipocytes, yet this activation is weak in comparison to the activation caused by TNF-α.

4.7. Cytokine secretion

TWEAK and LIGHT have been investigated concerning their ability to stimulate the secretion of pro-inflammatory cytokines in different cellular systems. Four of the pro-inflammatory cytokines described in the literature to be expressed in adipose tissue were used to determine the effects of TWEAK and LIGHT on their secretion from preadipocytes and adipocytes. Furthermore, the two well-described adipokines adiponectin and leptin were investigated in their response to TWEAK and LIGHT.

4.7.1. Adipokines

Leptin is exclusively secreted by adipocytes (246). Leptin secretion increases with adipocyte differentiation (57;247). Serum leptin values are increased in obesity and this increase is potentially responsible for resistance to leptin occurring in parallel with increased leptin serum levels in obesity (248).

Adiponectin is another well characterized protein secreted by mature adipocytes. Adiponectin serum levels are decreased during human obesity resulting in insulin resistance and the metabolic syndrome (249). Adiponectin expression and secretion increases with differentiation from preadipocytes to adipocytes (232). To determine the effects of TWEAK or LIGHT on leptin or adiponectin secretion, adipocytes differentiated for 14 days were incubated with 1000 ng/ml TWEAK or 100 ng/ml LIGHT for 24 hours. Levels of leptin and

adiponectin in the cell culture supernatants were determined by ELISA as described in methods.

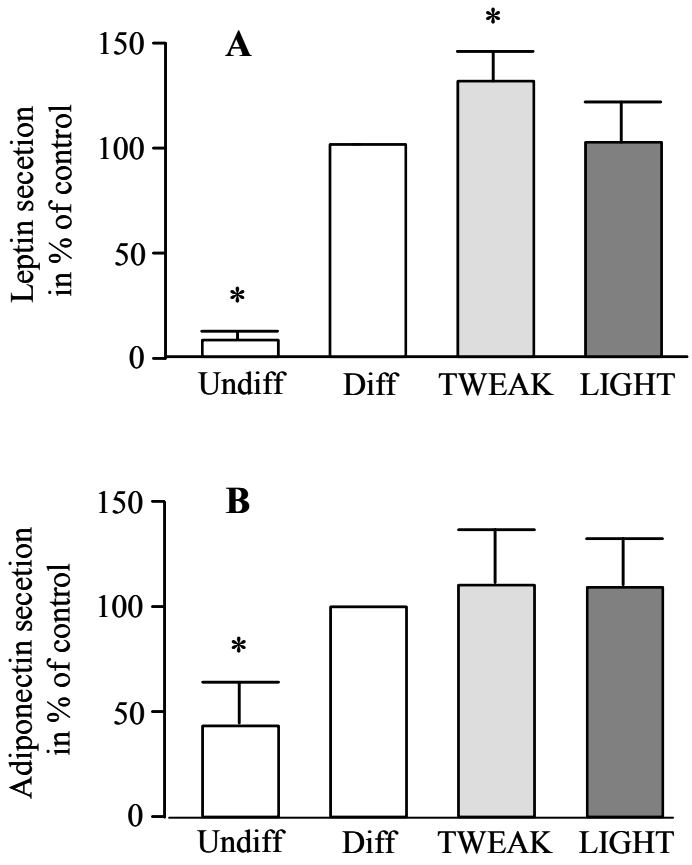


Figure 39: Leptin (A) and adiponectin (B) secretion from human primary differentiated (Diff) and undifferentiated (Undiff) adipocytes. Leptin and adiponectin content in the supernatant of differentiated cells incubated with 1000 ng/ml TWEAK or 100 ng/ml LIGHT for 24 hours was determined with ELISA. n = 5, values for each donor were determined in duplicate. * p 0.05 vs. differentiated untreated control.

Levels of adiponectin and leptin were approximately 4 ng/ml and 120 pg/ml, respectively, in untreated control cells. LIGHT had no effect on adiponectin or leptin secretion in differentiated human primary preadipocytes (Fig. 39). Contrary to this finding, although TWEAK also had no effect on adiponectin secretion, leptin secretion was significantly stimulated by 30 ± 14 % in human newly differentiated adipocytes.

4.7.2. Pro-inflammatory cytokines

IL-6 and RANTES have been chosen to determine the effect of TWEAK and LIGHT on secretion of typically pro-inflammatory cytokines. Secretion of both proteins was described to be stimulated by TWEAK in human dermal fibroblasts (148). Furthermore, TWEAK was

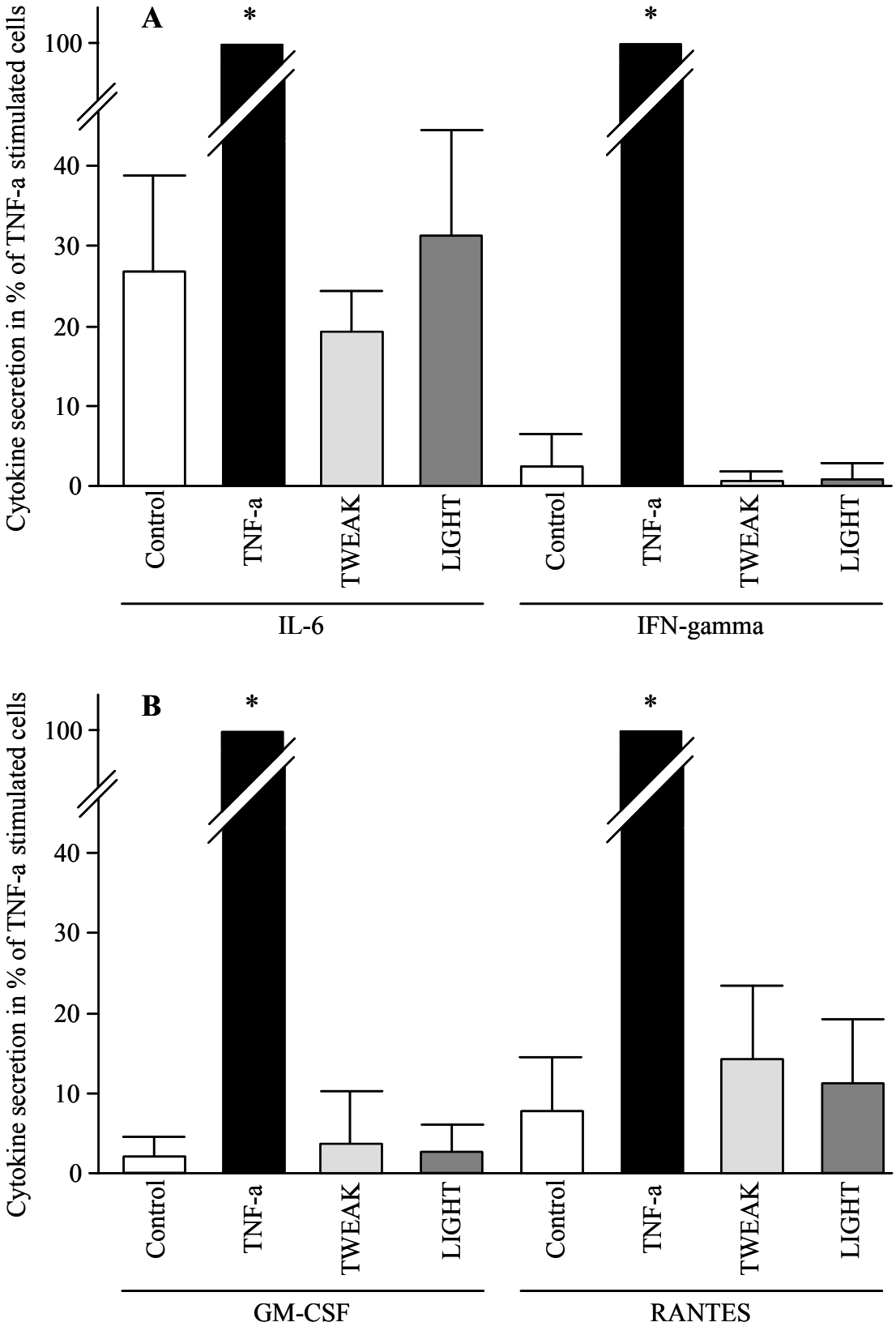


Figure 40: Secretion of pro-inflammatory cytokines from freshly isolated mature adipocytes. Cytokine content was determined with ELISA for IL-6 (A) and with the Bioplex bead-based multiplex technology for IFN-γ (A), GM-CSF and RANTES (B). n = 5. * $p < 0.05$ vs. untreated mature adipocytes.

found to stimulate GM-CSF secretion from human bronchial epithelial cells (115). A stimulation of IL-6 by LIGHT secretion has been found in primary macrophages from RA patients (250). GM-CSF and additionally IFN-γ secretion have been found to be stimulated by LIGHT in human T-cells. Furthermore, LIGHT stimulated RANTES release from a human RD cell line (202). To investigate the effects of TWEAK and LIGHT on the secretion of pro-inflammatory cytokines, human primary preadipocytes as well as freshly isolated mature adipocytes were incubated for 24 hours with 1000 ng/ml TWEAK, 100 ng/ml LIGHT or 100 ng/ml TNF-α as positive control. In freshly isolated human mature adipocytes, TNF-α had a pronounced effect on the secretion of all four investigated pro-inflammatory cytokines (Fig. 40), stimulating IL-6 secretion to approximately 2.5 ng/ml and IFN-γ secretion to about 220 ng/ml. GM-CSF secretion was stimulated by TNF-α to about 20 ng/ml and RANTES secretion to about 100 ng/ml. Neither TWEAK nor LIGHT had any significant effect on the secretion of the four pro-inflammatory cytokines. These results were confirmed in human primary preadipocytes (data not shown): TNF-α again had a pronounced and significant effect on cytokine secretion, while neither TWEAK nor LIGHT affected cytokine secretion under these conditions. In addition to the data described above, cells were also investigated for TNF-α secretion. No stimulation of TNF-α secretion was observed in both cells types, i.e. preadipocytes and mature adipocytes after 24 hours of incubation with 1000 ng/ml TWEAK or 100 ng/ml LIGHT (data not shown). In conclusion, TWEAK and LIGHT, contrary to TNF-α, had no stimulatory effect on the secretion of the selected pro-inflammatory cytokines in human primary preadipocytes or freshly isolated mature adipocytes.

5. Discussion

5.1. Screening principle

Using a high-throughput screening platform, two proteins with inhibitory effects on adipose differentiation, TWEAK (TNFSF12) and LIGHT (TNFSF14), have been found based on the inhibition of differentiation of murine 3T3-L1 preadipocytes into mature adipocytes. The effects of both proteins were subsequently examined in human primary preadipocytes. A total of approximately 200 000 clones from two human libraries were screened: a commercially available full length-clone collection and a metabolic library including, among others, cDNA from adipose tissues from lean and obese individuals. Supernatants of Hek293 cells over-expressing the respective proteins were transferred to differentiating 3T3-L1 preadipocytes to test their potential to inhibit adipose differentiation. With this approach, substances produced in a soluble form could be studied. The rationale behind this approach was to identify novel factors that are expressed in the human organism and that have a potential inhibitory effect on adipose tissue development not known so far. One advantage of this approach - in comparison to screening methods employing pooled biologicals from plants or other sources - is the fundamental compatibility with human physiology. In addition, the approach ensures that most of the soluble hits are proteins allocating through the vascular system to adipose tissue as the site of potential therapeutic application. Nevertheless, one disadvantage is the delivery of proteins that are expressed in the human body with certain, possibly unwanted biological effects. This is definitely true for one of the hits, TNF-α, confirming the effectiveness of the screening approach. TNF-α is a well known inhibitor of adipocyte differentiation, but is also described as having unfavorable effects in the human body, e.g. induction of insulin resistance (68;79;97-99). Therefore a thorough *in vitro* and *in vivo* investigation of proteins discovered in this assay – TWEAK and LIGHT - as well as an in-depth literature research for already known effects are mandatory prerequisites for further assessing potential therapeutic applications of the proteins in obesity. Thus, the action of both proteins on adipocyte metabolism needed to be investigated, since reduced staining of adipocytes with Nile Red, as applied in the high-throughput approach, may reflect heterogeneous alterations in cellular function. A decrease in the Nile Red signal may result from inhibited differentiation (as described above) with subsequent decrease in lipid accumulation. However, a decrease in the Nile Red signal may also be caused by a cytotoxic or apoptotic effect of either protein mediating a disappearance of differentiated preadipocytes. Cells undergoing a normal differentiation process might yet display a disturbed uptake of metabolites such as glucose or

an augmented catabolism, i.e. increased lipolysis. Hence, the mechanisms causing a reduced Nile Red signal need also to be thoroughly investigated.

5.2. The effects of TWEAK and LIGHT on human visceral and subcutaneous adipose tissue: general considerations

Visceral adipose tissue differs from subcutaneous adipose tissue in several aspects as described in the introduction. It is more closely associated with the development of metabolic disturbances, reacts differently to hormonal factors and also differs in various secretory aspects. It should be noted that most experiments for this thesis were conducted in human subcutaneous adipocyte cultures. This seemed rational for two main reasons. Firstly, subcutaneous adipose tissue constitutes more than 80 % of human WAT and is substantially expanded in human obesity. Secondly, the risk for comorbidities does not depend on the type of obesity - and therefore the type of adipose tissue - if the BMI exceeds a threshold of 30 kg/m^2. Furthermore, omental adipose tissue from healthy individuals is not available in sufficient amounts, since it is mostly obtained from abdominal surgery due to severe diseases. Subcutaneous adipose tissue is more easily available from healthy young patients and offers an ideal model to study the effects of new factors on adipose tissue function. To rule out potential differing effects of TWEAK and LIGHT on the two main types of adipose tissue, the results obtained in subcutaneous adipose tissue need to be confirmed in omental adipose tissue.

5.3. TWEAK and its effects on adipose tissue

In the present study, TWEAK was described to inhibit the differentiation of human subcutaneous preadipocytes in culture when present from the day of induction for the whole period of differentiation. The protein displayed a concentration-dependent effect with an EC50 of approximately 200 ng/ml, i.e. 10 nM, in human primary preadipocytes. Similar effects were found in human primary omental preadipocytes and in the human SGBS cell strain. Inhibitory effects on subcutaneous preadipocyte differentiation were also found when the protein was added on day four after induction and was present for the remaining eight days of differentiation, yet no inhibition was observed when the protein was added on day eight after induction of differentiation. These results indicate that TWEAK has an influence on early steps of initiation of adipocyte differentiation rather than on metabolic parameters of

lipid filling during terminal adipocyte maturation. Inhibitory effects of TWEAK on differentiation have been described for other cell types originating, like preadipocytes, from the mesenchymal lineage. TWEAK inhibited osteo- and chondrogenesis in suitable human *in vitro* systems (128) and it prevented differentiation in a human mesenchymal cell line and in murine primary myoblasts and stimulated their proliferation (144). Contrary to these findings, TWEAK was also shown to have a stimulatory effect on differentiation: osteoclastogenesis in a human macrophage/monocyte cell line was stimulated by TWEAK (116).

The activation of early key transcription factors of adipogenesis, such as PPAR-γ, leads to the emergence of the typical adipocyte phenotype and metabolism, and mature adipocytes lose the ability to proliferate (41;42). mRNA expression of the transcription factor PPAR-γ was investigated in differentiating preadipocytes cultured under adipogenic conditions for four days and in adipocytes cultured under the same conditions for 12 days. PPAR-γ mRNA expression was significantly reduced under both conditions, yet expression on day 4 was higher than in untreated cells or in cells treated with TNF-α. These results indicate that the differentiation process was initiated by the adipogenic conditions, but was inhibited partially by TWEAK via interference with early transcription factor expression. For two markers of mature adipocytes, HSL and adiponectin, mRNA expression was markedly decreased in cells differentiated in the presence of TWEAK for 12 days. Adipocyte differentiation was markedly inhibited when cells were incubated with TWEAK for the whole period of differentiation.

TWEAK had a pronounced stimulatory effect on preadipocyte proliferation comparable to EGF and stimulatory effects on proliferation were also found in endothelial cells and tumor cells; however, contrary to these results, TWEAK inhibited the proliferation of certain tumor cells and in murine mesangial cell lines (107;112;122;147). No effect on proliferation could be detected in human primary dermal fibroblasts (154). In the model of myogenic differentiation described above (144), TWEAK inhibited differentiation. In parallel, TWEAK was found to increase proliferation and expression of a number of genes involved in cell cycle progression. It was suggested that the disruption of the cell cycle arrest, as important first step of the differentiation process, is responsible for the inhibitory effect of TWEAK on cell differentiation. As described for the murine myoblasts, human primary preadipocytes also need to retract from cell cycle progression to enter the differentiation process (43;44). In the experiments presented in this thesis, TWEAK increased proliferation in the fibroblast-like human primary preadipocytes more than 10-fold in cells cultured in the absence of FBS and more than 3-fold in cells cultured in the presence of 5 % FBS. Further investigation is necessary to accurately determine the participation of both effects, the stimulation of

proliferation and the inhibition of differentiation, in the final appearance of fibroblast-like, spindle-shaped cells.

Besides the effects on proliferation and differentiation, TWEAK was initially described as a weak pro-apoptotic factor (107). In sensitive cell lines, the protein stimulates apoptosis on its own, but in most cellular systems stimulation of apoptosis requires co-incubation with sensitizers like IFN-γ (107;118). Human primary and SGBS preadipocytes can undergo apoptosis in the presence of TNF-α and the biosynthesis inhibitor CHX. Both cell types are rather insensitive to death receptor ligand-induced apoptosis, with mature adipocytes being more resistant to apoptosis than preadipocytes (236). Surprisingly, TWEAK alone caused a weak activation of caspase-3 and caspase-7 activation in differentiated adipocytes, but not in undifferentiated preadipocytes when given to the cells for 24 hours. The almost 4-fold increase in apoptosis is weak in comparison to the more than 50-fold increase of apoptosis following co-incubation of TWEAK and CHX. No differences were found between the results for TNF-α and CHX and the results for TWEAK and CHX. The weak pro-apoptotic effect of TWEAK in differentiated adipocytes needs to be confirmed *in vivo* and has to be taken into account when discussing a potential therapeutical application of the protein in obesity.

TWEAK has also been described to have cytotoxic effects in different cellular settings: it augments cell death in several tumor cell lines (108;118;134). In human primary proliferating and differentiating preadipocytes, TWEAK, after 24 hours of incubation at the high dose of 2000 ng/ml, did not alter LDH secretion as a measure of cell stress and cell lysis. The number of viable cells was slightly increased, and this increase was significant in differentiating cells. It can be concluded from these results that TWEAK exerts no gross cytotoxic effects on human primary preadipocytes and adipocytes *in vitro*. TWEAK´s weak apoptotic effect described above might be hidden behind its proliferation-promoting effect on preadipocytes that may be partly present among differentiated adipocytes.

After describing TWEAK´s potential effect on cell viability, proliferation and differentiation, the protein's effect on the metabolism of newly differentiated human adipocytes was examined. TNF-α was used as a reference because, within the TNFSF, it not only inhibits adipocyte differentiation, but also influences important metabolic pathways. Lipolysis and glucose uptake were investigated as two key metabolic characteristics of adipocytes. TNF-α is known to interfere with insulin-signaling (and consequently also with glucose uptake) and regulation of lipolysis (97;98;100). These well described effects were confirmed in the human primary adipocyte system. Contrary to the effects of TNF-α, TWEAK did not alter AKT phosphorylation and, as therefore expected, glucose uptake. Lipolysis was also unaffected.

These findings allow the conclusion that TWEAK does not alter important metabolic pathways in human adipocytes.

TWEAK binding to human primary preadipocytes and the presence of the Fn14 receptor have already been shown in the literature with hFc-TWEAK and the ITEM-4 antibody, respectively (144). Presence of the specific receptor in human primary preadipocytes and in differentiated adipocytes was confirmed by FACS analysis as well as at the mRNA level. Supporting information on Fn14 as the only functional receptor was obtained from experiments with agonistic and antagonistic antibodies. However, recent data suggested that a second, yet unknown receptor for TWEAK may exist (251). Treatment of cells with a specific agonistic antibody against the TWEAK receptor Fn14 under conditions similar to that for the treatment of TWEAK caused a concentration-dependent effect comparable to TWEAK incubation. These data confirmed the results from the FACS and mRNA experiments concerning receptor expression and led to the conclusion that Fn14 is sufficient to mediate TWEAK`s effects in human primary preadipocytes. To obtain more information on the proposed unique receptor, cells were co-incubated with TWEAK and an antagonistic receptor antibody specific for Fn14. This receptor antibody almost completely blocked TWEAK`s inhibitory effect on adipocyte differentiation and led to a Nile Red signal comparable to cells treated with the receptor antibody alone. These results may suggest that the Fn14 is sufficient and uniquely responsible for TWEAK´s inhibitory effects on adipocyte differentiation in the human system.

Concerning the signaling pathways employed by TWEAK only limited information is available so far as depicted in the introduction. Activation of both NF-κB pathways has been described (157), but in the human adipocyte system only a weak activation of the classical NF-κB pathway, i.e. RelA and p50, could be found. None of the three MAP kinases was activated by TWEAK under the chosen conditions. NF-κB activation through TWEAK has been connected to the pro-inflammatory effects, i.e. the secretion of pro-inflammatory cytokines mediated by the protein (115;147). Despite weak activation of the classical NF-κB pathway in human adipocytes and the known stimulatory effect of TWEAK on the secretion of some pro-inflammatory cytokines in other cell types (115;148), no stimulatory effect of TWEAK could be observed on the secretion of these cytokines in preadipocytes or freshly isolated mature adipocytes. Additionally, effects of TWEAK on the secretion of the adipokines adiponectin and leptin was investigated in differentiated adipocytes. While no effect on adiponectin secretion was noticed following a 24 hour incubation with TWEAK, leptin secretion was significantly increased by approximately 30 %. Taking the

hyperleptinemia and the leptin resistance into account, that both correlate with obesity (246), this effect is unfavorable and needs to be further investigated

The presence of TWEAK in human serum and in adipose tissue was investigated to obtain further information about the role of the protein in humans. To date, no information is available concerning the physiological role of TWEAK in the human body or the normal serum levels in healthy individuals. mRNA expression of TWEAK and its receptor Fn14 has already been investigated in human adipose tissue (136). Fn14 mRNA expression was only detectable in extremely obese patients, but neither TWEAK nor Fn14 expression correlated with BMI. Concerning these results, it has to be taken into account that TWEAK mRNA expression is not a suitable indicator for protein expression (139).

TWEAK mRNA expression was investigated in primary undifferentiated human preadipocytes and differentiated adipocytes. mRNA was clearly expressed in preadipocytes, and there was a trend towards a decreased expression in differentiated adipocytes. Since TWEAK mRNA expression is not sufficient for the prediction of protein expression, Western blots were performed. On the protein level, TWEAK could be detected in preadipocytes and in differentiated as well as in freshly isolated mature adipocytes. Bands varied in intensity between cell types and donors, and several cleavage bands were observed, the sizes of which were between that of the trimer and the monomer. From two sets of undifferentiated preadipocytes that were studied before and after macrophage isolation a major production of TWEAK in macrophages in the SVF is rather unlikely.

TWEAK serum levels were on average 105 ± 31 pg/ml in a group of 25 normal-weight individuals and were reduced to 84 ± 34 pg/ml in non-diabetic obese patients. These results are insofar surprising, since TWEAK is widely regarded as a pro-inflammatory cytokine, and obesity considered as a state of chronic, mild inflammation (6;139;252). In the obese state, serum values of IL-6 as typical pro-inflammatory cytokine correlate with the BMI (61). One would therefore expect an increase in TWEAK serum levels if adipose tissue is a major source for the cytokine and if the cytokine is causally involved in the pro-inflammatory consequences of obesity. Yet, TNF-α serum levels in obese individuals are rather low or at least not markedly increased despite the established pro-inflammatory nature of the protein and the well investigated increase of TNF-α expression in adipose tissue from obese subjects (79;83).

To summarize, TWEAK is a potent inhibitory factor for the differentiation of preadipocytes to adipocytes in the murine and the human system. These effects are probably mediated via an early interference with the differentiation process as indicated by a diminished mRNA

expression of PPAR-γ and typical late markers of adipocyte differentiation. In addition to the effects on cell differentiation, TWEAK has also a stimulatory effect on preadipocyte proliferation and weakly stimulates apoptosis in differentiated adipocytes. A cytotoxic effect of TWEAK could not be observed even at rather high concentrations of the protein. Contrary to findings in other cell systems or *in vivo*, TWEAK does not stimulate the secretion of pro-inflammatory cytokines from preadipocytes or adipocytes nor does it alter the secretion of the anti-inflammatory adipokine adiponectin. However, a weak stimulatory effect on leptin secretion was observed. No alterations in the important metabolic parameters glucose uptake and lipolysis could be found. Although weak activation of the classical NF-κB pathway, that is also present in adipocytes, has been described, no activation of the MAP kinases was found. TWEAK circulates in the serum of healthy individuals, and serum levels are decreased in obesity. mRNA and protein expression could be measured in cells of the SVF before and after differentiation as well as in freshly isolated mature adipocytes. The only receptor described so far for TWEAK, Fn14, is expressed at the mRNA and protein level in human adipose tissue and is responsible and sufficient for the TWEAK-mediated effects on adipose tissue.

5.4. LIGHT and its effects on adipose tissue

LIGHT was found in the present study as an inhibitor of preadipocyte differentiation. This inhibitory effect was exerted when the protein was present for the whole period of differentiation (starting with the day of induction) under adipogenic conditions, as well as when the protein was added on day 4 after induction of differentiation. Differentiation of human primary subcutaneous and omental as well as of SGBS preadipocytes was inhibited in a concentration-dependent manner. The EC50 was calculated as 10 ng/ml, i.e. 0.04 nM in human primary subcutaneous preadipocytes. Since the protein did not alter adipocyte differentiation when added on day 8, a role of LIGHT in the early steps of differentiation was suggested. As described for TWEAK, for LIGHT repressive effects were observed on mRNA expression of typical late markers of differentiated adipocytes as well as of the key transcription factor PPAR-γ, which strengthens a significant role of the protein for adipose differentiation. PPAR-γ mRNA was reduced to the level of undifferentiated control cells after a 12-day incubation of primary subcutaneous preadipocytes under adipogenic conditions. In contrast to the findings in this study, LIGHT was found to have augmenting effects on the differentiation processes in other cell types. Dendritic cell and B-cell maturation is stimulated by LIGHT (179;180). Treatment of PBMC with LIGHT induces osteoclastogenesis and the

protein stimulates changes in RD cells that are part of the muscle cell differentiation process (188;202). To my knowledge, no inhibitory effects of LIGHT on differentiation processes have been described so far.

As mentioned before, a decrease of the Nile Red signal may also be caused by direct effects of LIGHT on adipocyte metabolism. Yet, no effects of LIGHT on two crucial parameters of adipocyte metabolism, i.e. glucose uptake and lipolysis, could be observed. In one recent study, LIGHT was described to alter lipid metabolism when over-expressed in T lymphocytes (215). The exact mechanisms for this finding are not clear. A role for T-cell-bound LIGHT in hepatic lipase (HL) expression was suggested. Effects on adipose tissue or adipocytes were yet not investigated, since the effects of membrane-bound LIGHT on HL expression were considered to be sufficient to explain the disturbing effects on lipid metabolism

Another reason for a decreased Nile Red signal may be an attenuated viability of preadipose cells after incubation with the protein. Inconsistent effects of LIGHT on cell viability have been described in the literature. LIGHT from tumor-secreted microvesicles promotes T-cell apoptosis and causes apoptotic effects in several human tumor cell lines (163;244). Contrary to these finding, LIGHT displays anti-apoptotic or protective effects in synovial fibroblasts and hepatocytes under certain conditions (163;183;190;244). In the human adipocyte system, LIGHT did not influence cell viability as judged by LDH secretion as marker of cell membrane integrity and by the MTS assay for the number of viable cells. To further verify these findings, an apoptosis assay was performed. This assay did not reveal any influence of LIGHT on cell viability and therefore confirmed the results from the cytotoxicity assays. When LIGHT was co-incubated with CHX, effects comparable to those of TNF-α/CHX co-incubation were observed.

LIGHT was found to stimulate cell proliferation in different cellular systems. A stimulatory effect on T-cell proliferation has been described in many publications, and proliferative effects have been found in vascular smooth muscle cells (166;176;179;237). In human primary preadipocytes and adipocytes, LIGHT did not exert any influence on cell proliferation.

Expression of the receptors for LIGHT was reported in recent studies for different cell types. While HVEM is mainly expressed on cells of lymphoid origin, the LTßR is predominantly found on cells of stromal mesenchymal origin (170;171;178-180;182). HVEM and LTßR co-expression has been detected in various cell types (163;183-185). Expression of both receptors at the mRNA level was detected in human subcutaneous preadipocytes and differentiated adipocytes. FACS assays confirmed these findings at the level of cell-surface

protein expression. The presence of HVEM is surprising and may be attributed to immune cells present in adipose tissue (35;37;253). These assumption needs to be further verified, e.g. in co-staining FACS experiments for HVEM and immune cells markers. To gain more information about the role of the receptor, agonistic antibodies were applied to human primary subcutaneous preadipocytes under conditions similar to those for LIGHT protein incubation. Unfortunately, no agonistic antibodies were available at that time for HVEM. From the results with the agonistic LTßR antibody it is obvious that the LTßR is present on human primary subcutaneous preadipocytes and is sufficient to mediate the effects of LIGHT in these cells. siRNA experiments could provide more information about the contribution of both receptors to the effects of LIGHT in human adipose cells.

LIGHT mRNA expression was investigated in human primary subcutaneous preadipocytes and differentiated adipocytes. LIGHT was weakly expressed in differentiated adipocytes and slightly stronger in undifferentiated preadipocytes. Weak LIGHT mRNA expression was also found in other studies in peripheral lymphoid tissues and in major organs (161). Protein expression was described for activated T-cells and immature dendritic cells, but no protein expression was found in cells of stromal origin (161;163;170;176;177). Therefore, an expression in the stromal-vascular fraction of adipose tissue is surprising. Protein expression was investigated by Western blot analysis in preadipocytes of two donors before and after macrophage isolation. LIGHT was slightly stronger expressed after macrophage depletion. In the Western blot experiments, no bands of the monomer were detected, yet bands of the expected size of the trimer and possibly multiple cleavage products were observed. To obtain further information about the cells type expressing LIGHT in adipose tissue, FACS analysis after co-incubation with antibodies for specific immune cell markers and for LIGHT would be helpful.

LIGHT serum levels in humans have been investigated before. Serum levels were around 120 pg/ml in RA and around 170 pg/ml in OA patients (190). In a set of 12 healthy donors, LIGHT plasma levels varied between 5 and 75 pg/ml (187). No information is available on the body weight of these donors. In the present study, LIGHT serum levels in a group of 22 normal-weight, non-obese donors were found to be very low, with an average below 1 pg/ml. In obese patients, serum levels were significantly increased, with values of approximately 6 pg/ml independent of the presence or absence of diabetes. These values are still low compared to the serum values found in RA and OA patients. The increased values in obesity need to be further evaluated since LIGHT is widely described as a pro-inflammatory cytokine, and obesity is regarded as an inflammatory status (6;170). An unfavorable effect of

a possible pro-inflammatory protein in the already inflammatory state of obesity needs to be excluded.

To obtain more information on a possible pro-inflammatory role of LIGHT in adipose tissue, cytokine secretion was investigated in human primary preadipocytes and freshly isolated mature adipocytes following 24 hours of incubation with LIGHT. Despite the suggested pro-inflammatory properties of LIGHT described in previous publications, LIGHT did not alter secretion of the investigated cytokines under the chosen conditions. All four investigated cytokines had been described to be affected by LIGHT. IL-6 secretion was increased in macrophages from the synovial fluid of RA patients (250). In this model, LIGHT also stimulated TNF-α secretion, an effect not observed in human adipose tissue cells. IFN-γ and GM-CSF secretion was stimulated in murine primary T-cells (212). LIGHT was found to stimulate RANTES secretion from a human RD cell line (202). Therefore, the results for the effects of LIGHT on the secretion of pro-inflammatory cytokines in adipose cells differ from the obvious pro-inflammatory effects of the protein described in the literature. For the present study, effects of LIGHT on the secretion of two important adipokines were investigated in differentiated primary adipocytes. No effect on adiponectin or leptin secretion could be observed following 24 hours of incubation with LIGHT.

Finally, the signaling pathways addressed by LIGHT in human primary differentiated adipocytes were investigated. As mentioned for TWEAK, LIGHT was described to activate the classical and the alternative NF-κB pathway as well as the MAP kinases in different cellular systems (166;183;185;202;217;218;220). NF-κB activation by 10 ng/ml LIGHT was described to be weaker than activation induced by the same amount of TNF-α in synovial fibroblasts from RA patients (190). These results were confirmed in differentiated adipocytes following 30 minutes of incubation with LIGHT. While activation of the classical NF-κB pathway, i.e. RelA and p50 activation, was observed, no augmentation of the alternative NF-κB or the MAPK pathway was found under the chosen conditions in human adipocytes.

To summarize, LIGHT is highly effective in the inhibition of differentiation in human and murine preadipocytes. These effects on adipose differentiation are suggested to be caused by an alteration of the transcriptional program in adipocytes following the induction of differentiation. This is concluded from the decreased mRNA expression of the adipogenic key transcription factor PPAR-γ and typical late markers of differentiation. LIGHT does not alter cell viability or proliferation. Cytotoxic effects were not observed even at the high concentration of 2000 ng/ml. Most important, LIGHT did not alter the secretion of pro-inflammatory cytokines from preadipocytes and adipocytes nor glucose uptake and

lipolysis as crucial metabolic parameters of differentiated adipocytes. LIGHT modestly activates the classical NF-κB pathway in adipose tissue. Surprisingly, LIGHT protein expression was detected in preadipocytes and adipocytes, and serum levels are low and close to the detection limit in healthy individuals, but are increased in obesity. Both LIGHT receptors are present on the surface of human primary preadipocytes and adipocytes, and activation of the LTßR is sufficient to mediate the effects of LIGHT on adipocyte differentiation.

5.5. Potential therapeutical application of TWEAK and LIGHT in human obesity: chances and risks

As described in the first chapter, the initial treatment of obesity includes a decrease in energy intake via dietary intervention and behavioral alterations and an increase in energy expenditure via physical exercise (3;9). Since long-term results of these strategies are not convincing, and since in some cases obesity needs to be treated more effectively to prevent life-threatening comorbidities of the disease, pharmacological treatment is an additional option (3;25). Existing anti-obesity drug therapies aim at two main mechanisms, which are the regulation of food intake and satiety and the uptake of nutrients in the gastrointestinal tract (254). Sibutramine and rimonabant are two substances that act centrally by targeting the mechanism of food intake via influencing serotonine re-uptake or the cannabinoid-receptor system, respectively. Orlistat functions in the periphery by inhibiting gastric lipase and thereby impairing lipid uptake in the gut.

For the present study, a different approach was chosen: inhibitors of adipocyte differentiation directly target adipose tissue as pivotal organ of energy storage. Obesity is initially associated with an augmented lipid storage in existing adipocytes, yet new adipocytes are recruited from the pool of preadipocytes when existing fat cells obtain a critical size (54). Therefore, targeting adipocyte differentiation might offer an alternative tool to limit weight gain in extremely obese patients or in overweight patients with severe comorbidities. However, this method has one obvious limitation. The main reason for obesity, besides an undeniable genetic predisposition, is a positive energy balance, i.e. energy intake exceeds energy consumption (1;20). Therefore, in most obese patients with increasing body weight a positive energy balance is most likely. Excessive energy is stored in adipose tissue, and this leads to the question about the disposition of excessive energy when the above described critical fat cell size is achieved, yet the development of new fat cells is inhibited by a potential

therapeutic intervention. The worst case scenario arising from this situation can be observed in the syndromes of lipoatrophy or lipodystrophy. These diseases can be acquired or inherited and have the common property of missing or insufficient adipose tissue mass (255-257). These patients suffer from severe dyslipidemia and excessive lipid accumulation in muscle and liver. Severe diabetes and, as a consequence of decreased production due to limited adipose tissue mass, hypoleptinemia and hypoadiponectinemia develop. These unfavorable effects accompanying lipoatrophy in HIV patients have been partly accredited to the impairment of adipocyte differentiation by antiviral drug therapy (258). Although the exact mechanisms for the development of obesity-associated type-2-diabetes are not clear, a common feature and a possible link between both diseases is ectopic lipid storage and dyslipidemia (259). Increased serum values of triglycerides and free fatty acids (FFA) may convey insulin resistance and storage of lipids in muscle and liver. All these findings would argue against the application of differentiation inhibitors in the treatment of obesity. Yet, is has to be considered that cases of lipodystrophy and –atrophy, rare and both severe metabolic disorders, are only partially comparable to the obese state. Concerning the question of compatibility of the existing disturbances in lipid metabolism in the obese state and the implications of an inhibition of differentiation, animal data will provide more precise answers. Preliminary data in mice ectopically expressing TWEAK and LIGHT showed no metabolic disturbances under a high caloric diet (data not shown).

Next to the prevention of excess weight gain, both proteins might also be interesting in the prevention of the weight re-gain after phases of weight loss. Adipocyte delipidation is supposed to take place during weight loss, and the subsequent regain in body weight is partially connected to the re-differentiation of these de-differentiated preadipocytes (260;261).

In conclusion, it seems valuable and feasible to further develop this approach for the modification of weight gain after these promising *in vitro* results. Both, TWEAK and LIGHT, did effectively inhibit adipocyte differentiation and yet did not display adverse effects on the investigated metabolic parameters or on the secretion of pro-inflammatory cytokines.

5.5.1. Potential therapeutical application of TWEAK

As discussed above, the possible systemic consequences of TWEAK administration need to be investigated *in vivo* in adequate animal models, if TWEAK is to be used to prevent further weight gain in obesity by inhibiting the differentiation of adipocytes. This would cover animals in the phase of weight gain under a normo- and high-caloric diet as well as obese animals with both types of diet. Besides this necessary further research, a possible systemic or

local utilization of TWEAK or of agonistic antibodies for Fn14 needs to be discussed in the light of the protein's effects observed in cell culture and those described in the literature (*in vivo* and *in vitro*), and in the light of the literature about obesity.

As already specified, the increase in fat mass occurs during obesity via fat cell hypertrophy and hyperplasia (54). New fat cells develop from preadipocytes present in adipose tissue, and proliferation of these preadipocytes takes place during whole life. The stimulatory effect of TWEAK on human preadipocyte proliferation needs to be thoroughly investigated in adequate *in vivo* models to rule out an unwanted hyperplastic effect of the protein. The weak pro-apoptotic effect of the protein on differentiated adipocytes might be also an interesting feature. Preadipocyte and adipocyte apoptosis is most probably involved in the reduction of adipose tissue mass after weight loss (54). The contribution of both effects to the regulation of adipose tissue mass in physiological and pathophysiological states can only be precisely evaluated in *in vivo* animal models.

An unfavorable effect of TWEAK is the weak increase in leptin secretion from differentiated adipocytes. Hyperleptinemia is a common feature of obesity and is, as depicted above, probably involved in the development of leptin resistance (246). Contribution of the stimulatory effect of TWEAK on leptin secretion observed *in vitro* needs to be further investigated *in vivo*.

One might argue that TWEAK acts like TNF-α with just weaker activity. Both proteins inhibit differentiation of human preadipocytes to mature adipocytes, but TNF-α is actually more potent since the undifferentiated preadipocyte phenotype is fully preserved after a 16-day differentiation period in the presence of 1 nM TNF-α (68). An almost complete inhibition of differentiation is achieved with TWEAK at a much higher concentration (1000 ng/ml, i.e. 55 nM). All experiments in the present study were performed at this concentration. Both proteins are comparable with regard to the effects on adipocyte differentiation, but are different with regard to their effects on the other adipocyte parameters investigated. Discussing the effectiveness of TWEAK it should be taken into account that the protein used here was obtained from E. coli. Glykosylation of proteins is not possible in this system, yet has been described for TWEAK and might influence protein's activity.

As described above, TWEAK´s probable involvement in a variety of diseases was investigated in human and animal models *in vivo* and *in vitro*. Its possible involvement in human disease states was only partially demonstrated in the human system. TWEAK mRNA and protein expression were increased in some human tumors in comparison to normal, healthy tissues, and Fn14 expression was up-regulated in atherosclerotic plaques

(124;133;134). TWEAK expression is increased in a T-cell subset of LE patients, and urinary TWEAK levels correlate with disease strength in LE patients with nephritis (125;126). Hints for the participation of TWEAK in the development of rheumatoid arthritis and central nervous system diseases have been obtained from human cell culture or from animal models *in vitro* and *in vivo* (128;140;145;150). Yet, to my knowledge, no information is available from the literature about the physiological role of TWEAK in the human body. It is also not clear from the reported data if TWEAK expression is altered as a reaction to different disease states or if the protein is causally involved in the development of these diseases.

The pro-angiogenic effect described in the literature needs to be further evaluated *in vivo* for a possible application (154). An increase in adipose tissue vascularization might stimulate adipose tissue blood supply and growth. Since TWEAK expression has been described for a subset of activated T-cells in LE patients and for IFN-γ activated monocytes, effects of a possible systemic application might mimic immune cell activation and this possibility needs also to be evaluated in adequate models (118;125).

Hints for different roles of TWEAK and Fn14 in physiological and pathophysiological states can be found in some publications, and this raises the question about the conclusions that can be drawn from these findings. One example is that mRNA expression of Fn14 in murine primary neurons is increased after dissection, yet Fn14 expression is not altered in normal nerve development (119). Another example are TWEAK's pro-inflammatory effects in isolated human primary astrocytes; yet TWEAK transcripts are also found in the normal brain (145).

Knock-out animal models do not indicate a critical role for TWEAK or Fn14, but suggest a limiting role for TWEAK in the reaction of the innate immune system (123;144). In murine models of TWEAK over-expression, the effects are largely dependent on the site and amount of protein expression. Muscle specific over-expression was found to have severe negative consequences on muscle mass, body mass and viability (142). Liver-specific over-expression does not alter fertility or viability, but stimulates liver progenitor proliferation. Yet, his stimulation stays within healthy limits (141).

To conclude, TWEAK is a promising tool for the inhibition of differentiation of preadipocytes in human adipose tissue. Previous findings concerning the role of TWEAK are limited to disease models and do not deliver information about a possible physiological role of the protein or about the consequences of a systemic administration of soluble protein. Since the protein does not exert pro-inflammatory properties in human preadipocytes and adipocytes and leaves important metabolic parameters unaltered, a further evaluation for a possible

therapeutical application of the protein appears to be justified. This evaluation needs to include a confirmation of the positive effects *in vivo* as well as the exclusion of the above described possible negative effects in suitable animal models.

5.5.2. Potential therapeutical application of LIGHT

As postulated for TWEAK, a potential therapeutical application of LIGHT needs to be thoroughly investigated in suitable animal models to rule out adverse effects of the inhibition of adipose differentiation. LIGHT proved to be more potent in its inhibitory effect on adipocyte differentiation and to have less unfavorable effects than TWEAK. Neither an effect on cell viability or proliferation nor on secretion of adipokines or pro-inflammatory cytokines were observed.

The possible argument about LIGHT´s similar but weaker role in comparison to TNF-α can be refuted by the following findings: The EC50 of 0.04 nM is well below the concentration of 1 nM described for a complete inhibitory effect of TNF-α. All experiments were performed with a concentration at least 10-times higher than the EC50, and no adverse effects were observed at these concentrations. It can be concluded that the effects of LIGHT clearly differ from the effects of TNF-α in adipose cells.

LIGHT used for the present study was obtained from a production in Hek293. As for TWEAK, glykosylation has also been described for LIGHT (161). In vivo experiments with both proteins should therefore be carried out with recombinant protein produced in mammal cell lines.

Though LIGHT may seem more promising than TWEAK considering the results from the human cell culture experiments, results described in the literature define the protein as important player in the development and function of the immune system. As for TWEAK, no data is available on the physiological role of LIGHT in humans, yet there is convincing data about the involvement of LIGHT in several disease states. Participation of LIGHT in the development of the immune system has been discussed for negative selection of thymocytes and the development of self-tolerance (198;199). LIGHT can re-establish an almost normal immune system in LT-α deficient mice (201). LIGHT stimulated physiological responses of monocytes and neutrophils to bacterial infection (203). As expected from these findings, LIGHT is probably also involved in the development of auto-immune diseases. In animal models, LIGHT was involved in the development of RA (206). An important finding for the present study is a possible contribution of LIGHT to the development of type-1-diabetes (165;207). Obese patients frequently suffer from type-2-diabetes, also called non-insulin-

dependent diabetes mellitus (3). While in type-1-diabetes, pancreatic β-cells are destroyed in an auto-reactive process with the consequence of severe insulin deficiency, type-2-diabetes is caused by insulin resistance and impaired insulin secretion (231). Nevertheless, a possible effect of LIGHT on pancreas function needs to be studied extensively. Yet, the results in the first of the above mentioned studies were obtained with HVEM-Ig. HVEM reacts with other members of the TNFSF (169;178) and it was not specified in the study which of these possible HVEM-binding partners were detected. From the second study, it can be concluded that the LTßR is important for the development of type-1-diabetes in susceptible mice, but no information is available about the respective ligands. From the described results, a focus should be put on the effects of systemic LIGHT application on pancreas function. Another organ suggested to be affected by LIGHT is the liver. The LIGHT-LTßR axis is responsible for disease severity in a murine hepatitis model (214). LIGHT knock-out animals do not display alterations in the liver, but mice over-expressing LIGHT in T-lymphocytes suffer from hepatomegaly, and LIGHT has been implicated in liver regeneration (262).

As for TWEAK, it can be said for LIGHT that differing and contrary functions have been described. LIGHT was found to stimulate the development of RA in a murine model (206). In another mouse model, the injection of LTßR-Ig caused an increase in RA severity and duration. These results point to a protective role of LIGHT (263). A weakness of the results is the missing discrimination between LIGHT and LT in both models. Apoptotic effects of LIGHT strongly depend on the cell type and the conditions applied. LIGHT causes apoptosis in a stably LIGHT-transfected breast cancer cell line and upon IFN-γ co-stimulation in HT-29 cells (163;164). In contrast, a protective effect could be found for LIGHT in primary hepatocytes upon ConA/TNF-α induced apoptosis (183). In the same study, this protective effect was not observed in hepatic tumor cell lines. It must be concluded from these and other studies that the effects of LIGHT are largely dependent on the type of application, the chosen conditions and the selected models. Most of the results were obtained with membrane-bound LIGHT, and these results might vary from the findings obtained with systemic or local application of soluble LIGHT. Agonistic antibodies specifically targeting one of the receptors for LIGHT might offer a valuable tool with effects differing from those obtained with membrane-bound protein.

Animal models provided contradictory outcomes concerning the role of LIGHT. Knock-out animal do not suggest a crucial role for LIGHT. The animals are viable and have only a slight alteration in T-cell proliferation (192-195). Over-expression of LIGHT leads to severe

alterations in the immune system (197-200). These results coincide with findings for the involvement of LIGHT in immune system development and function.

As described in the introduction, LIGHT has been implicated in human diseases. Soluble LIGHT was detected in the bronchoalveolar secretion of scleroderma patients with inflammation in contrast to healthy patients (189).

Macrophages of RA patients express LIGHT at higher levels than macrophages from OA patients (250). As for TWEAK, no information is available so far on the physiological role of LIGHT in humans or animals. Furthermore, a systemic application was not performed.

In conclusion, LIGHT is a promising tool for the inhibition of differentiation in adipocytes. In human adipose cells, the protein does no exert the pro-inflammatory effects or the effects on cell viability as was described in previous studies using other cell types. Adipokine secretion as assessed for leptin and adiponectin remains unaltered and important metabolic parameters are not influenced by LIGHT. A further evaluation of the protein for a potential therapeutic application in obesity is therefore justified. It must include the evaluation of systemic effects in suitable animal models as a next step.

5.6. Future perspectives and further investigations

Animal models should provide further important information about the consequences of systemic or local application of the protein or agonistic antibodies and also about the open question on the role in lipid metabolism when administered in obese patients.

siRNA experiments could be helpful tools to judge the importance of the signaling pathways investigated. Yet a limitation for such experiments is the low transfection efficacy in primary human cells and the need for a subsequent near to normal differentiation process that is restricted following transfection. One alternative is the implementation of these experiments in SGBS cells, a human preadipocyte cell strain which is particularly suitable for transfection studies.

To obtain further information, these signaling pathways might also be investigated concerning the temporal effects of TWEAK and LIGHT. Inhibitors of NF-κB pathways could elucidate the role of these pathways in adipocytes. All effects found for the two proteins should be confirmed in suitable animal models.

5.6.1. TWEAK

To further specify the contribution of TWEAK's pro-proliferative effect to its inhibitory action in preadipocyte differentiation, a gene expression array for typical proteins of cell cycle progression and of differentiation could provide valuable information. The contribution of this and of the pro-apoptotic effects need to be as thoroughly investigated in adequate animal models as the impact on the leptin response.

5.6.2. LIGHT

The presence of the HVEM receptor in the SVF of human adipose tissue needs to be further evaluated: FACS analysis applying co-staining for the HVEM and for typical immune cells markers should give further information about the suggested receptor presence on infiltrating immune cells. Agonistic antibodies against the HVEM could deliver important information about the contribution of either LIGHT receptor to the effects mediated by the recombinant protein on human preadipocytes and adipocytes. siRNA experiments would be another option to determine the role of the two receptors for the action of LIGHT in adipose tissue.

6. Conclusion

The two proteins investigated in this study, TWEAK and LIGHT, turned out to be potent inhibitors of adipocyte differentiation in the murine and the human system. They display the described inhibitory effect probably through interference with the transcriptional activation of adipocyte differentiation. LIGHT does not exert any negative or unwanted effects on adipocyte metabolism or secretory function as investigated in this thesis in the human primary cell culture system. TWEAK is comparable to LIGHT in its effects on adipocyte metabolism, but does modestly stimulate leptin secretion. TWEAK also has a significant growth promoting effect in preadipocytes seeded at low density and a weak pro-apoptotic effect in differentiated adipocytes. The proteins are members of the TNFSF as is TNF-α, a well known inhibitor of adipocyte differentiation. TNF-α exerts its inhibitory effects on adipocyte differentiation in parallel to disturbing effects on adipocyte metabolism and unwanted effects on secretory function. TWEAK and LIGHT differ from TNF-α in the important feature that they do not exert the negative effects on metabolic functions. Both proteins are therefore interesting tools for a potential therapeutical application in obesity. The idea behind this strategy is that an inhibition of adipocyte differentiation through systemic or local application of TWEAK or LIGHT may prevent a further weight gain in obesity. Both proteins may fulfill the basic preconditions for the suggested therapeutical application in cell culture experiments. Yet, the suggested application requires a thorough investigation of the described effects *in vivo* in suitable animal models. A further investigation must also clarify the consequences of a systemic or local application of TWEAK and LIGHT or respective agonistic antibodies. Animal models furthermore need to deliver a proof of concept for the therapeutical idea presented in this study. In combination with this proof, possible negative effects on lipid metabolism, especially in the liver and the skeletal muscle, need to be excluded. The results obtained in this study may provide a realistic basis for further investigation of both proteins *in vivo*.

7. Summary

Two members of the TNF superfamily, TWEAK (TNFSF14) and LIGHT (TNFSF12) were found in a high through-put screening based on cDNA libraries for the discovery of novel inhibitors of adipocyte differentiation in murine 3T3-L1 preadipocytes. Subsequent studies in the human system confirmed the findings for subcutaneous and omental primary preadipocytes as well as for the SGBS cell strain applying the Nile Red assay as read-out. The inhibitory effects were found to be concentration-dependent with an EC50 of 200 ng/ml for TWEAK and an EC50 of 10 ng/ml for LIGHT. Inhibition of differentiation occurred only when the proteins were added at an early time point of the differentiation process. Both proteins decreased mRNA expression of the key transcription factor PPAR-γ and of the late markers of adipocyte differentiation adiponectin and hormone-sensitive lipase. Neither TWEAK nor LIGHT had a negative effect on cell viability as measured by LDH secretion and determination of viable cell number. Contrary to LIGHT which showed no effect on apoptosis or proliferation, TWEAK stimulated proliferation of preadipocytes and weakly caused apoptosis in differentiated adipocytes. No major effects of both proteins were observed on the secretion of proinflammatory cytokines as determined by ELISA and the Bioplex multiplex bead-based technology. Leptin and adiponectin secretion was unaltered following incubation with LIGHT, while TWEAK weakly stimulated leptin secretion. Both proteins did not alter two central metabolic function of adipose tissue, i.e. glucose uptake and lipolysis, in human primary adipocytes. Receptors for both proteins, Fn14 for TWEAK and HVEM as well as LTßR for LIGHT, were expressed at the mRNA level as observed with RT-PCR. Receptor expression on the cell surface was confirmed with FACS experiments and with agonistic antibodies for Fn14 and LTßR. Fn14 was confirmed to be the only receptor mediating effects of TWEAK in human primary adipocytes using agonistic and antagonistic antibodies. LTßR was found to be sufficient for the LIGHT-mediated effects in this system using agonistic antibodies. Both proteins were found to activate the classical NF-κB pathway in adipocytes by an ELISA for NF-κB binding activity.

In conclusion, both proteins offer a valuable tool for the inhibition of adipocyte differentiation without altering important metabolic or secretory functions of the adipocytes. A further evaluation of both proteins in suitable *in vivo* models for the potential therapeutical application for the prevention of weight-gain in obesity is feasible.

8. Zusammenfassung

Zwei Mitglieder der TNF Superfamilie, TWEAK (TNFSF14) und LIGHT (TNFSF12), wurden in einem High-Throughput Screening Ansatz als Inhibitoren der Adipozytendifferenzierung in murinen 3T3-L1 Präadipozyten entdeckt. Nachfolgend wurde die inhibitorische Wirkung im humanen System für subkutane und omentale Präadipozyten sowie für die SGBS Zelllinie durch Anwendung des NileRed Assays bestätigt. Die hemmenden Effekte waren konzentrationsabhängig mit einer EC50 von 200 ng/ml für TWEAK und von 10 ng/ml für LIGHT. Die Hemmung der Adipozytendifferenzierung zeigte sich nur bei einer Zugabe der Proteine während eines frühen Zeitpunktes im Differenzierungsprozess. Beide Proteine verminderten die mRNA Expression des entscheidenden Transkriptionsfaktors PPAR-γ sowie die Expression von Adiponektin und hormonsensitiver Lipase als späten Markern der Adipozytendifferenzierung. Weder TWEAK noch LIGHT hatten eine negativen Einfluss auf die Zellviabilität wie sich aus der Messungen der LDH-Freisetzung und der Bestimmung der Zahl lebender Zellen erkennen ließ. Im Gegensatz zu LIGHT, das keinen Einfluss auf die Proliferations- oder Apoptoserate hatte, zeigte TWEAK einen deutlichen stimulatorischen Effekt auf die Proliferation von Präadipozyten und verursachte eine schwache pro-apoptotische Wirkung in differenzierten Adipozyten. Beide Proteine hatten nahezu keine Wirkung auf die Sekretion proinflammatorischer Zytokine, die mit ELISAs und Bioplex Mulitplex Assay bestimmt wurde. Die Leptin- und Adiponektinsekretion war unter dem Einfluss von LIGHT unverändert, während TWEAK die Leptinsekretion schwach stimulierte. Beide Proteine hatten keinen Einfluss auf die Glukoseaufnahme oder die Lipolyse als zwei zentrale metabolische Funktion humaner Fettzellen. Die Rezeptoren für beide Proteine, Fn14 für TWEAK und HVEM sowie LTßR für LIGHT, werden auf der mRNA Ebene exprimiert, wie sich in RT-PCR Experimenten erkennen ließ. Die Expression der Rezeptoren auf der Zelloberfläche wurde mit FACS-Experimenten und durch den Einsatz agonistischer Antikörper bestätigt. Fn14 konnte mit agonistischen und antagonistischen Antikörpern als einziger Rezeptor für TWEAK auf den humanen primären Fettzellen bestätigt werden. Der LTßR ist für die von LIGHT vermittelten Auswirkungen in dem beschriebenen System ausreichend. Die Aktivierung des klassischen NF-κB Signalweges konnte für beide Proteine mit einem ELISA für die NF-κB Bindungsaktivität gezeigt werden.

Zusammenfassend bieten beide Proteine ein interessantes Potential zur Hemmung der Adipozytendifferenzierung, ohne dabei wichtige metabolische oder sekretorische Funktionen der Fettzellen zu verändern. Eine weitere Beurteilung der potentiellen therapeutischen

Anwendung beider Proteine zur Vermeidung von Gewichtszunahme bei Adipositas in passenden *in vivo* Modellen erscheint sinnvoll.

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10. Appendix

10.1 List of abbreviations

3T3-L1	Murine embryonic fibroblast cell line
A375	Human melanoma cell line
aa	Amino acid
AKT	Proteinkinase B
aP2 (ALBP)	Adipocyte lipid-binding protein
APS	Ammonium persulfate
ATP	Adenosintriphosphate
bFGF	Basic fibroblast growth factor
bHLHzip	Basic-helix-loop-helix leucine zipper protein
BMI	Body mass index
BrdU	5-bromo-2`-deoxyuridine
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
CHX	Cycloheximide
CRD	Cysteine-rich domain
DAG	Deutsche Adipositas Gesellschaft
DNA	Deoxyribonucleic acid
DC	Dendritic cells
DcR	Soluble decoy receptor
DD	Death domain
DEXA	Dual-Energy X-Ray Absorptiometry
DHAP	Dihydroxyacetone phosphate
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalitis
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Eph4	Mammalian breast tumor cell line
Erk	Extracellular regulated kinases
FACS	Fluorescence-activated cell sorting
FAS	Fatty acid synthase
FasL	Fas ligand
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fn14	Fibroblast growth factor-inducible-14
FOXO	Drosophila forkhead transcription factor
GAPDH	Glycerinaldehydphosphate dehydrogenase
GH	Growth hormone
GLUT	Glucose transporter
GM-CSF	Granulocyte-monocyte colony-stimulating factor
GPDH	Glycerinphosphate dehydrogenase
GSK	Glycogen synthase kinase
HCC	Human hepatocellular carcinoma cell line

HEK293	Human embryonic kidney cell line
HeLa	Human epithelial carcinoma cell line
Hrc	Human recombinant
HRP	Horseradish peroxidase
HSL	Hormone-sensitive lipase
HT-29	Human adenocarcinoma cell line
HUVEC	Human umbilical vein endothelial cells
HVEM	Herpesvirus entry mediator
IBMX	Isobutylmethylxanthine
ICAM	Intracellular adhesion molecule
IFN	Interferon
IKK	Inhibitor of κB-kinase
IL	Interleukin
IOTF	International Obesity Task Force
IR	Insulin receptor
IRS	Insulin receptor substrate
Iκ-B	Inhibitor of κB
JNK	c-Jun N-terminal kinase
KATO-III	Human gastric adenocarcinoma cell line
kDa	Kilo Dalton
KLF	Kruppel-like factor
KRP	Krebs-Ringer phosphate buffer
LDH	Lactate dehydrogenase
LE	Lupus erythematosus
LIGHT	Lymphotoxin-like, exhibits inducible expression and competes with HSV glycoprotein D (gD) for HVEM, a receptor expressed by T-lymphocytes
LPL	Lipoproteinlipase
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTβR	Lymphotoxin-β receptor
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
NADH	Nicotinamide adenin dinucleotide
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NK	Natural killer cell
NP40	Nonidet P40
Om	Omental
PAI	Plasminogen activator-inhibitor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC12	Rat neuron cell line
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PI3K	Phosphatidylinositol-3 kinase
PMSF	Phenyl methyl sulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvenyl difluoride
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF-κB ligand

RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RAW264.7	Human macrophage/monocyte cell line
RD	Human rhabdomyosarkome cell line
RNA	Ribonucleic acid
RXR	Retinoic X receptors
Sc	Subcutaneous
SDS	Sodium dodecyl sulphate
SGBS	Simpson-Golabi-Behmel syndrome
SREBP	Sterol regulatory element-binding protein
SVF	Stromal-vascular fraction
T_3	Triiodothyronine
TBS	Tris-buffered saline
TEMED	Tetramethylethylendiamin
TGF	Transforming growth factor
TIM	TRAF-interaction motif
TNF	Tumor necrosis factor
TNFRSF	TNF receptor superfamily
TNFSF	TNF superfamily
TRADD	TNF-associated death domain
TRAF	TNF receptor-associated factor
Tris	Trishydroxymethyl aminomethan hydrochlorid
TWEAK	TNF-like weak inducer of apoptosis
VCAM	Vascular endothelial cell adhesion molecule
WAT	White adipose tissue
WHO	World Health Organization

10.2. List of figures

10.3. List of tables

10.4. Chemicals and Consumables

10.4.1. Chemicals

2-Deoxy-D-[1-^{3}H] glucose	GE Healthcare, Munich, Germany
Absolute™ QPCR SYBR® Green ROX Mix	AB Gene, Hamburg, Germany
Acrylamide 30 %	Roth, Karlsruhe, Germany
Alexa Fluor® 488 donkey anti-goat IgG	Invitrogen, Karlsruhe, Germany
Alexa Fluor® 488 goat anti-mouse IgG	Invitrogen, Karlsruhe, Germany
Ammonium persulfate (APS)	Sigma-Aldrich, Munich, Germany
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling, Danvers, USA
Biotin	Roth, Karlsruhe, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Munich, Germany
Bromphenol blue	VWR, Darmstadt, Germany
$CaCl_2$	Merck, Darmstadt, Germany
Collagenase	Biochrom, Berlin, Germany
Complete Mini Proteaseinhibitor	Roche, Penzberg, Germany
Coomassie Brilliant Blue G-250	Serva, Heidelberg, Germany
Cycloheximide (CHX)	Sigma-Aldrich, Munich, Germany
Dihydroxyacetone phosphate (DHAP)	Sigma-Aldrich, Munich, Germany
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe, Germany
Dithiothreitol (DTT)	OLS, Hamburg, Germany
DMEM/Ham´s F12 colorless	Sigma-Aldrich, Munich, Germany
DMEM/Ham´s F12 liquid SGBS	Invitrogen, Karlsruhe, Germany
DMEM/Ham´s F12 powder primary culture	Invitrogen, Karlsruhe, Germany
D-Pantothenat	Sigma-Aldrich, Munich, Germany
Ethylenediaminetetraacetate (EDTA)	Merck, Darmstadt, Germany
Fetal bovine serum (FBS)	Biochrom, Berlin, Germany
Gentamicin	PAA, Pasching, Austria
Glycerokinase	Sigma-Aldrich, Munich, Germany
Glycerol	Merck, Darmstadt, Germany
Glycin	Merck, Darmstadt, Germany
Goat anti-human LTßR antibody	R&D Systems, Wiesbaden, Germany
Human recombinant Cardiotrophin-1	R&D Systems, Wiesbaden, Germany
Human recombinant EGF	R&D Systems, Wiesbaden, Germany
Human recombinant Endothelin-2	Alexis, Grünberg, Germany
Human recombinant FGF-16	R&D Systems, Wiesbaden, Germany
Human recombinant IL-11	R&D Systems, Wiesbaden, Germany
Human recombinant IL-6	R&D Systems, Wiesbaden, Germany
Human recombinant LIGHT/TNFSF14	Alexis, Grünberg, Germany
Human recombinant Lymphotoxin α1/ß2	R&D Systems, Wiesbaden, Germany
Human recombinant Lymphotoxin α2/ß1	R&D Systems, Wiesbaden, Germany
Human recombinant TGF-ß1	R&D Systems, Wiesbaden, Germany
Human recombinant TWEAK/TNFSF12	R&D Systems, Wiesbaden, Germany
Human recombinant TNF-α/TNFSF1A	R&D Systems, Wiesbaden, Germany
Hydrocortisone	Sigma-Aldrich, Munich, Germany
IGEPAL	Sigma-Aldrich, Munich, Germany
Insulin	Sigma-Aldrich, Munich, Germany
Isobutylmethylxanthine (IBMX)	Serva, Heidelberg, Germany
Isoproterenol	Sigma-Aldrich, Munich, Germany
K_2HPO_4	VWR, Darmstadt, Germany

KCl	Merck, Darmstadt, Germany
Kodak Developer for autoradiography	Sigma-Aldrich, Munich, Germany
Kodak Fixer for autoradiography	Sigma-Aldrich, Munich, Germany
Mercaptoethanol	Sigma-Aldrich, Munich, Germany
Methanol	Roth, Karlsruhe, Germany
$MgCl_2$	Merck, Darmstadt, Germany
$MgSO_4$	Merck, Darmstadt, Germany
Mouse anti-human Fn14 agonistic antibody ITEM-1	eBioscience, San Diego, USA
Mouse anti-human Fn14 agonistic antibody ITEM-4	eBioscience, San Diego, USA
Mouse anti-human Fn14 antagonistic antibody ITEM-2	eBioscience, San Diego, USA
Mouse anti-human HVEM antibody MAB356	R&D Systems, Wiesbaden, Germany
Mouse anti-human LTßR agonistic antibody AF629	Alexis, Grünberg, Germany
Mouse IgA Isotype Control	eBioscience, San Diego, USA
Mouse IgG1 Isotype Control	R&D Systems, Wiesbaden, Germany
NaCl	Merck, Darmstadt, Germany
NaF	Sigma-Aldrich, Munich, Germany
NaH_2PO_4	Merck, Darmstadt, Germany
$NaHCO_3$	Merck, Darmstadt, Germany
NH_4Cl	Merck, Darmstadt, Germany
Nicotinamidadenindinucleotide (NADH)	Sigma-Aldrich, Munich, Germany
Nile Red	Invitrogen, Karlsruhe, Germany
Nonidet P40 (NP40)	Sigma-Aldrich, Munich, Germany
Normal Goat IgG	R&D Systems, Wiesbaden, Germany
Phosphate-buffered saline (PBS)	Sigma-Aldrich, Munich, Germany
Penicillin/Streptomycin	Invitrogen, Karlsruhe, Germany
Phenyl methyl sulfonyl fluoride (PMSF)	Sigma-Aldrich, Munich, Germany
Precision Plus Protein™ Standard	Bio-Rad, Munich, Germany
Rabbit anti-human Akt Antibody	Cell Signaling, Danvers, USA
Rabbit anti-human phospho-Akt Antibody	Cell Signaling, Danvers, USA
Rosiglitazon	Cayman, Ann Arbor, USA
Scintillation liquid Rotiszint eco plus	Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Serva, Heidelberg, Germany
Sodium pyruvate	Sigma-Aldrich, Munich, Germany
Taqman Universal PCR Mastermix	Applera, Darmstadt, Germany
Tetramethylethylendiamin (TEMED)	Roth, Karlsruhe, Germany
Transferrin	Sigma-Aldrich, Munich, Germany
Triethanolaminhydrochlorid	Sigma-Aldrich, Munich, Germany
Triiodothyronine (T_3)	Sigma-Aldrich, Munich, Germany
Trishydroxymethyl aminomethan hydrochlorid (Tris)	Merck, Darmstadt, Germany
Trypan blue	Sigma-Aldrich, Munich, Germany
Trypsin/EDTA 10 x	PAA, Pasching, Austria
Tween 20	Sigma-Aldrich, Munich, Germany
Wortmannin	Sigma-Aldrich, Munich, Germany
Dexamethasone	Sigma-Aldrich, Munich, Germany
Ethanol	Roth, Karlsruhe, Germany
Phosphoric acid	Roth, Karlsruhe, Germany
Pyruvate	Sigma-Aldrich, Munich, Germany

10.4.2. Consumables

15 ml and 50 ml tubes	TPP, Trasadingen, Switzerland Corning, Schiphol-Rijk, The Netherlands
Absolute QPCR Seal	AB Gene, Hamburg, Germany
Bottle top filter 0.22 µm PES	Corning, Schiphol-Rijk, The Netherlands
Cell culture dishes 96-well, 12-well, 6-well, 6 cm	BD Bioscience, Heidelberg, Germany TPP, Trasadingen, Switzerland Corning, Schiphol-Rijk, The Netherlands
Cell scraper	TPP, Trasadingen, Switzerland
EPTwin PCR plate 96 skirted	Eppendorf, Hamburg, Germany
Filter 70 µm	BD Bioscience, Heidelberg, Germany
Filter membrane 2000 µm, 250 µm, 150 µm	VWR, Darmstadt, Germany
Gel blotting paper	Schleicher&Schuell, Dassel, Germany
Hybond P PVDF transfer membrane	GE Healthcare, Munich, Germany
Hyperfilm ECL chemiluminescence film	GE Healthcare, Munich, Germany
Sterivex sterile filter unit 0.22 µm	Millipore, Billerica, USA
Scintillation cups	Perkin Elmer, Waltham, USA
Single use syringe filter	Zefa, Harthausen, Germany
White microtiter plate	Thermo, Schwerte, Germany

10.4.3. Kits

ATP Kit SL 144-041	BioThema, Haninge, Sweden
BCA Protein Assay Kit	Pierce, Rockford, USA
Bio-Plex Cell Lysis Kit	Bio-Rad, Munich, Germany
Bio-Plex Multiplex Cytokine Assay	Bio-Rad, Munich, Germany
Bio-Plex Phosphoprotein Assay	Bio-Rad, Munich, Germany
Bio-Plex Total Target Assay	Bio-Rad, Munich, Germany
Caspase-Glo® 3/7 Assay	Promega, Mannheim, Germany
Cell Proliferation ELISA, BrdU (colorimetric)	Roche, Penzberg, Germany
CellTiter 96® AQ_{UEOUS} One Cell Proliferation Assay	Promega, Mannheim, Germany
ECL Advance Western Blot Detection Kit	GE Healthcare, Munich, Germany
Human Adipocyte Lincoplex Kit	Linco, Missouri, USA
Human Adiponektin Quantikine® ELISA	R&D Systems, Wiesbaden, Germany
Human IL-6 ELISA	eBioscience, San Diego, USA
Human Leptin Quantikine® ELISA	R&D Systems, Wiesbaden, Germany
Human LIGHT/TNFSF14 Quantikine® ELISA	R&D Systems, Wiesbaden, Germany
Human TWEAK Instant ELISA	Bender MedSystems, Vienna, Austria
iScript™cDNA Synthesis Kit	Bio-Rad, Munich, Germany
Total RNA Isolation Kit	Macherey-Nagel, Dueren, Germany
TransAm™ NFκB Family Kit	Active Motif, Carlsbad, USA

10.5. Buffers and Media

All buffers and media were prepared with aqua bidest.

10.5.1. Cell isolation and culture

PBS
1 Tablet PBS per 200 ml water
Sterile filtration

DMEM/Ham´s F12 primary culture
DMEM/Ham´s F12 powder
33 µM Biotin
17 µM D-Pantothenat
14 mM $NaHCO_3$
Adjustment to pH 7.3, sterile filtration

Basal medium primary culture
DMEM/Ham´s F12 primary culture
50 µg/ml Gentamicin

Differentiation medium
DMEM/Ham´s F12
66 nM Insulin
1 nM T_3
100 nM Hydrocortisone
10 µg/ml Transferrin
50 µg/ml Gentamicin

Induction medium primary culture
Differentiation medium
2 µM Rosiglitazon
0.5 mM IBMX

Erythrocyte lysis buffer
155 mM NH_4Cl
5.7 mM K_2HPO_4
0.1 mM EDTA x 2 H_2O
Adjustment to pH 7.3, sterile filtration

DMEM/Ham´s F12 SGBS
DMEM/Ham´s F12 liquid
33 µM Biotin
17 µM D-Pantothenat

Basal medium SGBS
DMEM/Ham´s F12 SGBS
100 U/ml Penicillin
100 µg/ml Streptomycin

Induction medium SGBS
Differentiation medium
2 µM Rosiglitazon
25 nM Dexamethasone
0.5 mM IBMX

Trypsin solution
PBS
0.5 mg/ml Trypsin
0.22 mg/ml EDTA

Krebs-Ringer phosphate buffer (KRP)
154 mM NaCl
154 mM KCl
11 mM $CaCl_2$
154 mM $MgSO_4$
100 mM NaH_2PO_4
Adjustment to pH 7.4, sterile filtration

10.5.2. Assays

GPDH

GPDH harvest buffer
0.05 M Tris-HCl pH 7.4
1 mM EDTA
1 mM Mercaptoethanol

Protein content

Bradford reagent
0.01 % (w/v) Coomassie Brilliant Blue G250
4.8 % (v/v) Ethanol
8.5 % (v/v) Phosphoric acid
Sterile filtration

GPDH reaction buffer
0.12 M Triethanolamin-HCl pH 7.5
3 mM EDTA
1.4 mM NADH
0.6 mM Mercaptoethanol
2.4 mM DHAP

LDH assay

LDH assay buffer
81.3 mM Tris-HCl pH 7.2
203.3 mM NaCl
0.244 mM NADH

LDH pyruvate buffer
81.3 mM Tris-HCl pH 7.2
203.3 mM NaCl
9.76 mM Pyruvate

Lipolysis

DMEM/Ham´s F12 colorless
DMEM/Ham´s F12 colorless powder
1 mM Sodium pyruvate
14 mM $NaHCO_3$
2 % BSA
Adjustment to pH 7.3, sterile filtration

Real time PCR

RT PCR with Taqman primers/probes
3.5 µl cDNA
10.0 µl Taqman Universal PCR Mastermix
1.0 µl primer/probe
5.5 µl H_2O

RT PCR with designed primers
3.5 µl cDNA
10.0 µl Absolute SYBR Green ROX mix
1.0 µl each primer (100 pmol/µl)
4.5 µl H_2O

FACS

EDTA solution
PBS
2.5 mM EDTA

Nile Red assay

Nile Red solution
PBS
4 µg/ml Nile Red
40 % (v/v) DMSO

Glucose uptake

IGEPAL lysis buffer
0.5 % IGEPAL
50 mM Tris-HCl pH 8.0
140 mM NaCl
1.5 mM $MgSO_4$

Western blot

Ripa buffer
50 mM Tris-HCl pH 7.4
1 % NP40
0.25 % SDS
150 mM NaCl
1 mM EDTA
1 mM PMSF
1 mM DTT
10 mM NaF
1 pill/10 ml Complete mini

Laemmli buffer
250 mM Tris-HCl pH 6.8
30 % Glycerol
4 % SDS
10 % Mercaptoethanol
0.01 % Bromphenolblue

Running gel
10 % Acrylamide
150 mM Tris-HCl pH 8.8
1 % SDS
1 % Ammonium persulfate (APS)
0.04 % (v/v) TEMED

Stacking gel
5 % Acrylamide
50 mM Tris-HCl pH 6.8
1 % SDS
1 % APS
0.1 % (v/v) TEMED

Antibody solution
PBS
0.5 % BSA

ELISA NF-κB

Digman C buffer
20 mM Hepes pH 7.9
0.42 M NaCl
1.5 mM $MgCl_2$
0.2 mM EDTA
25 % (v/v) Glycerol
1 mM DTT
1 mM PMSF

Running buffer
250 mM Tris
1.92 M Glycin
1 % SDS

Transfer buffer
25 mM Tris-HCl pH 8.5
192 mM Glycin
20 % (v/v) Methanol

TBST
20 mM Tris-HCl pH 7.2
138 mM NaCl
0.1 % Tween 20

Stripping buffer
62.5 mM Tris-HCl pH 6.7
1 % SDS
100 mM Mercaptoethanol

10.6. Primer

10.6.1. Taqman gene expression assays (primer/probe)

Coded protein	*Gene accession number*	*Catalogue number*
Adiponektin	NM_004797	Hs00605917_m1
GAPDH	NM_002046	Hs99999905_m1
Hormone sensitive lipase (HSL)	NM_005357	Hs00193510_m1
LIGHT (TNFSF14)	NM_003807	Hs00187011_mq
PPAR-γ	NM_138711	Hs00234592_m1
TWEAK (TNFSF12)	NM_003809	Hs00611242_m1

10.6.2. Primer designed with Vector NTI software

Coded protein	*Gene accession number*	*Primer sequence*	*Amplicon size*
Fn14/ TNFRSF12A	NM_ 016639	Forward 5´-AAGTTCACCACCCCCATAGAGG-3´ Reverse 5´-ACATTGTCACTGGATCAGCGC-3´	72 bp
HVEM/ TNFRSF14	NM_ 003820	Forward 5´-AGAGGGTGCAGAAGGGAGGC-3´ Reverse 5´-GGGCAGTTCTGACACAGGGTG-3´	55 bp
LTßR/ TNFRSF3	NM_ 002342	Forward 5´-TGCCTCCATATGCGTCGG-3´ Reverse 5´-TGAGACATAGGTGCCTGGCG-3´	56 bp

10.7. Equipment

96-well-plate shaker Tiramax 100	Heidolph, Schwabach, Germany
Biophotometer	Eppendorf, Hamburg, Germany
Bio-Plex Suspension array system	Bio-Rad, Munich, Germany
Centrifuge 5810, 5810 R, 5417R, 5415, MiniSpin	Eppendorf, Hamburg, Germany
Chemistry vacuum pumping unit MZ2CE	Vacubrand, Wertheim, Germany
CO_2-Inkubator HERAcell 240	Kendro, Hanau, Germany
CO_2-Inkubator SteriCycle	Thermo, Schwerte, Germany
Electrophoresis Power Supply EPS301	GE Healthcare, Munich, Germany
Electrophoresis System Hoefer Mini Ve	GE Healthcare, Munich, Germany
FACS BD LSR II	BD Bioscience, Heidelberg, Germany
Fluorescence microscope DMIL and camera DC300	Leica Microsystems, Wetzlar, Germany
Fluoroskan Ascent multiwell fluorometer	Thermo, Schwerte, Germany
Inolab pH meter	WTW, Weilheim, Germany
Laboratory balance	Denver Instruments, Denver, USA
Luminoskan Ascent multiwell luminometer	Thermo, Schwerte, Germany
Mastercycler gradient	Eppendorf, Hamburg, Germany
Mastercycler® ep realplex	Eppendorf, Hamburg, Germany
MultiScreen Separations System 96-well suction	Millipore, Billerica, USA
Neubauer Chamber	Brand, Wertheim, Germany
Photometer DU 800 with thermoblock	Beckmann Coulter, Krefeld, Germany
Pumpdrive 5001 pump for sterilfiltration	Heidolph, Schwabach, Germany
Safety Cabinet HeraSafe	Kendro, Hanau, Germany
Scintillation counter 1450 Microbeta	Perkin Elmer, Waltham, USA
Scissors, forceps	VWR, Darmstadt, Germany
Shaker Polymax 1040	Heidolph, Schwabach, Germany
Shaking water bath	GFL, Burgwedel, Germany
Thermoleader dry block heat bath	Uniequip, Munich, Germany
Transfer System Semi-Dry-Blot Maxi	Roth, Karlsruhe, Germany
Ultrasound homogenator Sonopuls HD 2070	Bandelin, Berlin, Germany
Varioskan multiwell photo- and luminometer	Thermo, Schwerte, Germany
Vortex gene 2	Scientific Industries, Bohemia, USA
Water bath with heating circulator ED190	Julabo, Seelbach, Germany

10.7.1. Software

Office 2003	Microsoft, Munich, Germany
Reference Manger 11	Adept Scientific, Herts, UK
GraphPad Prism 4	GraphPad, San Diego, USA
Vector NTI	Invitrogen, Karlsruhe, Germany
FCS express	De Novo, Thornhill, Canada