Molecular and functional analysis of the cross-talk between human preadipocytes, adipocytes and endothelial cells *in vitro*

Isabelle Mack

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1 Introduction

1.1 Obesity

Obesity is becoming a key and rapidly growing public health problem in adults and children (Hotamisligil, 2006). Its prevalence is increasing worldwide with epidemic levels having been reached in Western industrialized societies (Van Gaal et al., 2006). Obesity is defined as an excess gain in white adipose tissue (WAT) which increases the risk of developing a number of medical problems, ranging from features of the metabolic syndrome to sleep apnoea, structural problems with joints and malignancies (Molavi et al., 2006). Several methods are available for measuring the degree of obesity but the commonest and simplest way for classification is the worldwide accepted body mass index (BMI) which is defined as the weight in kilograms divided by the square of the height in metres (kg/m$^2$). According to the World Health Organisation (WHO), the normal weight range falls between a BMI of 18.5 and 24.9. An individual is regarded as being underweight if the BMI is < 18.5 kg/m$^2$, overweight if the BMI is $\geq$ 25 kg/m$^2$, obese if it is $\geq$ 30 kg/m$^2$ and morbidly obese if the BMI is $\geq$ 40 kg/m$^2$ (http://www.who.int/nut/#obs). A BMI between 22.5 and 25 kg/m$^2$ is associated with the highest life expectancy in both sexes, while mortality of individuals with a BMI between 20 to 22.4 kg/m$^2$ is even higher than in individuals with a BMI between 25 and 27.4 kg/m$^2$, whereas a BMI $\geq$ 30kg/m$^2$ not only increases the risk of several diseases dramatically, but also the risk of death (Whitlock et al., 2009). Since the BMI alone is not a good indicator for the health risk assessment in overweight and obese individuals, the estimation of the adipose tissue distribution is extremely important (Eisele and Hauner, 2006; Hauner, 2009; Manolopoulos et al., 2010). In practice, the waist circumference is measured because the abdominal type of obesity (“apple-shaped”) is especially linked to a high cardiovascular and metabolic risk. The risk is heightened for waistline circumferences over 92 cm for men and over 80 cm for women and significantly heightened for waistline circumferences over 102 cm for men and over 92 cm for women (Hauner, 2007). In contrast, a gluteofemoral body fat distribution (“pear-shaped”), may be associated with a protective lipid and glucose profile, as well as a decrease in cardiovascular and metabolic risk (Manolopoulos et al., 2010). However, independent of the type of body fat the risk for concomitant diseases is increased when a BMI of 30 kg/m$^2$ or more is reached (Skurk and Hauner, 2002). The rise in obesity can be explained by the growing trend of physical inactivity, coupled with a high calorie intake of easily available and highly palatable energy-rich foods. Susceptibility to obesity is thought to be genetically
dependent throughout the population by a factor differing between 30% and 70% in individuals (Berthoud, 2002; Lyon and Hirschhorn, 2005). Such a wide range makes it difficult to generalise susceptibility due to factors such as diet and lifestyle unless the genetic susceptibility can be more accurately determined for an individual or group (Baskin et al., 2005; Weyer et al., 1999). Nevertheless, only a few obesity cases are attributed to a rare single genetic cause (Berthoud, 2002; O'Rahilly, 2009). Therefore, the development of obesity involves the interplay of a large number of susceptible genes with obesigenic environmental factors.

1.1.1 Prevalence of obesity

The most recent figures available for Germany indicate that 66% of men and 51% of women are either overweight or obese. One individual in five has a BMI equal or above 30 kg/m² (Nationale Verzehrsstudie II, 2008; http://www.was-esse-ich.de/). According to the National Health and Nutrition Examination Survey 33% of the adult Americans are overweight, 34% are obese and almost 6% are morbidly obese (http://www.cdc.gov/obesity/data/index.html). Additionally, the prevalence of childhood obesity is increasing at alarming rates. Referring to the International Obesity TaskForce report around 15% of the children aged 5-17 years in Europe are overweight and 5% are obese. For America the figures are worse, with about 25% of the children being overweight and about 8% being obese (http://www.iotf.org/childhoodobesity.asp). Bearing in mind, that weight, BMI and waist circumference which are used to define obesity continually rise with increasing age in both sexes (Nationale Verzehrsstudie II, 2008; http://www.was-esse-ich.de/), health care costs for obesity associated diseases will foreseeable increase further. As yet, the annual expenditure of the German health care system for the treatment of obesity’s comorbidities, primarily type 2 diabetes, is estimated to be about 13 billion euros (Hauner, 2006) clearly a serious economic strain.

1.1.2 Obesity-associated diseases

The metabolic syndrome is a cluster of medical disorders which increase the risk of developing cardiovascular disease (CVD). Depending on the definition, obesity or central obesity may be one component out of three of the following criteria: glucose intolerance (impaired glucose tolerance, impaired fasting glycaemia or diabetes), dyslipidaemia
(depressed high-density lipoprotein [HDL] cholesterol and/or elevated blood triacylglycerols [TAG]), hypertension, insulin resistance and microalbuminuria (Alberti et al., 2006; Zimmet et al., 2005). According to the US National Health Statistic Report, the prevalence of the metabolic syndrome among adults 20 years of age and above is 34 % on average when the National Cholesterol Education Program/Adult Treatment Panel III (NCEP/ATP III) criteria are applied and increases with the age but even more strikingly with rising BMI (Ervin, 2009). For Germany no appropriate studies are available but the prevalence is assumed to be around 25 % or 18 % when estimated by the WHO or NCEP/ATP III criteria, respectively (Wirth, 2007).

Type 2 Diabetes is generally a combined disturbance of insulin secretion by the pancreas and the inability of the tissues in the body, mainly muscle, adipose tissue and liver, to respond appropriately to insulin, the latter being the prominent feature if the individual is obese (Hauner, 2007). Obesity and increased abdominal fat distribution are the greatest risk factors for developing type 2 diabetes, other factors include genetic predisposition, age, hypertension, dyslipidaemia, glucose intolerance during pregnancy and physical inactivity. The main complications of type 2 diabetes are micro- and macroangiopathies and neuropathies. It is estimated that around 5 million individuals in Germany, equivalent to 5-6 % of the population, have a diagnosed type 2 diabetes (Hauner, 2007) causing the health care system enormous costs as addressed in chapter 1.1.1

Endothelial dysfunction of the micro- and macrovasculature (see chapter 1.2.4.1) is central to the development of CVD (Krentz et al., 2009; Singhal, 2005) which is strongly associated with obesity (Whitlock et al., 2009). According to a recently published meta-analysis which included 57 prospective studies, ischemic heart diseases, stroke and other vascular diseases are the most common cause of premature death in obese people (Whitlock et al., 2009).

Obesity also appears to be associated with an increased mortality from cancer, with a higher incidence of certain cancers, including the colorectum, (post menopausal) breast, endometrium, kidney, pancreas and oesophagus (Anderson and Caswell, 2009). Obesity is also linked to orthopaedic problems, respiratory diseases (Molavi et al., 2006) and renal disorders (Nguyen and Hsu, 2007; Wahba and Mak, 2007).
1.1.3 Obesity as an inflammatory state

Obesity is associated with a state of chronic low-grade inflammation which possibly provides a connection between obesity, insulin resistance and the other aspects of the metabolic syndrome (Trayhurn and Wood, 2004). More precisely, this state of low-grade inflammation also contributes to the micro- and macrovascular endothelial dysfunction and vascular remodelling (Hotamisligil, 2006; Singer and Granger, 2007; Van Gaal et al., 2006); leading to hypertension, atherosclerosis and microvascular complications (Nguyen and Hsu, 2007; Van Gaal et al., 2006). Changes in the microcirculation might even be a cause of the development of insulin resistance and the metabolic syndrome (Wiernsperger et al., 2007). The systemic inflammation, involves the elevated circulation of inflammatory cytokines and acute phase proteins such as C-reactive protein (CRP), interleukin (IL) 6, plasminogen activator inhibitor-1 (PAI-1), P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and fibrinogen (Van Gaal et al., 2006). Their source has been suggested to be the liver and also WAT (Van Gaal et al., 2006), since WAT expansion is linked to increased levels of several adipokines e.g. IL-6 and PAI-1 within the tissue (Wellen and Hotamisligil, 2005). Many adipokines may act locally in WAT, but others, typically leptin and adiponectin also reach the circulation and thus, it is suggested that some adipokines contribute to the state of the systemic chronic low-grade inflammation (Molavi et al., 2006; Trayhurn, 2005).

The question of the origin of inflammation in obesity is not clear but four main mechanisms are discussed which are likely to increase the production of proinflammatory molecules in WAT; hypoxia, endoplasmatic reticulum (ER) stress, oxidative stress and immune cell accumulation (Bourlier and Bouloumie, 2009; Hotamisligil, 2006; Trayhurn and Wood, 2004; Wellen and Hotamisligil, 2005).

Hypoxia is likely to occur in expanding WAT, which appears to lead to an inflammatory response in adipocytes to increase blood flow and stimulate angiogenesis (Trayhurn and Wood, 2004; Ye, 2009) as addressed in chapter 1.2.6. Obesity also generates conditions that increase the demand on the ER, especially in WAT due to tissue remodelling and metabolic turnover. As result ER stress may occur leading to the activation of inflammatory pathways including the stress-activated phospho-kinases/c-Jun N-terminale Kinase (SAPK/JNK) and Nuclear factor kappa B (NfKB) pathways (Wellen and Hotamisligil, 2005). Relevant for the initiation of inflammation in obesity may be also oxidative stress. ROS (radical oxygen species) produced by adipose tissue endothelial cell mitochondria due to hyperglycaemic
conditions could result in oxidative damage and the activation of inflammatory signalling pathways. Activated and/or damaged endothelial cells then may enforce immune cell attraction in WAT. Additionally, hyperglycaemia may also increase ROS production in adipocytes leading to a further enhanced production of proinflammatory adipokines (Wellen and Hotamisligil, 2005). Finally, once immune cell accumulation has occurred in WAT this further may amplify cytokine production, secreted by immune cells and other cells present in WAT (Bourlier and Bouloumie, 2009; Wellen and Hotamisligil, 2005).

1.2 Adipose Tissue

1.2.1 Adipose tissue function

Adipose tissue in the bodies of women and men of normal weight generally consists of 20 to 30% and 12 to 20% respectively, of their total body weight (Klaus, 2001). Whole adipose is categorized in three different types according to their location and function: 1) WAT, 2) brown adipose tissue (BAT) which plays an essential role in thermogenesis, especially in rodents, and 3) bone marrow fat which serves among other things as energy reservoir (Klaus, 2001). In the following the focus lies on WAT, since in humans, it quantitatively plays the most important role (Tanzi and Fare, 2009). WAT is in the first place an energy reservoir and essential for energy homeostasis. During periods of fuel surplus, glucose and fatty acids (FA) are taken up by adipocytes and converted into triglycerides for storage. De novo FA synthesis appears to play only a minor role in humans due to their habitual high fat intake (Löffler, 1998). The term lipogenesis encompasses the processes of fatty acid synthesis and triglyceride synthesis. During periods of fuel deprivation, the TAG are broken down by the adipocytes into FA and glycerol and released into the circulation; this process is termed lipolysis (Boschmann, 2001). WAT is also an important endocrine and secretory organ as discussed in chapter 1.2.2 and necessary for mechanical and thermal insulation (Klaus, 2001). For desert and marine mammals WAT may be, in addition to its property as energy reservoir important as source of metabolic water (Klaus, 2001).
1.2.2 WAT secretory function

WAT has been recognized as a critical secretory organ, releasing a wide range of molecules. The main groups of these molecules with examples are presented in figure 1, some of them will be addressed in detail in the following. Notably, FA are quantitatively the primary released substance from WAT, which are produced during the lipolysis of TAG.

Figure 1 Secretory products of white adipose tissue

White adipose tissue secretes a wide range of factors with the majority being synthesized de novo and only some molecules being converted from precursor molecules. This figure represents a number of selected molecules secreted by WAT. MT, metallothionein; SAA, serum amyloid A; TNF-α, tumour necrosis factor-α; IL, interleukin; MIF, macrophage migratory inhibitory factor; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation, normally T-expressed, and presumably secreted; SDF-1α, stromal cell-derived factor 1 alpha; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; LPL, lipoprotein lipase; ZAG, zinc-α2-glycoprotein; PG, prostaglandin (Bing and Trayhurn, 2009; Fischer-Posovszky et al., 2007; Kintscher et al., 2008; Peeraully et al., 2004; Skurk et al., 2009; Trayhurn, 2005).
1.2.2.1 Leptin

The gene of leptin was discovered by positional cloning in mice (Zhang et al., 1994). Leptin is a 16 kDa, cytokine-like hormone and is primarily secreted by WAT but its expression is also found in other organs (Trayhurn and Beattie, 2001). In obesity leptin expression is elevated and strongly correlates with body fat mass (Trayhurn et al., 2001). Leptin is a key regulatory protein in energy balance, being also involved in haematopoiesis, reproduction, the immune system and angiogenesis (Beltowski, 2006; Trayhurn and Beattie, 2001). In obesity, when circulating leptin levels are high, it may also contribute to endothelial dysfunction and atherosclerosis (Beltowski, 2006). Leptin promotes endothelial cell proliferation, migration and the formation of fenestrated capillaries and upregulates vascular endothelial growth factor (VEGF) expression (Beltowski, 2006; Christiaens and Lijnen, 2009). Its role in endothelial cell activation in the context of leukocyte infiltration is discussed controversially in the literature (Curat et al., 2004; Skilton et al., 2005). Leptin has several receptors belonging to the obese receptor (Ob-R) family. In endothelial cells leptin signals via the Ob-Rb receptor leading in the activation of the janus kinase signal transducer and activator of transcription (JAK/STAT) pathway (Sierra-Honigmann et al., 1998; Suganami et al., 2004). Leptin expression is upregulated by insulin, glucocorticoids, tumor necrosis factor alpha (TNF-α) and oestrogens and is suppressed by noradrenaline and adrenaline. The sympathetic nervous system which innervates WAT plays an important role in leptin regulation (Bartness and Bamshad, 1998; Trayhurn et al., 2001; Wabitsch et al., 1996).

1.2.2.2 Adiponectin

Adiponectin is an abundant circulating protein, is mainly secreted by mature adipocytes and exists in several distinct forms (Whitehead et al., 2006). Its expression is decreased in obesity and even further reduced in insulin resistance and type 2 diabetes (Christiaens and Lijnen, 2009). There also exists a sexual dimorphism in humans, with women having higher adiponectin plasma levels then men (Nishizawa et al., 2002). Besides its important role in metabolism (Yamauchi et al., 2002) it has anti-inflammatory properties and mediates beneficial effects in the vasculature in vivo and in vitro (Goldstein and Scalia, 2004). Adiponectin may function pro- and anti-angiogenic (Christiaens and Lijnen, 2009). It is downregulated by glucocorticoids, TNF-α, IL-6 and β-adrenoceptor agonists and upregulated by peroxisome proliferators-activated receptor gamma (PPARγ) agonists, e.g.
thiazolidindiones (Fasshauer and Paschke, 2003). Adiponectin signals via the adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) (Whitehead et al., 2006).

1.2.2.3 IL-6

IL-6 is a cytokine which is produced by many cell types and also by those of WAT. It is important in the regulation of inflammatory processes (Jones, 2005) but it also plays a role in carbohydrate and lipid metabolism (Glund and Krook, 2008). In obesity and insulin resistance, IL-6 plasma levels and WAT IL-6 expression are increased (Kim et al., 2009) and thus, it can contribute to the state of chronic low-grade inflammation. IL-6 is downregulated by glucocorticoids and upregulated by insulin, TNF-α and β-adrenoreceptor agonists (Fasshauer and Paschke, 2003). Its signalling is mediated via a tyrosine kinase associated receptor involving the JAK/STAT pathway (Kim et al., 2009).

1.2.2.4 Vascular endothelial growth factor (VEGF)

VEGF is necessary for vasculogenesis and angiogenesis during development and postnatal e.g. during pregnancy, wound healing and pathophysiologic conditions, including cancer, rheumatoid arthritis and cardiovascular disease. The complexity of the VEGF family and its receptors allows a broad range of biological actions not only in endothelial cells but also many other cell types (Ferrara and Davis-Smyth, 1997). The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-F and placenta growth factor (PIGF) and its receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) and belong to the family of receptor tyrosine kinases (Ferrara et al., 2003; Zachary and Gliki, 2001). VEGF-A is the most abundant form and VEGFR-2 appears to play a major role in endothelial VEGF signalling. VEGF is produced by many cell types, including endothelial cells, vascular smooth muscle cells, epithelial cells, preadipocytes and adipocytes (Fain et al., 2004; Ferrara and Davis-Smyth, 1997; Ferrara et al., 2003). VEGF is extremely important for endothelial cell survival or anti-apoptotic signalling, proliferation, migration, nitric oxide (NO) and prostacyclin (PGI₂) induction and vascular permeability (Zachary and Gliki, 2001). VEGF expression is regulated by hypoxia, several cytokines, including IL-1 and IL-6 and cell differentiation and transformation (Ferrara and Davis-Smyth, 1997).
1.2.2.5 Chemokines

Obesity is connected with an increased level of infiltrating immune cells in WAT (Kintscher et al., 2008; Weisberg et al., 2003), the latter being also source of a range of chemokines such as monocyte chemoattractant protein-1 (MCP-1) (Fain and Madan, 2005), CCL5, also called RANTES (regulated upon activation, normally T-expressed and presumably secreted; Skurk et al., 2009), II-8 (Fain and Madan, 2005) and stromal cell-derived factor 1 alpha (SDF-1α) (Kintscher et al., 2008). The production of inflammatory chemokines occurs in response to inflammatory cytokines, including II-1, TNF and interferons and bacterial toxins in most tissues and immigrating leukocytes with the aim to recruit leukocytes to inflamed and infected tissues (Baggiolini, 2001).

1.2.3 WAT morphology

WAT depots are distributed throughout the body and can be roughly categorized as visceral and subcutaneous. Small quantities can also be found within and around organs and blood vessels (Cinti, 2005). WAT is not homogeneous, rather it displays depot and sex-specific metabolic properties and is subject to differential neural and endocrine regulation. Body fat distribution generally differs between men and women even at comparable body fat content. Women tend to depot subcutaneous adipose tissue in the gluteo-femoral area whereas men in the abdominal region and normally also have larger amounts of visceral fat mass than women (Boschmann, 2001).

Mature adipocytes represent about 50% of the total cell content of WAT (Hausman, 1985) and store the TAG which in turn constitute up to 85 % of tissue weight (Trayhurn et al., 2006). The remaining stromal vascular fraction consists of many cell types, including preadipocytes, fibroblasts, endothelial cells, pericytes, blood and endothelial cells, diverse precursor cells and immune cells, such as macrophages and T-lymphocytes. Increased immune cell infiltration occurs in obesity (Cinti, 2001; Rausch et al., 2007; Weisberg et al., 2003) as discussed in chapter 1.2.7. All cells of WAT are involved in autocrine, paracrine and endocrine processes and cross-talks (Hauner, 2005) but their differential contribution to the total pool of adipokines is not clear (Antuna-Puente et al., 2008; Fain et al., 2008; Fain et al., 2004; Weisberg et al., 2003). Adipose tissue is highly vascularized (see chapter 1.2.5) and innervated by the sympathetic (Cinti, 2001) and the parasympathetic (Kreier and Buijs, 2007) nervous system.
White adipocytes have a diameter ranging from 10 to 150 µm which is due to the ability of the cells to accumulate different amounts of TAG in a single vacuole. The nucleus is located peripherally in white adipocytes which also have less well-developed mitochondria compared with brown adipocytes (Cinti, 2001; Cinti, 2009).

1.2.4 WAT growth and vasculature

1.2.4.1 Blood vessels, endothelial cells and endothelial dysfunction

Blood vessels are very heterogeneous in architecture and function but can be roughly categorized as macrovascular and microvascular (Aird, 2003; Aird, 2007; Aird, 2007; Steinbeck, 2002). The inner lining of the circulatory system consists of a single layer of endothelial cells adhering to the basal lamina which are the gatekeeper between the blood and tissues. In the large blood vessels of the macrovasculature, the endothelial cells are surrounded by vascular smooth muscle cells followed by a third layer, the adventitia which consists of connective tissue and elastic elements. In contrast, microvascular endothelial cells, the cells of the capillaries, in general reside on a basal lamina and are only supported by sparsely distributed pericytes. Microvascular endothelial cells are either of the continuous, the fenestrated or sinusoidal type, exert organ-specific activities and are necessary for gas and nutrient exchange (Randall et al., 1997). Overall, resting endothelial cells have four main functions: they maintain blood fluidity, regulate blood flow, control vessel wall permeability and quiesce circulating leukocytes. Endothelial dysfunction describes the situation when endothelial cells fail to appropriately perform any of these basal functions, although this term is often used to describe only the failure of arterial endothelial cells to produce sufficient of the vasorelaxant NO (Pober and Sessa, 2007). Importantly, endothelial cells can be activated by stimuli from the environment such as inflammatory mediators leading to a series of changes in the cells which enables them to offer a new repertoire of activities and receptors which are further adapted during transition from acute to chronic inflammation. Upon activation, the blood flow and the vascular leakage of plasma proteins may be elevated and the endothelial cells control the recruitment of leukocytes into the underlying tissue (see chapter 1.2.7.1). This process generally occurs in the microvascular beds in tissues, an exception is found in atherosclerosis where monocytes transmigrate over the macrovascular aortic wall following initial lipoprotein entrapment in the subendothelial matrix. Additionally,
upon activation, the microvascular endothelial cells are subject to angiogenesis in wounding or inflammatory processes (Kuldo et al., 2005; Pober and Sessa, 2007; van Hinsbergh, 2001; Zimmerman et al., 1999).

1.2.4.2 WAT growth

WAT growth requires continuous remodelling of the vascular network, mainly the microvasculature, which is then subject to neovascularization and/or remodelling of existing capillaries (Christiaens and Lijnen, 2009). WAT can increase either due to hypertrophy of existing adipocytes or hyperplasia where the number of adipocytes is increased (Schling and Loffler, 2002). The determining factors of fat mass in adults are not completely understood but increased lipid storage due to hypertrophy appears to be most important (Bjorntorp, 1974; Hirsch and Batchelor, 1976). In contrast, hyperplasia and the determination of adipocyte number appear to occur during childhood (Knittle et al., 1979; Spalding et al., 2008), since in adults the adipocyte number remains unchanged in lean and obese individuals even after marked weight loss which had been stable for two years at the time of the measurements (Spalding et al., 2008). Nevertheless, if it comes to massive increases in body weight and adipocytes have reached a critical size, hyperplasia also appears to occur (Hirsch and Batchelor, 1976) which is achieved by inducing adipogenesis. In this process undifferentiated fibroblast-like preadipocytes which are found in adipose tissue along with pluripotent precursor cells that can be induced to follow the path of adipose cell lineage, differentiate to mature adipocytes (Gregoire, 2001). Adipogenesis also takes place in normal weight individuals since adipose tissue is a plastic organ with an annual turnover of adipocytes of approximately 10 % (Spalding et al., 2008).

1.2.4.3 Adipogenesis

Some of the key features of adipogenic differentiation can be summarized as follows. Adipogenesis is a complex and tightly controlled process which is accompanied by dramatic changes in the extracellular matrix (ECM). For conversion of preadipocytes into adipocytes clonal expansion is necessary which is the re-entry of growth-arrested preadipocytes into the cell cycle and the progression through at least two cell-cycle divisions. At early differentiation CCAAT-enhancer binding proteins (C/EBP)β and δ are expressed and important to stop clonal expansion and increase the expression of PPARγ2. PPARγ2 heterodimerizes with
retinoic X receptors (RXR) and activates C/EBPα which exerts positive feedback on PPARγ2 until both transcription factors reach maximum levels as differentiation proceeds. This is paralleled by declining levels of C/EBPβ and δ. PPARγ2 and C/EBPα coordinate the activation of many late and terminally expressed genes in the adipocyte differentiation process e.g. aP2, GLUT-4 and phosphoenolpyruvate carboxykinase (PEPCK). Additionally, the insulin receptor number and sensitivity increases and adipokines such as adiponectin are expressed. Sterol responsive element binding protein 1c, also called adipocyte determination and differentiation factor 1 (ADD1/SREBP1) can increase the transcriptional activity of PPARγ while Forkhead box C2 (FOXC2) can enhance the activity of C/EBPα, PPARγ, and ADD1/SREBP1 (Farmer, 2006; Gregoire, 2001; Gregoire et al., 1998; Reed et al., 1977; Reed et al., 1981; Rosen and Spiegelman, 2000; Valet et al., 2002).

1.2.4.4 WAT vasculature

Organ function is critically dependent on tissue perfusion. In organ growth this is generally associated with a compensatory increase in vascular supply (angiogenesis), otherwise this may lead to organ dysfunction as e.g. in cardiac hypertrophy with arterial hypertension (Tomanek et al., 1986). Angiogenesis is tightly regulated reducing the likelihood of different pathologies including rheumatoid arthritis and diabetic retinopathy (Koch, 2003).

WAT is generally well vascularized but depot differences (Bjorntorp, 1996; Crandall et al., 1997) and differences within the tissue have been observed (Gersh and Still, 1945; Rutkowski et al., 2009). This may be due to specific depot-dependent cell replication rate as shown for rat endothelial cells (Hausman and Richardson, 2004). Overall, subcutaneous WAT is less vascularized than visceral WAT (Bjorntorp, 1996). Additionally, variation from individual to individual and age dependency in angiogenic potential is likely, e.g. aging and diabetes in obese mice impaired the neovascular potential of adipose-derived stromal cells (El-Ftesi et al., 2009).
1.2.5 WAT blood flow

Adipose tissue blood flow varies between species, depots and during different metabolic conditions and is regulated via the nervous system, hormones, exercise and diet (Crandall et al., 1997). For example, subcutaneous WAT resting blood flow expressed as ml per 100 g tissue per minute varies extremely in dogs (3 to 19 ml), rats (5 to 40 ml), rabbits (1.37 to 22 ml) and humans (0.5 to 8.63 ml) (Di Girolamo et al., 1971; Larsen et al., 1966). Additionally, in humans total adipose tissue blood flow is elevated in the obese, reflecting the increased total adipose mass (Lesser and Deutsch, 1967). On the other hand, the blood flow per unit weight of adipose tissue in humans (Adams et al., 2005; Lesser and Deutsch, 1967) and in dogs (Di Girolamo et al., 1971) is reduced with increasing fat mass. An explanation for these observations is given in the following. In humans, moderate gain weight is mainly achieved by hypertrophy of adipocytes (Bjorntorp, 1974; Hirsch and Batchelor, 1976)(see chapter 1.2.4.2) and thus, it appears as if depots containing larger fat cells contain fewer fat cells per unit of wet weight. In vivo experiments in dogs (Di Girolamo et al., 1971), humans (Jansson et al., 1992) and other species (Crandall et al., 1997) show that when the blood flow is related to the number of adipocytes, the blood flow per cell is relatively constant, irrespectively of fat cell size. In humans the blood flow is 20-30 pl/cell/min (Blaak et al., 1995). These results are supported by the observation that each adipocyte appears to be in contact with at least one capillary in rats (Gersh and Still, 1945). Altogether, this explains the great variety of WAT resting blood flow between species and why, in obese individuals, the blood flow per unit weight of adipose tissue is decreased.
1.2.6 WAT hypoxia

A lack of oxygen availability, a state which is called hypoxia, leads to several adaptation processes in an organism. At cellular level, the affected cells counteract with adapting their energy metabolism by switching to anaerobic glycolysis and induce mechanisms in order to prevent cell death and to induce vascular supply (e.g. angiogenesis) (Brahimi-Horn et al., 2007; Semenza, 2002). The key transcription factor involved in the transmission of the hypoxic response is the hypoxia-inducible transcription factor 1 (HIF-1) (Brahimi-Horn and Pouyssegur, 2009) which is composed of one of the HIF-α-subunits (HIF-1α, HIF-2α and HIF-3α) and HIF-1β. HIF-1β is constitutively expressed but not regulated by oxygen. In contrast, HIF-1α, being the most prominent member of the α-subunits is constitutively expressed and degraded at the same time. Upon hypoxia the protein is stabilized and the gene expression is increased. HIF-1α has an oxygen-dependent degradation domain which contains two regulatory proline residues which are under normoxia hydroxylated by prolyl hydroxylase domain-containing enzymes (PHD) 1 to 4. This allows binding of von Hippel-Lindau tumor suppressor protein (pVHL) which forms an E3-ubiquitin ligase complex with co-factors which allows for subsequent poly-ubiquitination and degradation of HIF-1α in the proteasome. When oxygen and or Fe^{2+} are missing, the PHDs become inactive and HIF-1α protein accumulates (Brahimi-Horn et al., 2007; Brahimi-Horn and Pouyssegur, 2009). HIF-1 activates a series of genes, ranging from molecules being involved in cell proliferation, cell survival, angiogenesis, vascular tone, cell adhesion, cytoskeletal structure and extracellular matrix metabolism to energy metabolism (Semenza, 2003). Notably, HIF-1 is also alternatively regulated by heat shock proteins, cytokines, growth factors and oncogenes. For example, TNF-α has been found to accumulate and activate HIF-1 (Brahimi-Horn and Pouyssegur, 2009; Hellwig-Burgel et al., 2005). Therefore, HIF-1 may be upregulated in many biological situations in tissues but not due to hypoxia and so conclusions should be drawn carefully.

Hypoxia occurs in normal physiology (e.g. development of tissues and exercise) but also in pathophysiology (e.g. tumours and wound healing). It also occurs in the interstitial stromal areas of WAT, especially in obese mice as measured with in vivo oxygen sensor techniques and pimonidazole stainings (Rausch et al., 2007; Ye et al., 2007; Yin et al., 2009). An explanation may be that the increasing diameter of fat cells during adipose tissue growth, despite constant vascularization per adipocyte (Crandall et al., 1997), may exceed the oxygen
diffusion limit of 100 µm (Carmeliet and Jain, 2000; Rausch et al., 2007). Additionally, it is conceivable that the decreased WAT vascularization per weight may especially decrease the oxygen availability of the other homing cells in WAT located more than 100 µm away from the next capillary, since their vascular supply appears not to be cell number dependent. It is not known whether the observed hypoxia is intermittent or chronic. Supporting evidence for hypoxia in human WAT is an increased HIF-1α expression in adipose tissue of obese individuals which decreases after surgery-induced weight loss (Cancello et al., 2005) bearing in mind that this could be also due to other mechanisms than hypoxia. HIF-1α protein expression was also found to be enhanced in hypoxic human adipocytes (Wang et al., 2007) and the specific role of hypoxia in the induction of HIF-1α in adipose tissue was shown in mice (Ye et al., 2007). The glucose uptake is increased in hypoxic human adipocytes in vitro by increased GLUT-1 expression. This is due to increased demand of glucose because the occurring glycolysis during hypoxia produces less adenosine triphosphate (ATP) per glucose molecule (Wood et al., 2007). Adipogenesis appears to be regulated by oxygen sensitive mechanisms and preadipocyte differentiation is inhibited under hypoxia (Carriere et al., 2004; Lin et al., 2006; Macfarlane, 1997; Swiersz et al., 2004; Yun et al., 2002). Additionally, children possessing cyanotic heart disease had correlated less body fat (Baum and Stern, 1977). Rats exposed to hypoxia significantly lost weight (Tanaka et al., 1997). Importantly, human microvascular endothelial cells sustain human preadipocyte viability under hypoxia (Frye et al., 2005). It has been proposed, that hypoxia may lead to an inflammatory response in adipocytes to increase the blood flow and stimulate angiogenesis (Trayhurn and Wood, 2004). Severe hypoxia leads to a proinflammatory response in human and murine adipocytes in vitro (Trayhurn et al., 2008; Wang et al., 2007; Ye, 2009). Examples of the upregulated target genes and proteins include VEGF, leptin, PAI-1, migration inhibitory factor (MIF). Concurrently, adiponectin expression appears to be downregulated. A first direct relationship between hypoxia and insulin resistance has been shown in vitro for murine and human adipocytes where the autophosphorylation of the insulin receptor was impaired upon hypoxia exposure which was quickly reversed, after only 45 min reoxygenation (Regazzetti et al., 2009). Additionally, hypoxia may play a role in adipocyte death which preferentially occurs in WAT of obese mice and humans and is positively correlated with adipocyte hypertrophy in contrast to lean mice and humans where adipocyte death is seldom. In WAT the dead adipocytes are surrounded by macrophages which together form so-called crown-like structures (Cinti et al., 2005; Surmi and Hasty, 2008).
1.2.7 WAT immune cell infiltration

Many cell types reside in WAT, including immune cells such as T-lymphocytes and macrophages with their number being increased in obese mice (Weisberg et al., 2003; Xu et al., 2003) and humans (Cancello et al., 2006; Curat et al., 2006). It has been suggested that especially the macrophages in WAT induce insulin resistance by promoting a proinflammatory environment (Surmi and Hasty, 2008). As yet, it is neither clear what initiates the increased immune cell infiltration nor the sequence in which the different immune cell types appear in WAT. Severe hypertrophy of adipocytes, being mostly connected with fatty acid flux, hypoxia (see chapter 1.2.6), adipocyte cell death, increased leptin secretion (see chapter 1.2.2.1) and endothelial dysfunction (1.2.4.1) may be all contributing factors (Surmi and Hasty, 2008).

There is evidence, that ahead of monocyte infiltration into WAT, recruitment of neutrophils (Elgazar-Carmon et al., 2008) and T-lymphocytes (Kintscher et al., 2008) occurs. One study in mouse WAT showed that neutrophil recruitment transiently precedes monocyte infiltration as it is also known from several other inflammatory diseases. An important chemoattractant for neutrophils is IL-18 which is also expressed by WAT (Elgazar-Carmon et al., 2008; Skurk et al., 2005). The expression of RANTES and its receptor CCR5 is increased in WAT of obese mice and has been attributed for the paralleled elevated number of CD3+ T-lymphocytes (Rausch et al., 2007; Wu et al., 2007). Similar observations were made in WAT of obese individuals, where RANTES and CCR5 gene expression correlated with gene expression of macrophage markers (Wu et al., 2007). Other chemoattractants for the recruitment of T-lymphocytes besides RANTES, are MCP-1, interferon-inducible protein 10 (IP-10) and SDF-1α, known to be secreted by several cell types, including preadipocytes and adipocytes (Kintscher et al., 2008).

The number of T-lymphocytes in WAT correlates with the waist circumference of type 2 diabetes patients. Additionally, it was shown in a mouse model of high fat diet induced obesity, that T-lymphocyte infiltration precedes the accumulation of macrophages in WAT during the development of insulin resistance (Kintscher et al., 2008). At one stage during WAT growth monocytes infiltrate the tissue and differentiate to macrophages producing further cytokines such as TNF-α and IL-1 (Mosser, 2003), which are very potent proinflammatory activators of endothelial cells (Petzelbauer et al., 1993), adipocytes (Wang and Trayhurn, 2006) and other cell types. Additionally, an in vitro co-culture study between
macrophages and adipocytes showed that the macrophage TNF-α release interferes with adipocyte insulin signalling and induces fatty acid lipolysis (Suganami et al., 2005). These mechanisms altogether may result in a positive feedback loop and are visualized in figure 2.

Monocyte recruitment may be initiated by an activated endothelium and adipocytes, preadipocytes and other cells residing in WAT, producing chemoattractants such as MCP-1 and IL-8 (Fain and Madan, 2005; Fain et al., 2004; Gerszten et al., 1999). Additionally, T-lymphocytes secrete IFN-γ which in turn may stimulate other cell types, such as preadipocytes to increase their MCP-1 secretion and activate other cells, including endothelial cells and macrophages. Staining of human WAT showed that the majority of the T-lymphocytes were CD4⁺, the subset of lymphocytes producing IFN-γ (Kintscher et al., 2008).

Macrophages can be distinguished in M1- classically activated - and M2- alternatively activated - subpopulations, as well as intermediate types having M1 and M2 characteristics and are found in WAT (Bourlier et al., 2008; Lumeng et al., 2007; Zeyda et al., 2007). M1 macrophages are induced by inflammatory agents, have phagocytotic activity, predominantly secrete pro-inflammatory cytokines and are important in the initiation of inflammation. M2 macrophages were induced by IL-4 and IL-13 and secrete predominantly anti-inflammatory cytokines having a reparative and remodelling function by promoting the formation of new blood vessels and are important in terminating inflammatory process (Bourlier et al., 2008; Mosser, 2003). In WAT of lean mice the M2 polarized macrophages appear to play a dominant role while in WAT of obese mice the M1 polarized macrophages take over (Lumeng et al., 2007). In human WAT the macrophage population appears to be of the intermediate type, having both M1 and M2 characteristics. The M1 characteristics may be important in order to phagocytose dead adipocytes and explain the crown-like structures found in WAT (Cinti et al., 2005), while the M2 characteristics may contribute to repair and remodelling of WAT during expansion. Interestingly, phagocytosis of a dead cell can induce an M2 phenotype in macrophages (Kintscher et al., 2008).
I: Some adipokines (e.g. TNF-α) are able to activate the endothelium. This is characterized by the expression of selectins and adhesion molecules such as ICAM-1 and VCAM-1 and the release of chemokines e.g. from the Weibel-Pallade Bodies. II: Activated monocytes and other immune cells adhere to the adhesion proteins on the endothelium via integrins which is followed by transmigration. III: After the diapedesis the monocytes differentiate into macrophages. Activated macrophages release large amounts of TNF-α and IL-1 and several chemokines. IV: The released cytokines from macrophages activate the endothelium itself and trigger the adipocytes and preadipocytes to change their secretion to a more inflammatory pattern.

CAMs, cellular adhesion molecules; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein 1-alpha; RANTES, regulated upon activation, normally T-expressed, and presumably secreted; SDF-1α, stromal cell-derived factor 1 alpha; TNFα, tumour necrosis factor-α.
1.2.7.1 Mechanisms of leukocyte infiltration in tissues – Extravasation

The endothelium as the interface between circulating blood and immune cells and adipose tissue plays an interactive role in these infiltration and inflammatory processes (Pober and Sessa, 2007). In this context, the MAPK and JAK pathways mediating extracellular signals and the intracellular signal-regulated transcription factors STAT, NFκB and AP-1 play important roles (Hoefen and Berk, 2002; Kuldo et al., 2005; Pober and Sessa, 2007).

Interaction between leukocytes and the endothelial cell lining occurs in great complexity in physiologic and pathophysiologic conditions. During inflammation the microvascular endothelial cells tightly control the recruitment of leukocytes, including lymphocytes, monocytes and neutrophils into the underlying tissue, a process being termed extravasation (Randall et al., 1997) which consists of leukocyte rolling, activation, slow rolling, arrest/adhesion, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration through the basement membrane (Ley et al., 2007). The series of complex molecular and cellular interactions involved in this process is given schematically together with the known key molecules involved in each step in figure 3.

Activation of microvascular endothelial cells via potent inducers such as TNF-α, IL-1 and interferon gamma (IFN-γ) results in the presentation of a range of adhesion proteins and chemokines at their luminal surface. The rolling of leukocytes is mediated via selectins, with P-selectin (CD62P) and E-selectin (CD62E) being most important and expressed at the cell surface of endothelial cells which interact with glycoprotein ligands e.g. P-selectin glycoprotein ligand 1 (PSGL1) being expressed on almost all leukocytes and Sialyl-Lewis x being constitutively expressed on granulocytes and monocytes and activated b-lymphocytes and T-helper 1 (Th1) lymphocytes.

The rolling allows close contact between leukocytes and endothelium, the latter presenting chemokines e.g. MCP-1, IL-8, IL-6 and RANTES or other chemotactic compounds at the luminal surface; either released by the endothelial cells themselves, transported by them from their abluminal site or generated by proteolytic cleavage in activated mast cells and platelets, and delivered to endothelial cells through circulating microparticles or exocytosis of intracellular granules. These chemokines activate the leukocytes which involves the conformational change of integrins also called “activatable receptors” which are then
presented at the cells surface with high-affinity. Integrins also participate in rolling but especially mediate leukocyte arrest and firm adhesion together in the presence of chemokines. Depending on the leukocyte subset and the type of endothelial cells, different combinations of adhesion molecules, cytokines and chemokines are necessary for rolling, adhesion and transendothelial migration.

**Figure 3  The leukocyte adhesion cascade**

The leukocyte adhesion cascade consists of the following steps: capture, rolling, slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular transmigration. Key molecules involved in each step are indicated in boxes. CD99, cluster of differentiation 99; ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as αLβ2-integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; SRC kinases, sarcoma kinase; VAV, vav guanine nucleotide exchange factor; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as α4β1-integrin).

Well studied integrins are αLβ2 integrin also called lymphocyte function-associated antigen 1 (LFA-1) and CD11a-CD18, α4β1 integrin, also known as very late antigen 4 (VLA-4) and CD49d-CD29 and αMβ2 integrin, also known as macrophage antigen 1 (MAC-1) and CD11b/CD18. The leukocyte integrins then bind to immunoglobulin superfamily members, typically intercellular adhesion molecule 1 (ICAM-1; CD54) and vascular cell adhesion molecule 1 (VCAM-1; CD106) which are expressed by activated endothelial cells finally leading to leukocyte arrest.
LFA-1, expressed by monocytes, macrophages, T-lymphocytes and granulocytes and dencritic cells and MAC-1 expressed by monocytes, macrophages and granulocytes bind to ICAM-1 while VLA-4 is expressed on most leukocytes and binds to VCAM-1. Adhesion strengthening occurs by so-called outside-in signalling through integrins where the integrins mediate intracellular signals which regulate several cellular functions such as cell motility, proliferation and apoptosis. This is followed by leukocyte crawling mediated via MAC-1/ICAM-1 interactions, where in postcapillary venules monocytes and neutrophils crawl inside blood vessels in order to find preferred sites of transmigration/diapedesis. Although, leukocytes have to pass the endothelial cells, endothelial cell basement membrane and pericytes, transmigration is connected with minor disruption of the complex morphology of vessel walls. Transmigration preferentially occurs via the paracellular but also the transcellular route as shown in vivo and in vitro, the latter especially if crawling is impaired. The series of molecules involved in transmigration are presented in figure 2. In the tissue the leukocytes migrate along a chemokine gradient until the site of inflammation is reached and monocytes differentiate to macrophages (Imhof and Aurrand-Lions, 2004; Ley et al., 2007; Lim et al., 2003; Middleton et al., 2002; Vestweber, 2000).

ICAM-1 is the most extensive studied molecule of the ICAM family which consists of five members. ICAM-1 spans the cell membrane and contains a short cytoplasmic tail. It has five Ig-like domains, the first domain being responsible for LFA-1 and the third domain for MAC-1 binding. Besides its important role in leukocyte trafficking it also participates in signal transduction across cell membranes. Ligand binding may result in the activation of transcription factors, increased production of cytokines, cell membrane protein expression, ROS and cell proliferation (Hubbard and Rothlein, 2000). ICAM-1 is expressed constitutively at low levels on endothelial and other cells. Stimulation of endothelial cells with TNF-α, IL-1, IFN-γ, hydrogen peroxide (H₂O₂) and lipopolysaccharide (LPS) either alone or in combination leads to a strong upregulation of ICAM-1. Its expression is regulated mainly via protein kinase C (PKC), the mitogen-activated protein kinases (MAPK) extracellular-regulated kinases (ERK), SAPK/JNK and p38, the JAK/STAT and the NFκB intracellular signal transduction pathways. The major nuclear transcription factors for the activation of ICAM-1 expression are activator protein 1 (AP-1), NFκB, C/EBP, E-twenty six (Ets), STAT and Sp1. IFN-γ is the traditionally classic molecule which induces ICAM-1 gene expression via the JAK/STAT pathway (Roebuck and Finnegan, 1999).
1.2.8 Study of adipose tissue and adipocytes

The study of WAT physiology in vivo is generally conducted in animal models (Garofalo et al., 1996; Rayner, 2001) but also in humans by using the microdialysis technique, adipose tissue vein cannulation, and measurement of blood flow by $^{133}$Xenon washout and stable isotope tracers (Summers, 2006). In vivo experiments allow observing the full integrated physiological response to a stimulus. Consequently, certain investigations, such as the response to fasting, can only be performed using live animals.

Much of our knowledge of complex in vivo phenomena are based on studies performed in simplified in vitro model systems under controlled conditions (Kiely et al., 1999). In order to study the effects of different ascendants on isolated cells under defined conditions and to get insights how distinct cell types under different biological circumstances such as WAT expansion interact and how they contribute to the pool of pro-inflammatory and anti-inflammatory molecules, in vitro studies are indispensable (Farmer, 2006).

The study of WAT and adipocytes in vitro is performed by culturing tissue extracts (Fain and Madan, 2005) and primary mature adipocytes (Rodbell, 1964), primary preadipocytes (Hauner et al., 2001) or immortalized preadipocyte cell lines (Chen et al., 1989; Green and Meuth, 1974; Rodriguez et al., 2004), respectively.

Primary mature adipocyte culture is considered to be the most true-to-life in vitro system, since the adipocytes are derived directly from a donor. However, the separation process of mature adipocytes from the freshly dissected WAT by collagenase digestion (Rodbell, 1964) is also stressful, e.g. leading to an increase of inflammatory adipokine expression (Eisele et al., 2005; Ruan et al., 2003). In addition, the life span of these cells is limited from hours to several days.

The conversion of preadipocytes into adipocytes in vitro is achieved by exposing the cultured preadipocytes to a mixture of molecules which include insulin, glucocorticoids, isobutylmethylxanthine (IBMX) (Gregoire et al., 1998) and thiazolidinediones (Hausman et al., 2008). IGF-1, rather than insulin is the physiological inducer of adipocyte differentiation (Gregoire et al., 1998; Smith et al., 1988). However, in cell culture experiments generally unphysiologically high concentrations of insulin are used to activate the IGF-1 receptor, due to
insulins low binding affinity for this receptor which is expressed in high numbers on preadipocytes in contrast to a small number of insulin receptors which begin to increase only after induction of differentiation (Gregoire et al., 1998; Reed et al., 1977; Reed et al., 1981; Smith et al., 1988). IBMX functions via intracellular cAMP level accumulation and increases together with glucocorticoids C/EBP-β expression which is required for subsequent PPARγ expression (Gregoire et al., 1998; Wiper-Bergeron et al., 2007) whereas thiazolidinediones function as direct high-affinity ligands and activators of PPAR-γ (Hausman et al., 2008).

Primary preadipocytes are obtained from the stromal vascular fraction of collagenase-digested WAT (Hauner et al., 2001). They are then taken into culture conditions and differentiated into adipocytes. These cells can be passaged several times without losing their capacity of adipocyte differentiation (Skurk et al., 2007) before they stop proliferating. In contrast, the human Simpson-Golabi-Behmel syndrome (SGBS) cell strain, which consists of unmanipulated stromal vascular cells derived from WAT of an infant with the SGBS, can be passaged over several months and by the same time remain their capacity for adipocyte differentiation (Wabitsch et al., 2001).

Clonal cell lines are widely used in the study of adipocytes (Chen et al., 1989; Green and Meuth, 1974; Rodriguez et al., 2004). They provide a homogeneous cell population and can be ideally passaged indefinitely. In practice they are easier to use than primary cells. However, in contrast to primary cells, the distance from their original source is greater and they have changed at least some properties due to the genetic changes which allow for immortality (Masters, 2002). Murine clonal preadipocyte lines which derive from embryonic mouse fibroblasts are 3T3-L1 (Green and Meuth, 1974) and 3T3-F422A cells (Chen et al., 1989). A new human clonal cell culture system has been described using multipotent adipose-derived stem cells (hMADS) (Rodriguez et al., 2004).
1.2.9 Study of endothelial cells in vitro

Important findings on endothelial cell function come from *in vitro* experiments by culturing either microvascular or macrovascular endothelial cells derived from a variety of organs (Bouis *et al.*, 2001; Hillyer and Male, 2005) including WAT (Curat *et al.*, 2004). As primary endothelial cells often human umbilical vein endothelial cells (HUVECS) (Gimbrone, 1976) and human aortic endothelial cells (HAEC) are used (Krishnaswamy *et al.*, 1999). The best characterized vascular endothelial cell lines are ECV304 (Takahashi *et al.*, 1990), EA.hy926 (Edgell *et al.*, 1983) and human microvascular endothelial cells 1 (HMEC-1) (Ades *et al.*, 1992). The behaviour of endothelial cells strongly depends on their vascular origin. HUVECS, ECV304 and EA.hy926 are used for the study of macrovascular endothelial cells and HMEC-1, simian-virus-human cerebromicrovascular endothelial cells (SV-HCEC) and human telomerase reverse transcriptase – human dermal microvascular endothelial cells (hTERT-HDMEC) for the study of microvascular endothelial cells (Bouis *et al.*, 2001). HMEC-1 cells have been derived from human foreskins by transfection with large T antigens of simian-virus 40 (SV40) (Ades *et al.*, 1992). They have morphological, phenotypical and functional characteristics of microvascular endothelial cells (Bouis *et al.*, 2001). A comparison between HMEC-1, ECV304 and EA.hy926 with regard to induced expression and surface antigens in the context with leukocytes and transmigration found HMEC-1 to be most similar to the primary endothelium (Lidington *et al.*, 1999). It was found that for investigating microvascular endothelial cells HMEC-1 and hTERT-HDMEC are the most appropriate models (Bouis *et al.*, 2001).
1.2.10 Study of cross-talk between cell types

Coculture experiments are commonly conducted to study the molecular and cellular cross-talk between distinct cell types in vitro. There are three types of coculture techniques described and named as ‘coculture’ in the literature: firstly, coculture using transwell plates, here cells in two different compartments separated from each other via a membrane share the same medium. Secondly, coculture where cell types are cultured on the same surface or in a three-dimensional matrix which allows direct cell-cell interactions besides sharing the same medium. Thirdly, indirect coculture where the cell culture medium of cells of a distinct cell type, called conditioned medium (CM), is used to stimulate other cells, e.g. cells of another cell type (Armbrust and Rohl, 2008; Laterra et al., 1990; Lumeng et al., 2007). Since in coculture experiments both cell types either have to share the same medium or the CM of one cell type is used to stimulate the other, it is experimentally of importance to use or develop a cell culture medium which is suitable for both cell types.

To increase the knowledge in WAT biology it is important to understand how distinct cell types under different biological conditions such as WAT expansion interact and how they contribute to the pool of pro-inflammatory and anti-inflammatory molecules. The role of distinct cell types in orchestrating WAT immune cell infiltration is not clear (Surmi and Hasty, 2008) but the contribution of other cell types beside adipocytes is likely, representing 50 % of the total cell content (Hausman, 1985). In the context of immunological mechanisms, preadipocytes are of special interest due to their ability of displaying a macrophagic or endothelial potential according to their environment (Casteilla et al., 2005). Transcriptome profiling demonstrated a closer relationship between preadipocytes and macrophages than between preadipocytes and adipocytes and the conversion of preadipocytes into macrophages has been shown to be efficient and rapid (Charriere et al., 2003). Proliferating preadipocytes also develop phagocytotic activity towards microorganisms (Cousin et al., 1999; Villena et al., 2001).

An informative in vitro technique for studying the interactions between endothelial cells and immune cells in infiltration processes are adhesion assays (Kiely et al., 1999). In these assays the firm binding of leukocytes to the endothelium is observed. The degree of adhesion varies with the magnitude of the activation of either the endothelial cells, the leukocytes or both cell types (Ley et al., 2007). Human monocyte-endothelial cell-cell adhesion is studied commonly
by using human primary monocytes (Curat et al., 2004), peripheral blood mononuclear cells (PBMC) (Mills et al., 2006), the human histiocytic lymphoma cell line (U937 cells) (Sundstrom and Nilsson, 1976) or the human acute monocytic leukaemia cell line (THP-1 cells) (Tsuchiya et al., 1980) as sources of monocytes.

PBMC consist of a mixture of blood cells having a round nucleus, including lymphocytes and monocytes. PBMC are generally isolated from whole blood by the hydrophilic polysaccharide ficoll which separates blood to its cellular components. The buffy coat, consisting of monocytes and lymphocytes, is generated under a layer of plasma (Roitt et al., 2006).

U937 cells are derived from the histiocytic lymphoma of a 37 year old male patient (Sundstrom and Nilsson, 1976). These cells have been extensively characterized and are used to study monocytic behaviour, including the differentiation to macrophages (Harris and Ralph, 1985).

As yet, two papers have been published analyzing the effect of conditioned medium (CM) from mature adipocytes isolated from adipose tissue on monocyte-endothelial cell-cell adhesion. The study by Curat et al., 2004 investigated the increased macrophage accumulation in WAT of obese people. In the study by Kralisch et al., 2007 the influence of CM from mature adipocytes on macrovascular endothelial cell function was analysed in the context of obesity and atherosclerosis. In contrast, the impact of adipocytes differentiated from preadipocytes in vitro on endothelial cell activation has not been studied so far. The studies mentioned above neglected the role of preadipocytes in the context of the molecular and cellular cross-talk with endothelial cells and underlying molecular signalling pathways have only been sparsely investigated.

Overall, the nature of the preadipocyte and adipocyte-derived factors and the cellular and molecular cross-talk which mediate endothelial activation and promote the infiltration processes of immune cells into adipose tissue are rather poorly understood. Therefore, it will be important to apply different in vitro coculture systems between preadipocytes, adipocytes and endothelial cells and cell biological, molecular and biochemical methods in order to dissect the differential gene expression and secretion pattern of preadipocytes and adipocytes and their impact on endothelial cell function. This should also give new insights into the mechanisms of endothelial cell activation and dysfunction which occurs in WAT or other organs in the course of obesity and its related disorders.
1.3 Aim of the study

Obesity is associated with a state of chronic low-grade inflammation which is thought to contribute strongly to the development of features of the metabolic syndrome. A hallmark of inflammation is the infiltration of immune cells such as macrophages and T-lymphocytes into inflamed tissue. Obesity is connected with an increased number of immune cells, especially macrophages, in white adipose tissue (WAT) in humans and mice. The microvascular endothelium as the interface between adipose tissue and circulating immune cells in the blood plays an interactive role in these infiltration processes. Endothelial cell activation, which is a change in phenotype and/or function in endothelial cells in response to stimuli from the environment, is a prerequisite for controlling the recruitment of leukocytes into the underlying tissue. It is a regulated event in physiologic vascular responses which can lead if dysregulated under pathophysiologic conditions, to endothelial cell dysfunction. The mature and hypertrophic adipocytes are generally considered to be the major adipogenic cell type secreting proinflammatory cytokines in WAT. In contrast, the proinflammatory capacity of preadipocytes and their impact under different biological conditions on endothelial cell activation has been widely neglected in the research field so far. Therefore, the overall aim of this study was to gain new insights into the cellular interactions and molecular mechanisms and to identify factors involved in this cross-talk of preadipocytes, adipocytes and endothelial cells.

The primary experimental aim of this study was to establish a human coculture system and sensitive assays in order to mimic the processes mentioned above in vitro. This was achieved by establishing a co-culture system between the conditioned medium (CM) of human primary SGBS preadipocytes/adipocytes and human microvascular endothelial cells, HMEC-1 cells and measuring endothelial cell activation by monocyte endothelial cell-cell adhesion. The interactions were studied using the following methods: cell culture of HMEC-1 cells, SGBS cells, U937 cells and primary mature adipocytes, monocyte endothelial cell-cell adhesion assay, cell surface ELISAs, proliferation assay, ELISAs and multiplex bead-based Luminex® assays, reverse transcriptase-polymerase chain reactions (RT-PCR) and quantitative real time PCR (qRT-PCR), Western blotting, inhibitor experiments, functional analysis, and peptidomic analysis.
Use of these techniques should allow the analysis of the following:

1. adipokine expression and secretion of SGBS preadipocytes and adipocytes under different biological conditions e.g. normoxia and hypoxia

2. the impact of CM from non-stimulated and stimulated SGBS preadipocytes and adipocytes, mimicking different biological conditions, on endothelial cell function by investigating monocyte-endothelial cell-cell adhesion, endothelial cell proliferation and signalling pathways

3. functional analysis of mediators in the CM of preadipocytes and adipocytes responsible for changes in monocyte-endothelial cell-cell adhesion

4. the effect of CM from different-sized primary mature adipocytes on monocyte-endothelial cell-cell adhesion

5. detection of candidate peptides in the CM of SGBS cells, possibly involved in the molecular cross-talk between preadipocytes, adipocytes and microvascular endothelial cells, by a peptidomics approach.
2 Materials and Methods

2.1 Cell Culture

2.1.1 Primary cell culture and size fractionation of human mature adipocytes

2.1.1.1 Reagents

Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (DMEM/F12; Invitrogen)
MCDB 131 (Invitrogen)
Bovine serum albumin (BSA; Sigma-Aldrich)
Collagenase 250U/mg (Biochrom)
Krebs-Ringer phosphate (KRP) buffer
- 154 mM NaCl (Sigma-Aldrich)
- 154 mM KCl (Sigma-Aldrich)
- 11 mM CaCl$_2$ (Merck KgaA)
- 154 mM MgSO$_4$ (Sigma-Aldrich)
- 100 mM NaH$_2$PO$_4$ (Sigma-Aldrich)
- adjustment to pH 7.4, sterile filtration

2.1.1.2 Theoretical Background and Method

For theoretical background information refer to chapter 1.2.8. Adipose tissue was obtained from healthy patients undergoing elective plastic surgery or abdominal surgery after having obtained informed consent. There was no selection for BMI, age or gender. The tissue was collected under sterile conditions and stored in DMEM/F12 medium and was immediately transported to the laboratory. Mature adipocytes were isolated according to the protocol by Rodbell (Rodbell, 1964) with the following modifications on the day of surgery: connective tissue and blood vessels were excised. The remaining WAT was minced into small pieces. Collagenase digestion of WAT was performed in KRP buffer containing 4 % BSA and 100 U/ml collagenase at 37 °C for not more than 1 hr in a shaking water bath (80 rpm). Floating cells were aspirated and filtered twice, first through a 2 mm and next through a 250 µm mesh. The cells in the flow-through were washed three times with KRP buffer containing 0.1 % BSA and were finally collected by aspiration of the wash solution.
Separation of adipocytes by size was achieved by flotation as described recently (Skurk *et al.*, 2007). Briefly, 10 ml of isolated adipocytes were transferred to a 100 ml separating funnel filled with 50 ml KRP buffer containing 0.1 % BSA. The cell-buffer suspension was gently mixed by inversion and cells were allowed to float for 60 sec before 35 ml of the suspension, containing the small cells, was collected. Next, 35 ml KRP buffer containing 0.1 % BSA was added to the remaining suspension in the separating funnel followed by mixing as described above. The adipocytes of medium size were removed by allowing them to float for 45 sec and 30 sec, respectively, and 35 ml of suspension was discarded and then refilled with buffer after each step. The remaining large adipocytes in the KRP buffer containing 0.1 % BSA in the separating funnel were collected. The protocols were approved by the ethical committee of the Technische Universität München.

The cell diameters of 100 cells from the original sample and both fractions were determined by light microscopy in order to calculate the fat cell count, volume and cell surface area. The conditioned medium (CM) was generated by culturing 1 ml of cells in 5 ml MCDB 131 medium at 37 °C for 16 hrs in an incubator with a humidified atmosphere of 5 % CO₂ and 95 % air. The cell volume (V) and the cell surface area (A) were calculated from the cell diameter (d) using the formulas $V = \frac{1}{6}\pi d^3$ and $A = \pi d^2$, respectively.

### 2.1.2 SGBS cell culture

#### 2.1.2.1 Reagents

**Proliferation medium**
- DMEM/F12 (Invitrogen) supplemented with:
  - 100 U/ml penicillin 100 µg/ml streptomycin (Invitrogen)
  - 33 µM biotin (Roth)
  - 17 µM D-pantothenat (Sigma-Aldrich)
  - 10 % fetal calf serum (FCS; Invitrogen)

**Adipose medium**
- 1/3:2/3 mixture of DMEM/F12 and MCDB 131 (Invitrogen) supplemented with:
  - 100 U/ml penicillin 100 µg/ml streptomycin
  - 11 µM biotin
  - 6 µM D-pantothenat
  - 66 nM insulin (Sigma-Aldrich)
  - 1 nM triiodothyronine (T₃; Sigma-Aldrich)
  - 100 nM hydrocortisone (Sigma-Aldrich)
  - 10 µg/ml transferrin (Sigma-Aldrich)
Induction medium
- Adipose medium supplemented with:
  - 2 µM rosiglitazone (Cayman)
  - 25 nM dexamethasone (Sigma-Aldrich)
  - 0.5 mM Isobutylmethylxanthine (IBMX; Serva)

MCDB 131 (Invitrogen)

Phosphate buffered saline (PBS) w/o Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (Sigma-Aldrich)
- 1 tablet PBS per 200 ml dH\textsubscript{2}O yields
  10 mM phosphate buffer
  2.7 mM KCL
  0.137 M NaCl
- adjustment to pH 7.4, sterile filtration

Trypsin/EDTA solution (PAA)
- PBS w/o Ca\textsuperscript{2+} and Mg\textsuperscript{2+}
- 0.5 mg/ml trypsin
- 0.75 mM ethylenediaminetetraacetic acid (EDTA)

2.1.2.2 Theoretical Background and Method

For theoretical background information refer to chapter 1.2.8. Preadipocytes from the SGBS cell strain (Wabitsch et al., 2001), kindly obtained from Prof. Wabitsch (Department of Pediatrics and Adolescent Medicine, University of Ulm, Ulm, Germany) were cultured as described recently (Mack et al., 2009). In detail: SGBS cells were cultured at 37 °C in an incubator with a humidified atmosphere of 5% CO\textsubscript{2} and 95% air. In experiments with hypoxia the cells were cultured at 37 °C in an incubator with a humidified atmosphere of 1% O\textsubscript{2}, 5% CO\textsubscript{2} and 94% N\textsubscript{2} or 4% O\textsubscript{2}, 5% CO\textsubscript{2} and 91% N\textsubscript{2}, respectively (Hypoxia workstation, InVivo O2 400, IUL Instruments).

SGBS preadipocytes were proliferated in proliferation medium. Subconfluent cells were split by washing once with PBS and treatment with trypsin/EDTA solution at 37 °C for 5 min. The reaction was stopped by adding proliferation medium. The cells were plated at the following densities: 10,000 cells per well of a 6-well cell culture plate and 600 cells per well of a 96-well cell culture plate. The scheme of the cell culture strategy is shown in figure 10.

To obtain differentiated adipocytes, confluent preadipocytes were washed 3 times with PBS and induced with induction medium (d0). Preadipocytes serving as uninduced controls were cultured in adipose medium. After 4 days of induction of differentiation (d4), the media of all cells were replaced with adipose medium and the cells were fed twice per week for additional 10 days (d14). Adipocyte differentiation was detectable only in cell cultures that were initially supplemented with adipogenic differentiation inducers (induction medium). This was observed in 80% or more of all cells. To obtain CM from SGBS cells without any additional
supplements the cell culture conditions were shifted from adipose medium to MCDB 131 medium after 14 days of differentiation. Depending on the experimental conditions, MCDB 131 medium was changed to fresh medium at day 15 (d15), and the cells were kept under normoxic or hypoxic conditions for 24 hrs (d16) or were stimulated with TNF-α (d16). The corresponding supernatants representing the CM (d16) were centrifuged at 1000 g for 5 min and passed through a 0.25 µm nylon filter. CM derived from hypoxic cells were reoxygenated for at least 15 minutes and not more than 1 hr in 50 ml tubes under the cell culture hood. Then, CM were either used directly or concentrated for coculture experiments, or were immediately frozen in liquid nitrogen and stored at -80 °C.

For concentrating the CM, ultrafiltration centrifugal filter devices (Amicon Ultra centrifugal filter device) were applied. The procedure was carried out according to the manual instructions. Molecules larger than 5 kDa accumulated in the concentrate and the medium was exchanged by fresh MCDB 131 medium in order to remove any remaining molecules of less than 5 kDa. The permeate contained the non-concentrated molecules of less than 5 kDa.

2.1.3 HMEC-1 cell culture

2.1.3.1 Reagents

HMEC-1 medium
- MCDB 131 (Invitrogen) supplemented with:
  - 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen)
  - 10 % FCS (Invitrogen)
  - 2 mM L-glutamine (Invitrogen)
  - 10 ng/ml epidermal growth factor (EGF; Immunotools)
  - 2.8 µM hydocortisone (Sigma-Aldrich)

MCDB 131 (Invitrogen)

PBS w/o Ca²⁺ and Mg²⁺ (Sigma-Aldrich)
- 1 tablet PBS per 200 ml dH₂O yields
  10 mM phosphate buffer
  2.7 mM KCl
  0.137 M NaCl
- adjustment to pH 7.4, sterile filtration

Trypsin/EDTA solution (PAA)
- PBS w/o Ca²⁺ and Mg²⁺
- 0.5 mg/ml trypsin
- 0.75 mM EDTA
2.1.3.2 Theoretical Background and Method

For theoretical background information refer to chapter 1.2.9. HMEC-1 cells (Ades et al., 1992; Bouis et al., 2001), obtained from the Centers for Disease Control and Prevention, Atlanta, USA, were cultured as has been reported elsewhere (BelAiba et al., 2007; Mack et al., 2009). In detail: HMEC-1 cells were proliferated in HMEC-1 medium and only used up to passage 30 in order to maintain their phenotypic endothelial characteristics. Cell splitting was performed at subconfluency by one PBS washing step and trypsinisation at 37 °C for 5 min. The reaction was stopped with HMEC-1 medium. The cells were either plated on 6 cm dishes, 6-well cell culture plates or 96-well cell culture plates at cell densities ranging between 1 x 10^5 cells per ml and 1 x 10^4 cells per ml. Generally, the experiments were performed with confluent HMEC-1 cells. Ahead of experiments, HMEC-1 cells were starved in MCDB 131 medium overnight (14-16 hrs). In all experiments the cells were cultured at 37 °C in an incubator with a humidified atmosphere of 5 % CO_2 and 95 % air.

2.1.4 U937 cell culture

2.1.4.1 Reagents

U937 medium
- Roswell Park Memorial Institute 1640 medium (RPMI 1640; Invitrogen) supplemented with:
  - 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen)
  - 10 % FCS (Invitrogen)

2.1.4.2 Theoretical Background and Method

The U937 cell line was derived from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma (Sundstrom and Nilsson, 1976). U937 cells display many monocytic characteristics and can differentiate to macrophages by a series of stimuli (Harris and Ralph, 1985). Monocytic U937 cells, supplied by Helmut Laumen (Department of Nutritional Medicine, Technische Universität München, Freising, Germany) were cultured as has been published elsewhere (Mack et al., 2009). In detail: U937 suspension cells were cultured in U937 medium. Cell passaging was performed by centrifugation at 200 g for 5 min. The supernatant was discarded and the cells were resuspended in U937 medium at densities between 5 x 10^4 per ml and 5 x 10^5 cells per ml and subcultured. For experiments, the cell
number was adjusted to $5 \times 10^5$ cells per ml one day before the experiments. In all experiments the cells were cultured at 37 °C in an incubator with a humidified atmosphere of 5 % CO$_2$ and 95 % air.

2.1.5  **Coculture between CM of SGBS cells and HMEC-1 cells**

For detailed information of the coculture strategy refer to chapter 3.1.6. Starved HMEC-1 cells were treated under serum-free conditions with MCDB 131 medium (control), MCDB 131 medium supplemented with TNF-α, IL-6, VEGF, leptin or cocultured with fresh and concentrated CM of SGBS cells or CM of human primary mature adipocytes for different time periods. In inhibitor experiments, HMEC-1 cells were pretreated with JNK inhibitor II, SB-203580, PD-98059, or JAK inhibitor 1 for 45 min before the coculture with CM supplemented with the corresponding inhibitor at the appropriate concentration. When IL-6 inhibitor experiments were performed, the CM were preincubated with 5 mg/ml monoclonal anti-human IL-6 antibody or the corresponding immunoglobulin G1 (IgG$_1$) isotype control for 15 min. For blockade of TNF-α activity, the CM were preincubated for 10 min with Enbrel® (Tracey *et al.*, 2008), a soluble TNF-α receptor antagonist.

2.1.6  **Coculture and monocyte-endothelial cell-cell adhesion assay**

2.1.6.1  **Theoretical Background**

In various inflammatory processes, leukocytes adhere to and subsequently migrate across locally activated endothelial lining to form exudates. In order to study these monocyte endothelial cell interactions, a monolayer adhesion assay *in vitro* under static conditions is useful to characterize the adhesion-stimulatory or inhibitory properties of substances acting on either the leukocyte or endothelial cell. Moreover, ligand-receptor interactions involved in leukocyte adhesion can be analysed (Kiely *et al.*, 1999). In this assay the monocytes were labelled with the fluorogenic dye *Calcein green AM* (De Clerck *et al.*, 1994). *Calcein green AM* passes as nonfluorescent molecule through the cell membrane of viable cells. Inside the cell, it is hydrolysed by intracellular esterases producing a negatively charged green fluorescent calcein that is retained in the cytoplasm. Thus, the degree of *Calcein green AM* labelled monocytes adhering to treated and control endothelial cells can be determined by
comparing the fluorescence data. The fluorescence increases linearly after cell lysis with cell numbers ranging between 30 and 3000 U937 cells per well of a 96-well cell culture plate (see chapter 7.5.1) and without lysis with cell numbers ranging between 200 and 16 200 Hela cells per well of a 96-well cell culture plate (Manual: Calcein AM cell viability assay kit, catalog number: 30026, Biotium, Inc. [Hayward, USA]).

2.1.6.2 Reagents

PBS w/o Ca\(^{2+}\) and Mg\(^{2+}\) (Sigma-Aldrich)
- 1 tablet PBS per 200 ml dH\(_2\)O yields
  10 mM phosphate buffer
  2.7 mM KCl
  0.137 M NaCl
- adjustment to pH 7.4, sterile filtration

*Calcein green AM* stock solution
- 1mM *Calcein green AM* (Invitrogen) in dimethyl sulfoxide (DMSO; Sigma-Aldrich)

Lysis buffer
- 50 mM Tris-HCL (Sigma-Aldrich)
- 0.1 % sodium dodecylsulfate (SDS; Sigma-Aldrich) in dH\(_2\)O
- adjustment to pH 8.2 to 8.4

2.1.6.3 Method

Monocyte-endothelial cell-cell adhesion between HMEC-1 and U937 cells was carried out as described elsewhere (Kong *et al.*, 2004) with the following modifications (Mack *et al.*, 2009; Seebach, 2006): the assays were performed in 96-well cell culture plates. Confluent HMEC-1 cells were treated with 100 µl MCDB 131 medium (control), MCDB 131 supplemented with TNF-α, IL-6, VEGF or leptin, fresh/concentrated CM of SGBS cells or CM of human primary mature adipocytes for 6 hrs. For the adhesion assay U937 cells were washed twice with PBS, then loaded with *Calcein green AM* (5 µM per 5 x 10\(^6\) cells) at 37 °C for 30 min and again washed twice with MCDB 131 to remove extracellular *Calcein green AM*. Next, after 6 hrs treatment HMEC-1 cells were washed twice with MCDB 131 medium, and 2.5 x 10\(^5\) *Calcein green AM* labeled U937 cells per well were added to HMEC-1 cells and incubated at 37 °C for 1 hr. Non-adherend U937 monocytes were removed by washing four times with PBS. The remaining cells in the well were incubated in lysis buffer at 37 °C in the dark for 5 min. Lysed cells were centrifuged at 3500 g for 5 min, and the fluorescence of the supernatants was measured at 494 and 517 nm using a spectrometer (Varioskan multiwell...
photo- and fluorometer, Thermo Scientific). HMEC-1 cells and unlabeled U937 cells were used as blanks. Samples were analysed at least in four replicates. An example for the analysis of the monocyte-endothelial cell-cell adhesion data is given in chapter 7.5.2.

2.2 Cell surface expression analysis by cell-ELISA

2.2.1 Theoretical Background

Leukocyte adhesion to endothelial cells lining the vasculature is mediated by a series of adhesion molecules such as ICAM-1 and VCAM-1 (Ley et al., 2007). The cell-ELISA is a technique to study cell surface antigens under different stimulations (Morandini et al., 2001). Therefore, an antigen is detected on live or fixed cells using the ELISA principals (chapter 2.6.1.1). Comparative studies showed that the cell-ELISA technique can be as sensitive and specific as flow cytometry (Grunow et al., 1994; Ogino et al., 2003).

2.2.2 Reagents

PBS with Ca\(^{2+}\) and Mg\(^{2+}\)
- 1 tablet PBS per 200 ml dH\(_2\)O (Sigma-Aldrich)
- 0.84 mM MgCl\(_2\) (Merck KGaA)
- 0.72 mM CaCl\(_2\) (Merck KGaA)
- adjustment to pH 7.4, sterile filtration

4 % paraformaldehyde (PFA; Sigma-Aldrich) in PBS with Ca\(^{2+}\) and Mg\(^{2+}\), adjustment to pH 7.4

Washing buffer
- PBS with Ca\(^{2+}\) and Mg\(^{2+}\)
- 0.5 % Tween 20 (v/v; Sigma-Aldrich)

Blocking buffer
- PBS with Ca\(^{2+}\) and Mg\(^{2+}\)
- 1 % BSA (Sigma-Aldrich)
- 30 % donkey serum (Chemicon International)

Goat anti-human ICAM-1 antibody (R&D Systems)
Goat anti human VCAM-1 antibody (R&D Systems)
horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody (Dianova)
tetramethylbenzidine (TMB; Sigma Aldrich)
1 M H\(_2\)SO\(_4\) (Serva)
2.2.3 Method

The cell-ELISA assay was carried out as described elsewhere (Amin et al., 2006; Shiojima and Walsh, 2002; Zhu et al., 2003) with the following modifications (Mack et al., 2009): the experiments were conducted in 96-well cell culture plates. Confluent HMEC-1 cells were stimulated with MCDB 131 medium (control), TNF-α, IL-6, VEGF, leptin, fresh CM of SGBS cells or CM of human primary mature adipocytes for 6 hrs. The cells were washed with cold PBS supplemented with Mg$^{2+}$ and Ca$^{2+}$ (PBS + Mg/Ca) and then fixed with 4% PFA at 4 °C for 10 min. After two PBS + Mg/Ca washing steps, the cells were incubated with blocking buffer at RT for 1 h and then exposed to goat anti-human ICAM-1 or VCAM-1 antibody (1:200 dilution in blocking buffer) at RT for 1 hr. Next, the cells were rinsed twice and then washed three times with washing buffer for 5 min followed by incubation with HRP-conjugated donkey anti-goat antibody (1:5000 dilution in blocking buffer) at RT for 30 min. After the cells were rinsed twice and washed three times with washing buffer for 5 min, 200 µl TMB per well was added as substrate. The reaction was stopped after 15 min with 50 µl 1M H$_2$SO$_4$. The absorbance was measured at 450 nm and 690 nm using a spectrometer (Varioskan multiwell photo- and fluorometer, Thermo Scientific). The data were normalized to the blank controls, where at least two wells with TNF-α treated HMEC-1 cells were incubated without the first antibody (ICAM-1 or VCAM-1 antibody). Initial experiments with isotype-matched control antibodies did not detect non-specific binding from the secondary antibody. Samples were analysed at least in duplicate. The analysis of the cell-ELISA data was similar to the approach used for the monocyte-endothelial cell-cell adhesion data (see chapter 7.5.2).
2.3  Proliferation assay

2.3.1  Theoretical Background

5-bromo-2-deoxyuridine (BrdU) is used for the detection of cell proliferation by assessing DNA synthesis. BrdU is a synthetic nucleotide, an analogue of thymidine and can be incorporated into newly synthesized DNA of replicating cells during the S-phase. Using a specific antibody against BrdU, allows detection of replicating cells (Schutte et al., 1987; Schutte et al., 1987) with the ELISA technique (chapter 2.6.1.1).

2.3.2  Reagents

Cell Proliferation ELISA; BrdU (colorimetric; Roche)
1 M H₂SO₄ (Serva)

2.3.3  Method

DNA synthesis was assessed by BrdU-labelling according to the manufacturer´s instructions. In brief, HMEC-1 cells were seeded in 96-well cell culture plates at a density of 1500 cells per well and exposed to different media or factors, such as MCDB 131 (control), MCDB 131 containing IL-6, leptin or VEGF and CM, all supplemented with 1% FCS. After 48 hrs, BrdU was added at a final concentration of 10 µM for 16 hrs. Incorporated BrdU was detected with HRP-conjugated anti-BrdU antibody using TMB as a substrate. The reaction was stopped with 1M H₂SO₄ and the absorbance was measured at 450 nm and 690 nm using a spectrometer (Varioskan multiwell photo- and fluorometer, Thermo Scientific). At least three wells were incubated without the first antibody (BrdU) and served as blank controls.
2.4 RNA extraction, DNAse I treatment and RNA integrity check

2.4.1 Reagents

Trizol® (Invitrogen)
Chloroform (Roth)
Isopropanol (Roth)
75 % Ethanol (Roth)
Nuclease-free water (Sigma-Aldrich)
DNA-free™ kit (kit containing DNAse I; Ambion)

Reagents for agarose gel electrophoresis
- sample buffer [40 % glycerol (Merck KGaA), 60 % ddH₂O, 0.01 % bromphenol blue (Merck KGaA)]
- agarose (Sigma-Aldrich)
- 0.5x TBE [45 mM Tris (Roche), 4 mM boric acid (Merck KGaA), 1 mM EDTA (Merck KGaA), adjustment to pH 8]
- 0.5 µg/ml EtBr (Sigma-Aldrich)

2.4.2 Theoretical Background and Method

RNA isolation using Trizol® is based on the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006). It is a liquid-liquid extraction technique and allows isolating RNA, protein and DNA from the same sample. Total RNA was isolated from the cells using the Trizol® protocol according to the manufacturer’s instructions with the following modifications: for RNA extraction from cells, the media of 2 wells from a 6-well cell culture plate were aspirated and 1 ml of Trizol® added. In order to maximize the cell homogenisation, the Trizol®-cell mixture was triturated 12 times using a 1 ml syringe with a 23 gauge needle. This was followed by incubation with 200 µl chloroform and centrifugation at 12000 g at 4°C for 15 min, leading to the formation of three layers: aqueous, interphase and phenol. The aqueous layer containing the RNA was transferred to a new tube and incubated with 50 µl of isopropanol. After centrifugation at 12000 g at 4°C for 10 min the RNA in the supernatant is likely to contain less contaminating DNA. Next, 450 µl isopropanol was added to the supernatant in order to precipitate the RNA. After centrifugation at 12000 g at 4 °C for 10 min the RNA pellet was washed with 1 ml 75 % ethanol in order to remove salts. The RNA pellet was then dissolved in nuclease-free water.
and subjected to DNAse I treatment (Vanecko and Laskowski, 1961) according to the manufacturer’s instructions in order to minimize contaminating DNA in the sample.

RNA samples were quantitated by spectrophotometry at 260 nm using the Beer-Lambert law (Beer, 1852). The 260 to 280 nm absorbance ratio was between 1.7 and 1.9. RNA integrity was analysed by agarose gel electrophoresis of total RNA and visualization of the 18S and 28S ribosomal RNA (rRNA) bands. Briefly, 0.8 µg of total RNA was loaded onto a 1% agarose gel containing 0.5 µg/ml ethidium bromide (EtBr) and 0.5x tris-borate-EDTA (TBE) buffer. For RNA detection a UV transilluminator was used and the results recorded using a digital camera (INTAS UV Systeme, Intas Science Imaging Instruments) Distinct bands for 28S and 18S rRNA forming about a 2:1 ratio of staining intensities were observed. Additionally, the RNA integrity of some samples was confirmed by analysis with the Bioanalyser from Agilent Technologies. The RNA Integrity Number (RIN) was then between 9 and 10. The highest RIN which can be achieved is 10 (Schroeder et al., 2006).

2.5 Reverse transcription (RT), polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR)

2.5.1 Theoretical background

RT-PCR and qRT-PCR are powerful and sensitive techniques to detect gene expression. In the reverse transcription step the enzyme reverse transcriptase (Baltimore, 1995) extends an oligonucleotide primer hybridized to a single-stranded (ss) RNA or DNA template in the presence of deoxynucleotide triphosphates (dNTPs) producing a complementary DNA strand. These DNA strands are referred to as cDNA which is further used as template for the PCR. During this process, one complementary DNA (cDNA) of interest is amplified using gene-specific primers, dNTPs and the DNA Taq polymerase. RT-PCR is used for qualitative and qRT-PCR for both qualitative and quantitative detection of gene expression.

In general, the PCR process (Kleppe et al., 1971; Mullis, 1990) begins with a denaturation phase at 94 °C followed by 15 to 40 amplification cycles and in the qRT-PCR process (Giulietti et al., 2001; VanGuilder et al., 2008) by 40 amplification cycles. Each cycle consists of three steps: denaturation, annealing and extension. Denaturation at 94 °C results in the dissociation of double-stranded (ds) DNA into ssDNA. The annealing temperature (T_A) is
primer specific depending on primer length and GC content. The duration of the extension step increases with amplicon size, but is usually 20 to 60 s.

The PCR process is characterized by three phases: exponential, linear and plateau. In the exponential phase the amplicon ideally doubles every cycle, the reaction is very specific and precise. In the linear phase, the reaction components have been consumed and products start to degrade until finally, the plateau phase is reached where the reaction has stopped.

PCR products are analysed at the endpoint of the reaction by subjecting them to an agarose gel electrophoresis. For visualisation, EtBr intercalates into the DNA and fluorescence under UV light. In contrast, in qRT-PCR experiments the accumulation of the amplicon is detected during the reaction. The data for the quantitative analyses are acquired from the exponential phase of the reaction. Four different chemistries are available for qRT-PCR – TaqMan®, Molecular Beacons, Scorpions® and SYBR® Green. The TaqMan® and SYBR® Green chemistries were used in this work.

2.5.1.1 SYBR® Green

SYBR® Green intercalates into dsDNA and upon excitation emits light. Thus, the fluorescence increases with PCR product accumulation. In contrast to the TaqMan® chemistry, SYBR® Green also binds to primer dimers and other dsDNA unspecific PCR products, leading to enhanced fluorescent signals. This problem can be minimized with well designed primers and the performance of a melting curve analysis at the end of the reaction where the dissociation-characteristics of double-stranded DNA during heating are investigated. The DNA is slowly heated from 60 °C to 95 °C. dsDNA products are dissociated at a specific temperature, depending on their strand length, GC content and complementarity. Intercalated dye is released in parallel, leading to a decrease in fluorescence. The specific PCR products can be distinguished from the unwanted by-products by their higher melting temperature (Giulietti et al., 2001; VanGuilder et al., 2008).
2.5.1.2 TaqMan®

TaqMan® probes are oligonucleotides which have a fluorescent reporter dye attached to the 5’ end and a quencher coupled to the 3’ end. The proximity of both molecules prevents the detection of a fluorescent signal from the probe. TaqMan® probes are designed to hybridize to the internal region of the PCR product. The Taq polymerase has also a 5’-3’-exonuclease-activity. During PCR, the 5’- nuclease activity of the polymerase cleaves the probe. This leads to the decoupling of the fluorescent and quenching molecules and consequently, to increasing fluorescence with each cycle, proportional to the amount of probe cleavage (Giulietti et al., 2001; VanGuilder et al., 2008).

2.5.1.3 Primer design

Two different primer design strategies were followed. Firstly, the DNA-sequence of one primer of the primer pair spanned an intron/exon boundary of the specific gene of interest. Thus, the primers will anneal only to the cDNA synthesized from the spliced mRNAs, but not to cDNA from unspliced RNA or genomic DNA. Secondly, the primers flanked at least one intron. If the intron(s) are large enough only amplicons of the cDNA should be formed. Otherwise, the products amplified from the cDNA will be smaller than those amplified from genomic DNA. Thus, they can be distinguished by size.

2.5.2 RT

2.5.2.1 Reagents

High capacity cDNA reverse transcriptase kit (Applied Biosystems)

2.5.2.2 Method

For cDNA synthesis, 1 µg of total RNA was reverse transcribed using random primers, according to the manufacturer’s instructions. Controls without reverse transcriptase were carried out to exclude the possibility of DNA contamination. Subsequently, a PCR reaction
using primers specific for human β-actin was performed in order to control the success of the RT.

2.5.3 PCR

2.5.3.1 Reagents

HotStarTaq DNA Polymerase kit (Qiagen)

Reagents for agarose gel electrophoresis
- sample buffer [40 % glycerol (Merck KGaA), 60 % ddH₂O, 0.01 % bromphenol blue (Merck KGaA)]
- agarose (Sigma-Aldrich)
- 0.5x TBE [45 mM Tris (Roche), 4 mM boric acid (Merck KGaA), 1 mM EDTA (Merck KGaA), adjustment to pH 8]
- 0.5 µg/ml EtBr (Sigma-Aldrich)

Primer (Eurofins MWG, see appendix)

2.5.3.2 Method

PCR analysis was carried out in 20 µl reactions using cDNA equivalents of 20 ng of RNA. The reactions were carried out according to the manufacturer’s instructions. Detection of mRNA for the human β-actin served as endogenous reference gene expression for HMEC-1 cells and PBMCs. The PCR amplification parameters were one initial 15-min denaturation step followed by 26 (β-actin) or 35 cycles (gp130, IL-6Rα, Ob-Rb and VEGFR-2) of denaturation, annealing, and elongation (95 °C for 15 s, specific Tₐ for 30 s, and 72 °C for 30 s, respectively) and a final step at 72 °C for 10 min.

Finally, sample buffer was added to each sample prior to loading onto a 1 % agarose gel containing 0.5 µg/ml EtBr and 0.5x TBE as buffer. For detection of PCR products a UV transilluminator was used and the results recorded using a digital camera (INTAS UV Systeme, Intas Science Imaging Instruments).
2.5.4 qRT-PCR

2.5.4.1 Reagents

TaqMan® Universal PCR Master Mix, No Amp Erase UNG (Applied Biosystems)
SYBR® Green PCR Master Mix (Applied Biosystems)
TaqMan® Primer and Probes (Applied Biosystems; see appendix)
Primer for qRT-PCRs with SYBR® Green (Eurofins MWG; see appendix)

2.5.4.2 Method

Quantitative real-time PCRs were carried out in 20 µl reactions using cDNA equivalents of 20 ng of RNA. Reagents for qRT-PCR and the ABI 7700 sequence detection system were used according the the manufacturer’s instructions. 18S rRNA and the mRNA of the gene for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) served as endogenous reference for SGBS cells and HMEC-1 cells. Samples were analysed at least in duplicate using one initial activation-step at 95 °C for 10 min, and, subsequently, 40 cycles of denaturation, annealing and elongation (95 °C for 30 s, 60 ºC for 30 s, 72 ºC for 45 s, respectively). This was followed by melting curve analysis when SYBR® Green was used (95 °C for 15 s, 60 ºC for 15 s, gradual heating to 95 ºC within 20 min, 95 ºC for 15 s)

For quantitation of qRT-PCR data, the ΔΔCt method (Livak and Schmittgen, 2001) was employed where the expression of the target gene is normalized to the expression of the endogenous reference gene and compared with the expression of the untreated controls.
2.6 Measurement of adipokines in CM

2.6.1 Theoretical Background

Adipokine concentrations were measured in the CM by sandwich ELISAs (Engvall and Perlmann, 1971; Lequin, 2005; Van Weemen and Schuurs, 1971) and multiplex bead-based Luminex® assays (Elshal and McCoy, 2006; Horan and Wheeless, 1977; Morgan et al., 2004). Both methods are highly sensitive, specific and rapid immunochemical tests used to detect molecules that have antigenic properties in a given sample.

2.6.1.1 ELISA

The sandwich ELISA measures the amount of antigen between two layers of antibodies therefore two different antibodies (capture antibody and detection/primary antibody) are used. They are specific for the same antigen, but bind to different antigenic sites. First, the capture antibody is bound to an ELISA microplate surface; this is followed by a blocking step in order to prevent non-specific binding. Next, the antigen-containing sample is added to the plate. The antigen binds to the capture antibody and is immobilized. Extensive washing steps remove the unbound constituent parts of the sample. Next, the detection or primary antibody binds to the captured antigen and a “sandwich” is formed. Washing removes the excess of unbound primary antibody. An enzyme-linked secondary antibody (e.g. HRP-linked secondary antibody) is added which binds specifically only to the Fc-region of the detection antibody. A final washing step removes excess unbound secondary antibody. A suitable substrate (e.g. TMB) is applied which is converted by the enzyme into a colour or fluorescent signal. Next, the absorbance or fluorescence is measured.
2.6.1.2 Multiplex bead-based Luminex® assays

This technology is used to simultaneously detect secreted proteins or signal transduction molecules within a single sample and applies the principle of a sandwich ELISA. In contrast to the ELISA, the capture antibody is attached to polystyrene beads and not to a microplate well. The beads are internally dyed with different ratios of two spectrally distinct fluorophores (red and near-infrared). Such, they can be distinguished from each other (Probst et al., 2003). The detection of cytokine-bound beads is achieved with phycoerythrin (PE)-labelled streptavidin which binds to the biotinylated detection antibodies.

2.6.2 Reagents

ELISA kits for adiponectin (R&D Systems), leptin (R&D Systems), MCP-1 (eBioscience), PAI-1 (Technoclone), and IL-6 (eBioscience)

Bio-plex cytokine reagents (Bio-Rad)

Bio-plex cytokine assays for IL-8, VEGF 121 and 165, TNFα, IL-1α, IL-1β, IFNγ, RANTES, IL-4, IP-10 and SDF-1α (Bio-Rad)

2.6.3 Method

Adiponectin, leptin, MCP-1, PAI-1, and IL-6 were measured in the CM by ELISA, with the detection limits of 3.9 ng/ml, 15.6 pg/ml, 62.5 pg/ml, 3.8 ng/ml and 12.5 pg/ml, respectively. The assays were performed according to the manufacture’s instructions. The protein concentrations for IL-8, VEGF 121 and 165, TNFα, IL-1α, IL-1β, IFNγ, RANTES, IL-4, IP-10 and SDF-1α in the CM were measured by a multiplex bead-based Luminex® assay. The detection limits were depending on the analyte and varied between 1 and 10 pg /ml. The assay was performed according to the manufacture’s instructions. The data of the CM were normalized to the total protein content of the corresponding cells.

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2.7 Protein determination

2.7.1 Bicinchoninic acid (BCA) method

2.7.1.1 Reagents

BCA Protein Assay Kit including BSA protein standard (Pierce)

2.7.1.2 Theoretical Background and Method

The BCA method (Smith et al., 1985) is based on the Biuret method where a Cu$^{2+}$ ion forms a complex with a peptide bond in alkaline solution and is then reduced to a Cu$^{+}$ ion. The extinction of this violet complex can be measured and the protein concentration calculated according to the Beer-Lambert law (Beer, 1852). The sensitivity of the Biuret reaction can be increased in the presence of BCA. Two molecules of BCA chelate a single Cu$^{1+}$ ion, forming a purple water-soluble complex which strongly absorbs light at 562 nm. Protein detection ranges between 0.5 µg/ml and 2 mg/ml. The assay was carried out in 96-well microplates and protein detection was performed according to the manufacturer’s instructions. BSA was used for the protein standard curve.

2.7.2 Modified Bradford method

2.7.2.1 Theoretical Background

This method is based on the Bradford procedure (Bradford, 1976), but is carried out in 96-well microplates. It relies on the ability of Coomassie® Brilliant Blue G-250 to shift its absorbance maximum in an acidic solution from 465 nm to 595 nm when protein binding occurs. More precisely, Coomassie® Brilliant Blue G-250 primarily binds to arginine and to less extend to other aromatic and basic amino acid residues.
2.7.2.2 Reagents

Modified Bradford buffer stock
- 11.5 % H$_3$PO$_4$ (Serva)
- 4.75 % ethanol (Roth)
- 0.005 % Coomassie® Brilliant Blue G-250 (Merck KGaA)

Modified Bradford buffer
- Modified Bradford buffer stock
- 7 µl 10 M NaOH (Sigma-Aldrich)/ml Biorad buffer stock

BSA protein standard solutions ranging from 0.5 to 3 µg/µl (Pierce)

2.7.2.3 Method

The assay was carried out in 96-well microplates. 1 µl BSA standard or sample was pipetted at least in duplicate into the appropriate wells. Next, 250 µl of fresh Modified Bradford buffer was added. The plate was placed for at RT 20 min on a shaking platform. Finally, the absorbance was measured at 595 nm using a spectrometer (Varioskan multiwell photo- and fluorometer, Thermo Scientific)

2.7.3 Amido Black method

2.7.3.1 Reagents

Amido Black staining solution
- 0.1 % Amido Black 10B (Merck KGaA)
- 40 % methanol (Roth)
- 40 % acetic acid (Roth)
- 20 % H$_2$O

Amido Black decoulourizer
- 50 % methanol
- 50 % H$_2$O

BSA protein standard ranging from 0.5 µg to 4 µg (Sigma-Aldrich)

2.7.3.2 Theoretical Background and Method

Amido Black 10B is a diazo dye which is traditionally used to stain precipitated total protein on a membrane (Schaffner and Weissmann, 1973). Here, Amido Black 10B was used to
control and estimate the protein content of samples which were dissolved in sample-buffer prior loading onto a SDS-polyacrylamid-gel. Therefore, a polyvinylidene difluoride (PVDF) membrane was equilibrated in methanol. Next, 1 µl of BSA standard or sample were dotted on the membrane. When the membrane had dried, it was subjected to Amido Black staining solution for 2 min. Unspecific staining of the membrane was removed by Amido Black decolourizer. The comparison of the staining intensities between the samples and the standards by eye allowed estimating the protein content of the samples.

2.8 SDS-Polyacrylamidgelelectrophoresis (SDS-PAGE) and Western blot analysis

2.8.1 Theoretical Background

The Western blot technique (Burnette, 1981; Eckert and Kartenbeck, 1997; Towbin et al., 1979) is used for specific protein detection in a given sample of tissue, homogenate or extract. The proteins are separated by SDS-PAGE, transferred to a membrane and finally detected using antibodies. In the loading buffer, the proteins are initially denatured using heat, SDS and mercaptoethanol. SDS destroys hydrogen bonds and hydrophobic and ionic interactions and mercaptoethanol reduces disulfide bonds; thus, a straightened primary structure of the proteins results. After the breakdown of secondary structures, SDS binds to the proteins leading to an attachment of 1.4 g of SDS per 1 g of protein; thus, there is an equal proportion of mass to charge. The samples are then subjected to SDS-PAGE. By using this process the proteins separate according to their sizes.

The negatively charged proteins are transferred from the gel to a nitrocellulose or PVDF membrane by electroblotting (Burnette, 1981; Eckert and Kartenbeck, 1997). A specific primary antibody is then used which recognizes its antigen on the proteins on the blot. Next a secondary antibody binds to the constant region (Fc) of the bound primary antibody. The Fc of the secondary antibody is labelled with HRP. In the presence of H₂O₂ and alkaline conditions HRP oxidizes cyclic diacylhydrazides, such as luminol which is then in an excited state. This chemiluminescent reaction emits light at a maximum emission of 428 nm for up to one hour and can be detected by exposure of the membrane to a blue-light sensitive autoradiography film.
2.8.2 Reagents

Radioimmunoprecipitation assay buffer (RIPA-buffer)
- 50 mM tris-HCl pH 7.4 (Sigma-Aldrich)
- 1 % nonyl enoxyl-polyethoxylethanol (NP-40; Sigma-Aldrich)
- 0.25 % SDS (Sigma-Aldrich)
- 150 mM NaCl (Sigma-Aldrich)
- 1 mM EDTA (Merck KGaA)
- 1 mM phenylmethanesulfonyl-fluoride (PMSF; Sigma-Aldrich)
- 1 mM dithiothreitol (DTT; Omni Life Science)
- 10 mM NaF (Sigma-Aldrich)
- 1 tablet/10 ml Complete Mini (Roche)
- 1 tablet/10 ml PhosStop (Roche)

5 x Loading buffer
- 300 mM Tris-HCl pH 6.8
- 5 % SDS
- 40 % glycerol (Merck KGaA)
- 0.05 M DTT
- 2.5 mM EDTA
- 0.01 % bromphenol blue (Merck KGaA)

SDS-PAGE:

Running gel
- 7.5-10 % acrylamide (Roth)
- 375 mM Tris-HCl pH 8.8
- 1 % SDS
- 1 % Ammonium persulfate (APS; Sigma-Aldrich)
- 0.04 % (v/v) tetramethyl-ethylenediamine (TEMED; Roth)

Stacking gel
- 5 % acrylamide
- 125 mM Tris-HCl pH 6.8
- 1 % SDS
- 0.75 % APS
- 0.1 % (v/v) TEMED
- 0.005 % bromphenol blue

10x Running buffer (pH 8.3)
- 250 mM Tris (Roche)
- 2 M glycin (Merck KGaA)
- 1 % SDS

Transfer:

Transfer buffer anode 1 (pH 10.4)
- 0.3 M tris
- 20 % (v/v) methanol (Roth)

Transfer buffer anode 2 (pH 10.4)
- 25 mM tris
- 20 % (v/v) methanol
Transfer buffer cathode 1 (pH 9.4)
- 25 mM tris
- 40 mM aminocaproic acid (Merck KGaA)
- 20 % (v/v) methanol

Hybond P PVDF transfer membrane (GE Healthcare)

1 x TBST washing buffer
- 20 mM tris-HCl pH 7.2
- 140 mM NaCl (Sigma-Aldrich)
- 0.1 % Tween 20 (Sigma-Aldrich)

Ponceau S solution
- 0.5 % Ponceau S (Sigma-Aldrich) in 1 % acetic acid (Roth)

ECL advance blocking reagent (GE Healthcare)
ECL advance solution A and B (GE Healthcare)
Antibodies (see appendix)

2.8.3 Method

Whole cell lysates were prepared from HMEC-1 cells, SGBS cells and human PBMC. PBMC were obtained from whole blood of healthy individuals, collected in BD Vacutainer cell preparation tubes containing sodium heparin as an anticoagulant, and were processed according to the instruction from BD Biosciences. For whole cell lysates, cells were lysed in ice-cold RIPA-buffer containing protease and phosphatase inhibitors for protein extraction. After centrifugation at 12,000 g at 4 °C for 5 min the supernatants containing the total soluble proteins were dissolved in loading buffer and heated at 95 °C for 5 min according to Laemmli (Laemmli, 1970). Next, the protein samples were separated by SDS-PAGE. SDS-PAGE was performed by using generally 20 µg protein sample, 5 % acrylamide stacking gels, 7.5-10 % acrylamide running gels and 1x running buffer. Next, the proteins were semi-dry electroblotted onto a PVDF-membrane, using the anode 1-, anode 2-, and cathode 1- transfer buffers. Next, the membranes were stained with Ponceau S solution in order to assure protein transfer to the membrane and to visualize equal protein load of the samples. The next steps were carried out according to the manual instructions of the ECL-Advanced System. Briefly, after a blocking step at RT for 1h, the membranes were incubated with the primary antibody at 4 °C overnight. After extensive washing with TBST, the membrane was incubated with HRP-conjugated secondary antibody at RT for 1h and then again washed with TBST. Immunoreactive bands were visualised with the ECL Advanced solutions A and B. Densitometric scanning was performed to quantify the intensities of the bands (ImageQuantTL, Amersham Biosciences).
2.9 Methods to monitor adipocyte differentiation

2.9.1 Glycerinphosphate dehydrogenase (GPDH) assay

2.9.1.1 Theoretical Background

GPDH is an enzyme which catalyzes the reversible reaction between dihydroxyacetone phosphate and glycerol 3-phosphate with nicotinamide adenine dinucleotide (NADH+H+/NAD+) as coenzyme. GPDH activity rapidly increases upon differentiation of preadipocytes into adipocytes. Consequently, GPDH activity is used as major indicator for this process (Skurk et al., 2006; Van Harmelen et al., 2004). NADH+H+ has its maxima of adsorption at 260 nm and 340 nm and NAD+ only at 260 nm. A decrease in NADH+H+ is accompanied by a decrease in adsorption at 340 nm. Since the amount of dihydroxyacetone phosphate is proportional to the amount of converted NADH+H+ the turnover of dihydroxyacetone phosphate can be indirectly measured using the Beer-Lambert law.

2.9.1.2 Reagents

GPDH harvest buffer
- 0.05 M tris-HCl pH 7.4 (Roche)
- 1 mM EDTA (Sigma-Aldrich)
- 1 mM mercaptoethanol (Sigma-Aldrich)

GPDH reaction buffer
- 0.12 M triethanolamin-HCl pH 7.5 (Sigma-Aldrich)
- 3 mM EDTA
- 1.4 mM nicotinamidadenindinucleotide (NADH; Sigma-Aldrich)
- 0.6 mM mercaptoethanol

2.4 mM dihydroxyacetone phosphate (DHAP; Sigma-Aldrich)
2.9.1.3 Method

GPDH activity was determined as described elsewhere (Wise and Green, 1979) with the following modifications: SGBS preadipocytes at day 0 and day 16 and adipocytes at day 16 after induction of differentiation were detached in GPDH harvest buffer on ice with a cell scraper and sonicated. After centrifugation at 10 000 g at 4 °C for 10 min, GPDH activity was determined in the supernatants by measuring the NADH+H⁺ consumption. Therefore, the cell lysate (150 µl for preadipocytes and 10 µl for adipocytes) was mixed with water (280 µl for preadipocytes and 420 µl for adipocytes) and 65 µl GPDH reaction buffer. The reaction was initiated by adding 5 µl DHAP. The absorption at 340 nm was determined at 25 °C for 5 min using a spectral photometer. GPDH mU/ml was calculated from the linear NADH+H⁺ consumption related to the protein content (mg/ml) of the cell extracts which was determined by the Modified Bradford Assay.

2.9.2 Oil Red O/Haematoxylin staining

2.9.2.1 Theoretical Background

Oil Red O is a diazo lipophilic dye and stains neutral triglycerides and lipids deep red. This dye has been successfully used to monitor the differentiation process of adipocytes (Fukumoto and Fujimoto, 2002; Jaiswal et al., 2000). Metal-haematein complexes, haematein being oxidized haematoxylin, stain cell nuclei by binding to basophilic structures such as nucleic acids (Romeis and Böck, 1989).

2.9.2.2 Reagents

PBS with Ca²⁺ and Mg²⁺
- 1 tablet PBS per 200 ml dH₂O, sterile filtration (Sigma-Aldrich)
- 0.84 mM MgCl₂ (Merck KGaA)
- 0.72 mM CaCl₂ (Merck KGaA)
4 % PFA (Sigma-Aldrich) in PBS with Ca²⁺ and Mg²⁺
0.3 % Oil Red O (Sigma-Aldrich) in isopropanol (Roth), filtered
Mayer’s Hematoxylin solution (ready to use; Merck KGaA)
2.9.2.3 Method

The Oil Red O stain method was performed as described elsewhere (Lillie and Ashburn, 1943) and with the following modifications: SGBS cells were washed twice with PBS, fixed with 4 % PFA for 10 min and washed again twice with PBS. Next, the cells were incubated with Oil Red O for 1h on a shaking platform. Then, the cells were washed twice with water. Haematoxylin was added and the cells incubated for 5 min on a shaking platform. The stained cells were then repeatedly washed with hot water and examined under a microscope.

2.9.3 Nile Red

2.9.3.1 Theoretical Background

Nile Red is a hydrophobic, fluorogenic stain which may be used with live or fixed cells for the detection of intracellular lipid droplets (Greenspan et al., 1985). The dye also interacts with other hydrophobic structures and its fluorescence is strongly influenced by the polarity of its environment (Sackett and Wolff, 1987).

2.9.3.2 Reagents

PBS with Ca\(^{2+}\) and Mg\(^{2+}\)
- 1 tablet PBS per 200 ml dH\(_2\)O, sterile filtration (Sigma Aldrich)
- 0.84 mM MgCl\(_2\) (Merck KGaA)
- 0.72 mM CaCl\(_2\) (Merck KGaA)

4 % PFA (Sigma Aldrich) in PBS with Ca\(^{2+}\) and Mg\(^{2+}\)

Nile Red stock solution
- 1 mg/ml Nile Red (Invitrogen) in DMSO (Roth)

Nile Red working solution
- 1 µg/ml Nile Red in PBS

2.9.3.3 Method

This method was carried out as described elsewhere (Tiller et al., 2009) and with the following modifications. SGBS cells were washed twice with PBS, fixed with 4 % PFA for 10 min, washed again twice with PBS and incubated with 60 % isopropanol for 3-5 min. This was followed by incubation with the Nile Red working solution for 60 min. Next, the cells were washed twice with PBS. The cells were examined under the microscope (fluorescence
microscope DMIL and camera DC300, Leica Microsystems). Alternatively, the Nile Red stained SGBS cells were exposed to 100 % isopropanol for 15 min in order to dissolve the bound dye for quantification of the degree of adipocyte differentiation. The fluorescence was measured at 550 nm and 638 nm using a spectrometer (Varioskan multiwell photo- and fluorometer, Thermo Scientific). In these experiments, undifferentiated control cells were measured in parallel in order to determine the degree of unspecific binding of Nile Red to other hydrophobic cell structures. The data were corrected for this background fluorescence.

2.10 Peptidomics

2.10.1 Theoretical Background and Method

Peptidomics is defined as the systematic, comprehensive, qualitative and quantitative multiplex analysis of endogenous peptides in a biological sample at a defined time point and location. These peptides are either intact small molecules (hormones, cytokines, growth factors) or represent degradation products of proteins due to proteolytic cleavage (Schulte et al., 2005; Zucht et al., 2005). With the method as described elsewhere (Budde et al., 2005; Zucht et al., 2005), molecules with sizes between 1 kDa to 15 kDa can be detected. The peptidomic analysis of CM derived from SGBS preadipocytes and adipocytes was performed by the company Digilab BioVisioN (Hannover, Germany). For sample collection, SGBS adipocytes and control preadipocytes were washed once with 4 ml MCDB 131 w/o phenol red medium (PAN Biotech) and fed with 1 ml MCDB 131 w/o phenol red medium per well of a 6-well cell culture plate at day 15 after induction of differentiation. 24 hrs later, the medium was harvested and centrifuged at 1000 g at 4 °C for 10 min and filtered through a 0.2 µm filter. Next, 100 µl of the medium were removed for protein concentration analysis. To the remaining medium 5 µl/ml 30% HCL was added. The samples were snap frozen in liquid nitrogen and stored at -80 °C until shipment. The samples were “blinded” before being sent to the company with the following coding (#): #1: adipocyte-CM derived of cells under normoxia, #3: adipocyte-CM derived of cells under 1 % hypoxia, #4: preadipocyte-CM derived of cells under normoxia, #6: preadipocyte-CM derived of cells under 1 % hypoxia.

For the peptidomic-analysis, the complexity of the samples was reduced by the extraction of peptides and separation by liquid chromatography by hydrophilicity into 96 fractions using an 4-40 % (v/v) acetonitrile gradient on a conventional reverse phase column. Each fraction was then subjected to mass spectrometry using the ABI 4700 MALDI TOF-TOF mass
spectrometer. After mass spectrometry data processing the data of each sample was transferred into an “in house”-database which also contained information on the peptide display of WAT supernatants. The liquid chromatography and mass spectrometry data were visualized as 2D peptide displays containing the information of the mass to charge ratio of the peptides on the horizontal axis, the elution profile (96 fractions, separated by hydrophilicity) on the vertical axis and the signal intensity shown as colour. An overview of the process is given in figure 4. As quality control each sample was spiked with a mixture of standard peptides to monitor the sample processing. In order to obtain the 2D peptide display, the data were pre-processed which involved a baseline correction procedure and a mass to charge ratio of the peptide-recalibration of the mass spectrometric data.

Unfortunately the company went bankrupt and the sample analysis, including peptide sequencing, was delayed and finally stopped. However, it was possible to receive data of a few “in silico annotated” peptides. This means that the profile (defined mass and defined fraction number) of candidate peptides is matched to the profile of already identified peptides using the database. This database also contained information on peptides found in CM of human WAT. Since the identity of these annotated peptides is only a prediction, their biochemical identification must be confirmed in the respective material such as CM by a preparative approach and peptide sequencing of the isolated peptide spot. Further techniques to detect the candidate peptides in the CM or in case of proteolytic cleavage products of proteins are Western blot and other immuno techniques such as sensitive ELISAs or radioimmunoassays (RIA). The expression of the genes encoding the peptides or proteins can be analysed in the RNA of the corresponding SGBS cells by qRT-PCR.

2.11 Statistical analysis

Results were obtained by at least three independent experiments if not stated otherwise. All data are presented as means ± SE. Statistical comparisons between two groups were analysed by Student’s t-test and between several groups by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls t-test. Probability values of P < 0.05 were considered statistically significant. For data analysis the computer programs different versions of Microsoft Excel, SPSS 11 and GraphPad Prism 4 were used.
Figure 4  Overview of the peptide profiling process.

- Chromatographic fractionation: HPLC separation
- Mass spectrometry: MALDI-MS spectrum of one fraction
- Conversion of peak height in intensity and colour
- High resolution Peptide Display of 96 fractions
- Comparison of experimental groups
- Disease-specific changes in peptide composition: Differential Peptide Display
- Sample-specific peptide extraction e.g. of tissue, blood, urine
- Disease-specific changes in peptide composition: Differential Peptide Display

The picture was taken from the Digilab BioVisioN report from 30 May 2008
3 Results

3.1 Culture conditions of SGBS cells and HMEC-1 cells

3.1.1 Introduction

In order to study the cross-talk between human preadipocytes/adipocytes and microvascular endothelial cells an appropriate coculture system had to be established. SGBS cells were found to be suitable to study human preadipocytes and adipocytes (Wabitsch et al., 2001) and HMEC-1 cells to study human microvascular endothelial cells (Ades et al., 1992; Bouis et al., 2001). The indirect coculture strategy was chosen where the CM of SGBS cells was used to stimulate HMEC-1 cells. The initial experiments were performed to establish a cell culturing strategy which is suitable for both, endothelial cells and preadipocytes/adipocytes. In this respect, the following situation for SGBS and HMEC-1 cells had to be considered. The basal medium of HMEC-1 cells is MCDB-131 which contains 5 mM glucose. In contrast, the basal medium of SGBS cells, which is DMEM/F12 contains 25 mM glucose. Moreover, the media have different compositions of amino acids, vitamins and salts (Table 1). It is important to note that high glucose levels lead to endothelial dysfunction in vitro and in vivo causing there micro- and macrovascular complications (Bakker et al., 2009; Salameh et al., 1997; Stenina, 2005; Varma et al., 2005). Moreover, high glucose concentrations cause impairment of insulin stimulated glucose uptake, disturbance of the insulin signalling pathway and cytokine secretion pattern of adipocytes at least in vitro (Lin et al., 2005; Renstrom et al., 2007).
### Table 1  Comparison of the components of DMEM/F12 and MCDB 131 medium

<table>
<thead>
<tr>
<th>Components</th>
<th>DMEM/F12</th>
<th>MCDB 131</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acids</strong></td>
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<tr>
<td>Glycine</td>
<td>250 µM</td>
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</tr>
<tr>
<td>L-Alanine</td>
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</tr>
<tr>
<td>L-Arginine hydrochloride</td>
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</tr>
<tr>
<td>L-Asparagine-H2O</td>
<td>50 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>50 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>L-Cysteine hydrochloride-H2O</td>
<td>99.77 µM</td>
<td>/</td>
</tr>
<tr>
<td>L-Cystine 2HCl</td>
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<td>198.86 µM</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
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</tr>
<tr>
<td>L-Histidine hydrochloride-H2O</td>
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<td>200 µM</td>
</tr>
<tr>
<td>L-Isoleucine</td>
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<td>L-Leucine</td>
<td>450.76 µM</td>
<td>1000 µM</td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
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<td>994.54 µM</td>
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<tr>
<td>L-Methionine</td>
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<td>L-Phenylalanine</td>
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<td>100 µM</td>
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<td>L-Serine</td>
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<tr>
<td>L-Tyrosine</td>
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<td>100 µM</td>
</tr>
<tr>
<td>L-Tyrosine disodium salt dihydrate</td>
<td>213.76 µM</td>
<td>/</td>
</tr>
<tr>
<td>L-Valine</td>
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<td><strong>Vitamins</strong></td>
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<tr>
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<tr>
<td>Folic acid</td>
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<td>/</td>
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<tr>
<td>Folic acid calcium salt</td>
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<td>Nicotinamide</td>
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</tr>
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<td>Vitamin B12</td>
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<td>i-Insitol</td>
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<td><strong>Inorganic salts</strong></td>
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<td>Ferric sulfate (FeSO₄·7H₂O)</td>
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<td>1.02 µM</td>
</tr>
<tr>
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<td>301.47 µM</td>
<td>/</td>
</tr>
<tr>
<td>Magnesium sulfate (MgSO₄) (anhyd.)</td>
<td>0.41 mM</td>
<td>10.02 mM</td>
</tr>
<tr>
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<td>/</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>Nickelous chloride NiCl₂·6H₂O</td>
<td>/</td>
<td>0.3 nM</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
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<td>3.97 mM</td>
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<tr>
<td>Selenous acid H₂SeO₃</td>
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<td>29.5 µM</td>
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<tr>
<td>Sodium bicarbonate (NaHCO₃)</td>
<td>29.02 mM</td>
<td>14 mM</td>
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<tr>
<td>Sodium chloride (NaCl)</td>
<td>120.61 mM</td>
<td>110.86 mM</td>
</tr>
<tr>
<td>Sodium phosphate dibasic (Na₂HPO₄) (anhyd.)</td>
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<td>0.5 mM</td>
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<td>Sodium phosphate monobasic (NaH₂PO₄·H₂O)</td>
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<td>/</td>
</tr>
<tr>
<td>Sodium meta silicate Na₂SiO₃·9H₂O</td>
<td>/</td>
<td>9.86 µM</td>
</tr>
<tr>
<td>Zinc sulfate (ZnSO₄·7H₂O)</td>
<td>3000 nM</td>
<td>1 nM</td>
</tr>
<tr>
<td>Sodium selenite</td>
<td>29 nM</td>
<td>/</td>
</tr>
<tr>
<td><strong>Other Components</strong></td>
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<tr>
<td>Adenine</td>
<td>/</td>
<td>1 µM</td>
</tr>
<tr>
<td>D-Glucose (Dextrose)</td>
<td>25 nM</td>
<td>5 nM</td>
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<td>Ethanolamine</td>
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<td>Hypoxanthine Na</td>
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<td>/</td>
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<tr>
<td>Linoleic acid</td>
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<td>/</td>
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<td>Lipoic acid</td>
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<tr>
<td>Lipoic acid</td>
<td>21.52 µM</td>
<td>32.94 µM</td>
</tr>
<tr>
<td>Putrescine 2HCl</td>
<td>503 nM</td>
<td>1.2 nM</td>
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<tr>
<td>Sodium pyruvate</td>
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<td>1 mM</td>
</tr>
<tr>
<td>Thymidine</td>
<td>1508 nM</td>
<td>99.2 nM</td>
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</table>
3.1.2 HMEC-1 cell proliferation using different basal media

Firstly, HMEC-1 cell proliferation was examined using different mixtures of MCDB 131 and DMEM/F12 as basal media. Using light microscopy (Figure 5A) and BrdU analysis (Figure 5B) a cell growth decrease of HMEC-1 cells, morphology changes and cell death were observed with increasing amounts of DMEM/F12 as basal medium. Thus, it was necessary to proliferate the HMEC-1 cells according to the standard protocol using MCDB 131 as basal medium.

3.1.3 SGBS proliferation using different basal media

As for HMEC-1 cells, SGBS cells were proliferated in different mixtures of MCDB 131 and DMEM/F12 as basal media. Microscopy (Figure 6A) and BrdU (Figure 6B) analysis showed a decrease of SGBS cell growth with increasing concentrations of MCDB 131 as basal medium. Moreover, the differentiation rate of SGBS cells was impaired when the cells were proliferated in medium containing MCDB 131 as basal medium (observed data) and so SGBS cells were proliferated in the traditional medium containing DMEM/F12 as basal medium.

3.1.4 Differentiation capacity of SGBS cells using different basal media

In contrast, differentiation of SGBS cells in a mixture of 2/3 MCDB 131 and 1/3 DMEM/F12 did not affect the differentiation capacity of SGBS cells when those were proliferated ahead in the traditional proliferation medium containing DMEM/F12 as basal medium. This was examined by microscopy (Figures 7A and B) and measuring Nile Red fluorescence (Figure 7C) and GPDH activity (Figure 7D). Consequently, the SGBS cells were differentiated in later experiments in the above mentioned ratio of basal media mixture with a final glucose concentration of 11.6 mM. For 3T3-L1 cells it has been shown before that the differentiation capacity of preadipocytes to adipocytes does not decrease when lower glucose concentrations then 25 mM are used (Gagnon and Sorisky, 1998). However, the morphology of the adipocytes appeared to change. A greater number of small lipid droplets could be observed by microscopy using 5 mM glucose versus big lipid droplets using 25 mM glucose (data not shown). This trend was also found in this experimental setup (Figures 7A and B).
HMEC-1 cells were proliferated in HMEC-1 medium as positive control (a) or in different cell culture media mixtures containing 10% FCS (b-f). The media mixtures were as follows: b: MCDB 131 medium, c: MCDB 131/DMEM/F12 medium, ratio 3:1, d: MCDB 131/DMEM/F12 medium, ratio 1:2, e: MCDB 131/DMEM/F12 medium, ratio 1:3 and f: DMEM/F12 medium. Phase contrast microscopy (A) and BrdU incorporation as proliferation assay (B) were used for examination. For the analysis of the experiments in B the obtained fluorescence data from the positive control (a) were considered as 100%. Results are means ± SE (n=3). *P<0.05, **P<0.01.
SGBS cells were proliferated in different cell culture media mixtures containing 10 % FCS (a-e). The media mixtures were as follows: a: DMEM/F12 medium (positive control), b: DMEM/F12/MCDB 131 medium, ratio 3:1, c: DMEM/F12:MCDB 131 medium, ratio 1:2, d: DMEM/F12:MCDB 131 medium, ratio 1:3, e: MCDB 131 medium. Phase contrast microscopy (A) and BrdU incorporation as proliferation assay (B) were used for examination. For the analysis of the experiments in B the obtained fluorescence data from the positive control (a) were considered as 100 %. Results are means ± SE (n=3). **P<0.01, ***P<0.001.
Figure 7  SGBS cell differentiation capacity using different basal media

SGBS cells were examined after 16 days post-induction of differentiation. Cells fed post-induction using as basal medium either DMEM/F12 (a) or MCDB 131/DMEM/F12, ratio 3:1 (b). Fluorescence (A) and phase contrast (B) microscopy visualized the Nile Red stained cells. The degree of differentiation was assessed quantitatively by Nile Red fluorescence (C) and GPDH activity (D). For the analysis of the experiments in C and D, the obtained fluorescence data or GPDH activity respectively, from the condition a were considered as 100 %. Results are means ± SE (n=3).
3.1.5 Differentiation time course of SGBS cells

The efficient differentiation of SGBS cells cultured in a basal medium mixture of 2/3 MCDB 131 and 1/3 DMEM/F12 is shown in a differentiation time course (Figure 8). The confluent preadipocytes at day 0 (Figure 8A) were induced to differentiate from preadipocytes into adipocytes by the addition of induction medium. By day 4 (Figure 8B) the preadipocytes had started to develop into adipocytes, showing first signs of lipid accumulation. To visualize the lipid cells were specifically stained with Oil Red O. Lipid droplets appeared as red coloured dots in the cells under the microscope. By day 8 (Figure 8C) the accumulation of small lipid droplets within the cells was universal. The adipocytes continued to increase lipid accumulation and the size of lipid droplets (Figures 8D and E) reaching a maximum around day 14 to 16 (Figure 8F) at which point the lipid droplets were most pronounced. At that time, adipocyte differentiation was observed in ≥ 80 % of the cells.

3.1.6 Coculture between SGBS and HMEC-1 cells

In this study the indirect coculture approach was chosen by using CM from SGBS cells to stimulate starved HMEC-1 cells (Figure 9). “Starving” cells means to deprive them of FCS and other additives to force the cells to enter the same cell cycle phase, namely, the G0 phase. Moreover, pre-activated cells may decrease their level of activation. Thus, the cells may be more sensible and react more homogeneously on stimuli. HMEC-1 cells are generally starved ahead of experiments (BelAiba et al., 2007; Sapet et al., 2006; Wang et al., 2004). Since HMEC-1 cells need MCDB 131 as basal medium it was necessary that the SGBS-CM used in the coculture experiments with HMEC-1 cells was based on MCDB 131. SGBS cell differentiation was performed in serum-free 2/3 MCDB 131 and 1/3 DMEM/F12 as basal medium mixture. Therefore, a last adaptation step for SGBS cells before CM were taken, was necessary. In order to exclude stress effects on SGBS cells when changing the basal medium, they were cultured for several hours in MCDB 131 medium before the medium was changed again and left for generally 24 hours on the SGBS cells to generate the CM. Changing the media did not effect negatively the cell culture. A scheme of the cell culture strategy is shown in figure 10. Since differentiating SGBS cells are cultured over a period of 14 days in medium containing insulin, hydrocortisol, T3 and transferrin, control preadipocytes were cultured in...
parallel but were not induced to differentiate. The characterisation of these cells is shown in chapter 3.2.4 and 3.2.3.

Figure 8  
SGBS cell differentiation time course

Figure 8
Phase contrast microscopy of SGBS cells prior to induction of differentiation (day 0; A), and post-induction on day 4 (B), day 8 (C), day 10 (D), day 12 (E) and day 16 (F) stained with Oil Red O (red) and haematoxylin (blue).
HMEC-1 cells were proliferated in HMEC-1 medium. Pre-confluent cells at approximately 40 % confluence (A), 70 % confluence (B) and confluence (C) were visualized by phase contrast microscopy. The confluent HMEC-1 cells display the typical endothelial phenotype with clear and defined cell borders (cobblestone appearance).
Figure 10  Scheme of the cell culture strategy

SGBS cells were seeded at low densities until post-confluence occurred 10 days later. Next, the cells were exposed to induction medium and the control preadipocytes were exposed to adipose medium (d0). After four days (d4) the medium was changed to adipose medium until day 14 post-induction (d14). For adaptation, the cells were exposed from day 14 to day 15 to MCDB 131 medium. In order to generate conditioned media (CM), the cells were cultured from day 15 to day 16 for 24 hrs in MCDB 131 medium. The CM were either used for analysis or for treatment of starved HMEC-1 cells.
3.2 Gene expression and cytokine secretion of SGBS preadipocytes and adipocytes

3.2.1 Introduction

For further characterization and in order to get more insights into the secretory capacity of SGBS cells, adipokine-mRNAs and -proteins at different times and under different conditions were measured. Therefore, the analysis was focused on the following adipokines: leptin, adiponectin, PAI-1, IL-6, VEGF, MCP-1, IL-8, RANTES, SDF1-α, IL-4, IL-1α, IL-1β, TNF-α and IFN-γ. Leptin and adiponectin (see chapters 1.2.2.1 and 1.2.2.2) are typically secreted by WAT and reach the bloodstream. PAI-1 (Alessi and Juhan-Vague, 2006) is involved in vascular haemostasis and IL-6 (see chapter 1.2.2.3) in inflammatory processes. VEGF is important in vascular remodelling (see chapter 1.2.2.4). MCP-1, IL-8, RANTES, SDF-1α are chemoattractants (see chapter 1.2.2.5). IL-4 has anti-inflammatory features but can act in concert with TNF-α and IL-1 as endothelial cell activator (Petzelbauer et al., 1993). IL-1 and TNF-α are potent pro-inflammatory proteins and activator of endothelial cells (see chapter 1.2.7.1). Detailed information about the conducted experiments and fold-changes are presented in the corresponding figure legends.

3.2.2 Cytokine expression and secretion of SGBS preadipocytes and adipocytes

The measurements of selected adipokines using qRT-PCR, ELISAs and multiplex bead-based Luminex® assays, revealed that the mRNA expressions and/or protein secretions of PAI-1, IL-6, VEGF and the chemoattractants MCP-1, IL-8 (Figure 11A) and SDF-1α (Figure 11B) were significantly higher in preadipocytes than in adipocytes. The mRNA for RANTES (Figure 11A) was slightly stronger expressed at mRNA level in adipocytes compared with preadipocytes whereas at protein level it was the opposite. However, both observations were not statistically significant. In order to elucidate whether classical endothelial cell activators besides VEGF were expressed or secreted by preadipocytes and adipocytes a selected panel of molecules including TNF-α, IL-1, IFN-γ (Figure 12) and IL-4 (Figure 11) was analysed. IL-4 (Figure 11) protein secretion was higher in preadipocytes as compared with adipocytes. IL-1α gene expression in preadipocytes was robust after 30 amplification cycles by qRT-PCR.
compared with weak signals for adipocytes. However, at protein level IL-1α was at the detection limit. In contrast, IL-1β gene expression was measured after 25 amplification cycles in adipocytes versus 31 cycles in preadipocytes. As for IL-1α, IL-1β protein was also at the detection limit. TNF-α gene expression was weak in preadipocytes (after 36 cycles) and not observed in adipocytes. Moreover, in the supernatants of preadipocytes and adipocytes TNF-α and also IFN-γ protein were at the detection limit. As expected, adiponectin, an adipocyte marker, was measured at high levels at mRNA (after 20 cycles, data not shown) and at protein level in adipocytes (220 ng/ml), but not in preadipocytes (Figure 11B). Interestingly, leptin expression was detected, not only in adipocytes, but also in subconfluent preadipocytes. An increase in leptin expression was observed in confluent preadipocytes (data not shown), as reported previously for primary preadipocytes (Simons et al., 2005).

3.2.3 Adipokine secretion of SGBS cells cultured in the presence and in the absence of hormones and antibiotics

Analyses using specific ELISAs for PAI-1, IL-6 and MCP-1 were performed in order to get insights into the cytokine secretion of SGBS cells cultured from day 14 to day 16 either according to the standard protocol in adipose medium or according to the protocol used to generate the CM for coculture experiments. Adipose medium contains hydrocortisone, insulin, T3 and Pen/Strep. Insulin and other hormones are known to change the cytokine secretion pattern of several cell types, including adipocytes (Fain and Madan, 2005; Wabitsch et al., 1996). For coculture experiments, CM of SGBS cells deprived of hydrocortisone-, insulin-, T3- and Pen/Strep were used (see chapter 3.1.6). The protein concentrations of the three obesity relevant cytokines PAI-1, IL-6 and MCP-1 were measured in these CM and compared with the CM of SGBS cells cultured in adipose medium in the presence of the above mentioned hormones and antibiotics (Figure 13). PAI-1 secretion did not change in preadipocytes and adipocytes. Interestingly, IL-6 secretion increased in preadipocytes and adipocytes when the cells were deprived of the indicated hormones and antibiotics, although this effect was not statistically significant for the adipocytes. Moreover, MCP-1 secretion was significantly elevated in adipocytes when cultured in medium lacking hormones and antibiotics. The opposite trend was observed for the preadipocytes but was not statistically significant. It is important to note, that the applied cell culture protocols affected the cells in their behaviour of cytokine secretion. As a consequence and in order to allow a comparison between preadipocytes and adipocytes in gene expression, cytokine secretion and the effect of...
their CM on endothelial cell activation, the preadipocytes were always cultured in parallel in the same media as the adipocytes, except for the induction to differentiate.

3.2.4 Comparison of the morphology, GPDH activity and adipokine secretion in SGBS preadipocytes at confluence and after 16 days culture period

Since confluent preadipocytes (Pd0) were cultured for another 16 days (Pd16) in the same media as differentiating adipocytes except that they were not induced to differentiate (see chapter 3.1.6 and 3.2.3), it was investigated whether differences in morphology, GPDH activity and cytokine secretion between Pd0 and Pd16 occurred. Therefore, phase contrast microscopy was performed (Figures 14A and B) and GPDH activity (Figure 14C) and the adipokines PAI-1, IL-6 and MCP-1 (Figure 14D) measured by specific ELISAs. Small morphology changes between day 0 and day 16 preadipocytes were observed. Nevertheless, the preadipocytes kept the image of fibroblastic-like cells over the culture period (Figure 14A and B). Generally, no lipid accumulation was found which is in agreement with the GPDH results (Figure 14C). PAI-1 and IL-6 protein release (Figure 14D) was higher in the confluent preadipocytes (Pd0) in comparison with 16 days postconfluent preadipocytes (Pd16). For MCP-1 release (Figure 14D), no changes were observed.
**Figure 11**  Comparison of the preadipocyte and adipocyte gene expression and protein secretion of selected adipokines

**A**

- **PAI-1**
  - mRNA levels: 8-fold for Pd and 4.1-fold for Ad.
  - Protein release: 2-fold for Pd and 6-fold for Ad.

- **IL-6**
  - mRNA levels: 2.5-fold for Pd and 3-fold for Ad.
  - Protein release: 3-fold for Pd and 6-fold for Ad.

- **VEGF**
  - mRNA levels: 4.5-fold for Pd and 5.5-fold for Ad.
  - Protein release: 6-fold for Pd and 6-fold for Ad.

**B**

- **SDF-1α**
  - Protein release: 140-fold difference between Pd and Ad.

- **IL-4**
  - Protein release: 2.6-fold difference between Pd and Ad.

- **Adiponectin**
  - Protein release: 220 ng/ml measured for Ad-CM whereas adiponectin was not detectable in Pd.

---

**Figure 11**

A: adipokine mRNA levels were quantified by qRT-PCR and adipocyte (Ad) values are presented as relative expression compared with values of the corresponding preadipocytes (Pd). Adipokine concentrations in conditioned media (CM) were quantified by specific ELISAs or bead-based Luminex assays. The relative differences between the values for mRNA and protein of Pd and Ad are 8- and 4.1-fold for PAI-1, 6-fold and 6-fold for IL-6, 2.5- and 3-fold for VEGF, 4.5- and 5.5-fold for MCP-1, 20- and 8.9-fold for IL-8. No differences for RANTES were observed. **B**: a 140-fold difference was measured for SDF-1α protein and for IL-4 a 2.6-fold difference. Adiponectin concentration of 220 ng/ml was measured for Ad-CM whereas adiponectin was not detectable in Pd. Results are means ± SE (n=4). *P<0.05, **P<0.01, ***P<0.001.
Figure 12  Overview of the preadipocyte and adipocyte gene expression and protein secretion of IL-1α, IL-1β, TNF-α and IFN-γ

<table>
<thead>
<tr>
<th>Protein</th>
<th>RNA</th>
<th>Protein</th>
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<td></td>
<td>[image]</td>
<td>[image]</td>
<td>[image]</td>
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<tr>
<td></td>
<td>values at detection limit (below 10 pg/ml)</td>
<td>values at detection limit (below 10 pg/ml)</td>
<td>values at detection limit (below 10 pg/ml)</td>
</tr>
<tr>
<td>TNF-α</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>weak detectable in preadipocytes, not detectable in adipocytes</td>
<td>values at detection limit (below 10 pg/ml)</td>
<td>values at detection limit (below 10 pg/ml)</td>
</tr>
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<td>IFN-γ</td>
<td></td>
<td></td>
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<td>not detectable (detection limit: 10 pg/ml)</td>
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Figure 12  Adipokine mRNA levels were quantified by qRT-PCR and are presented as relative expression compared with values of the corresponding preadipocytes (Pd). The relative increase of IL-1α in Pd vs. adipocytes (Ad) was 18-fold. In contrast, IL-1β was increased in Ad compared with Pd by 53-fold. Adipokine concentrations in conditioned media were quantified by bead-based Luminex assays with detection limits below 10 pg/ml. Results are means ± SE (n=4). *P<0.05, ***P<0.001.
Figure 13  Adipokine release of SGBS cells cultured in the presence and absence of hormones and antibiotics

SGBS preadipocytes (Pd) and adipocytes (Ad) were cultured from day 15 to day 16 for 24 hrs in MCDB 131 medium lacking (Pd, Ad) or containing (Pd+, Ad+) 66 nM insulin, 100 mM hydrocortisone, 10 µg/ml T3, 100 U/ml penicillin and 100 µg/ml streptomycin. PAI-1, IL-6 and MCP-1 were measured in the CM by specific ELISAs. Relative differences for IL-6 are 1.3-fold for Pd vs. Pd+ and for MCP-1 1.7-fold for Ad vs. Ad+. Results are means ± SE (n=4). n.s. means statistically not significant. *P<0.05.
Figure 14  Comparison of the GPDH activity and adipokine secretion in SGBS preadipocytes at day 0 and 16 days culture period

SGBS cells were cultured until confluence (Pd0) and then kept for 16 days in adipose medium (Pd16). Phase contrast microscopy was used to visualize Pd0 (A) and Pd16 (B) cells. The GPDH values of Pd0 and Pd16 cells (C) are illustrated in comparison to differentiated cells (Ad16). Protein secretion by Pd0 and Pd16 cells was measured using specific ELISAs for PAI-1, IL-6 and MCP-1 (D). Relative differences for the secretion by Pd0 compared with Pd16 are 2.2-fold for PAI-1 and 3-fold for IL-6. Results are means ± SE (n=4). n.s. means statistically not significant. **P<0.01, ***P<0.001.
3.3 **Impact of preadipocyte- and adipocyte-CM on HMEC-1 cell activation and signalling pathways**

### 3.3.1 Introduction

In order to investigate the impact of SGBS preadipocyte- and adipocyte-CM on endothelial cell function, RNA and protein expression analysis, functional assays and signalling pathway experiments were conducted. The endothelium serves as the interface between circulating blood immune cells and adipose tissue and plays an interactive role in infiltration and inflammatory processes of immune cells. Therefore, a monocyte-endothelial cell-cell adhesion assay was used as main cell biological read-out system for endothelial activation, which is a change in phenotype or function in endothelial cells in response to stimuli from the environment (Zimmerman *et al.*, 1999). In addition to the monocyte-endothelial cell-cell adhesion assay, an ICAM-1 cell surface ELISA was chosen to characterize this process at the molecular level. The SGBS preadipocytes and adipocytes were subjected to different biologically relevant physiologic/pathophysiologic conditions, including normoxia, hypoxia and TNF-α treatment and their CM were tested as mentioned above. Finally, the impact of CM of mature adipocytes with different sizes isolated from human WAT was analysed on endothelial cell activation. Note, corresponding information about the experiments and fold-changes are presented in the figure legends.

### 3.3.2 Establishment of a monocyte-endothelial cell-cell adhesion assay and ICAM-1 and VCAM-1 cell surface ELISA

In order to analyse the impact of SGBS-CM on endothelial cell activation, a monocyte endothelial cell-cell adhesion assay and an ICAM-1 and VCAM-1 cell surface ELISA were developed as described in chapter 2.1.6 and 2.2. Microscopic analysis of *Calcein green AM* stained U937 monocytes and of U937 monocytes adhering to HMEC-1 cells is shown in figures 15A and B, respectively. ICAM-1 and VCAM-1 are known to mediate the leukocyte-endothelial cell-cell adhesion. Stimulation of endothelial cells with TNF-α is known to induce ICAM-1 and VCAM-1 cell surface expression and to promote leukocyte adhesion (Johnston and Butcher, 2002; Weber *et al.*, 1995). Therefore, to test TNF-α stimulation as positive control, HMEC-1 cells were treated with different TNF-α concentrations before applied to the assays. The levels of monocytic U937 endothelial cell-cell adhesion (Figure
16A) and ICAM-1 (Figure 16B) and VCAM-1 (Figure 16C) protein expressions increased with increasing TNF-α concentrations. Since stimulation of HMEC-1 cells with 25 ng/ml of TNF-α led to a robust maximum in monocyte adhesion, this concentration was used in further experiments as positive control.

**Figure 15**  U937 monocyte-HMEC-1 cell adhesion

Monocytes were stained with *Calcein green AM* and visualized using fluorescence microscopy (A). Endothelial cells were treated with 25 ng/ml TNF-α for 6 hrs before the monocyte-endothelial cell-cell adhesion assay was performed and phase contrast microscopy used for visualization (B). U937 monocytes (see arrows) adhere to the confluent endothelial cells (B).
Figure 16  Effect of TNF-α on microvascular endothelial cells

HMEC-1 cells were treated with different concentrations of TNF-α. Subsequently U937 monocyte-endothelial cell-cell adhesion (A) and ICAM-1 (B) and VCAM-1 (C) cell surface expression were analysed. Results are means ± SE (n=2).
3.3.3 Impact of SGBS preadipocyte- and adipocyte-CM on endothelial ICAM-1 cell surface expression and monocyte-endothelial cell-cell adhesion

To determine the functional impact of CM from SGBS cells on endothelial cell activation, the adhesion of U937 cells to HMEC-1 cells stimulated either with CM or control medium was measured. In addition, ICAM-1 and VCAM-1 endothelial cell surface expression was analysed. The adhesion of U937 cells was substantially increased after preadipocyte-CM treatment compared with control medium whereas after adipocyte-CM treatment, only a modest increase in adhesion was observed, which was statistically not significant. In contrast, TNF-α-stimulated HMEC-1 cells showed a high level of adhesion (Figure 17A). In order to rule out the possibility, that adipocyte-CM did not activate endothelial cells because of the long differentiation period (16 days of culture), the same experiments were performed with CM of adipocytes cultured for only 7 days in adipose medium (less mature adipocytes). No significant differences in endothelial cell activation between these two types of Ad-CM were observed (data not shown). Concordantly with the U937 adhesion experiments, endothelial ICAM-1 expression was significantly upregulated after stimulation with preadipocyte-CM and TNF-α but not with adipocyte-CM (Figure 17B). However, VCAM-1 cell surface expression was neither increased on HMEC-1 cells after stimulation with preadipocyte-nor adipocyte-CM (data not shown) in contrast to incubation with TNFα (Figure 16C). Since the CM mediated monocyte adhesion was independent of VCAM-1 cell surface expression this protein was not further investigated. Next, the endothelial expression of MCP-1 and IL-8 operating as strong activators of monocytes and important for adhesion was assessed (Gerszten et al., 1999). For preadipocyte-CM and adipocyte-CM a marked increase of gene expression for both target genes was measured (Figures 17C and D).
Figure 17  Effects of preadipocyte- and adipocyte-CM on microvascular endothelial cells

HMEC-1 cells were treated with control medium (Ctrl), preadipocyte-conditioned medium (Pd-CM) and adipocyte-CM (Ad-CM) and subsequently analysed for the adhesion of U937 cells to HMEC-1(A), ICAM-1 cell surface expression (B), and the gene expression of the monocytic activators IL-8 (C) and MCP-1 (D). 25 ng/ml TNF-α was used as a positive control (TNFα). Monocyte adhesion (A) and ICAM-1 (B) increased 1.8- and 1.4-fold after treatment with Pd-CM and 1.2- and 1.1-fold (n.s.) with Ad-CM, respectively, compared with the Ctrl-treatment. Monocyte adhesion and ICAM-1 differences between the treatments with CM were 1.5- and 1.3-fold, respectively. C and D: Gene expression levels of IL-8 (C) and MCP-1(D) increased in HMEC-1 after treatment with Pd-CM and Ad-CM 3- and 2.8-fold vs. 3.2- and 3.4-fold, respectively, compared with the Ctrl. Results are means ± SE (n≥3). n.s. means statistically not significant. *P<0.05, **P<0.01, ***P<0.001.
3.3.4 Impact of concentrated SGBS-CM on monocyte-endothelial cell-cell adhesion

To determine whether the concentration of CM by ultrafiltration lead to an increase in monocyte adhesion and whether the exerted effects could be attributed to low or high molecular substances further analysis was performed (Figure 18). 13x concentrated CM of both preadipocytes and adipocytes, containing only molecules of more than 5 kDa led to an increase in monocyte adhesion. During the concentration process protein or protein activity was lost as can be seen for the TNF-α positive control. 2.5 ng/ml TNF-α was concentrated 10x to achieve presumably a final concentration of 25 ng/ml. When compared with fresh 25 ng/ml TNF-α, the monocyte adhesion is decreased. The unconcentrated CM, only containing molecules of less than 5 kDa, had negligible effects on monocyte adhesion (Figure 18). Consequently, the exerted effects by the CM are likely to be attributed to molecules secreted by SGBS cells of more than 5 kDa which justifies focusing on the analysis of cytokines in the CM of SGBS cells. However, effects of molecules of less than 5 KDa on monocytes adhesion can not be ruled out, since they may act in concert with molecules of more than 5 kDa.
Figure 18  Impact of concentrated SGBS-CM on monocyte-endothelial cell-cell adhesion

HMEC-1 cells were treated with medium (Ctrl), preadipocyte-conditioned medium (Pd-CM) and adipocyte-CM (Ad-CM) and 10x concentrated (conc) CM, only containing molecules >5kDa (Pd-CM conc, Ad-CM conc) and CM containing only molecules <5kDa (permeate; Pd-CM permeate, Ad-CM permeate). 25 ng/ml TNF-α (TNF-α) was used as positive control. 2.5 ng/ml TNF-α was used in the CM-concentrating procedure to test the efficiency of this technique (TNF-α 2.5 10x conc and TNF-α permeate). Subsequently, the monocyte adhesion assay was performed. The relative increase in monocyte adhesion after stimulation with Pd-CM were 1.7-fold, with Pd-CM conc 2.4-fold, with Ad-CM 1.2-fold (n.s.) and with Ad-CM conc 2.1-fold, compared with the Ctrl-treatment. TNF-α exposure led to a 5.4-fold and with TNF-α 2.5 10x conc 3.7-fold elevation. The permeate treatments showed only baseline levels in monocyte adhesion. Monocyte adhesion differences between Pd-CM and Pd-CM conc were 40 % and between Ad-CM and Ad-CM conc 75 %, respectively. Differences between TNF-α and TNF-α 2.5 10x conc were 45 %. Results are means ± SE (n≥3). n.s. means statistically not significant. *P<0.05, **P<0.01, ***P<0.001.
3.3.5 Impact of TNF-α treated SGBS cells on monocyte endothelial cell-cell adhesion

TNF-α is expressed primarily by macrophages in WAT, can operate as negative regulator of fat storage and induces its own TNF-α gene expression in adipocytes (Hauner et al., 1995; Neels et al., 2006). To examine the direct influence of TNF-α on the endothelial activation capacity of SGBS cells in the absence of macrophages and other paracrine or juxtacrine disturbances, adipokine expression of TNF-α-treated SGBS cells and the impact of their CM on monocyte-endothelial cell-cell adhesion were assessed. TNF-α-stimulation induced a robust TNF-α mRNA expression in preadipocytes and adipocytes (Figure 19A). Fresh CM of TNF-α-stimulated SGBS cells substantially induced the U937 cell adhesion to HMEC-1 (Figure 19B). To rule out that exogenous TNF-α remaining in the CM after TNF-α-stimulation was functional, MCDB 131 medium in the absence of cells without and with TNF-α was kept in the incubator in parallel with SGBS cells as control media and then used in the adhesion assay. For these control media, only baseline levels of adhesion were detected. To test whether CM of TNF-α-induced SGBS cells contained functional TNF-α-activity, CM were pre-incubated with the potent TNF-α-antagonist Enbrel® (Tracey et al., 2008) or mock-pre-incubated before stimulating HMEC-1. The used Enbrel® concentration of 200 ng/ml was sufficient to achieve a maximum of blocking efficiency in the monocyte adhesion assay (Figure 19C). Enbrel®-pre-treatment significantly decreased the monocyte-endothelial cell-cell adhesion to 50% and 28% of the levels achieved with CM of TNF-α-stimulated preadipocytes and adipocytes, respectively (Figure 19D). In order to support these data, total and phosphorylated IκBα were analysed in HMEC-1 cells (Figure 19E) since TNF-α exerted effects in endothelial cells have been shown to be mediated via the NfκB-signalling pathway (Weber et al., 1995). IκBα phosphorylation was increased in HMEC-1 cells after treatment with CM of TNF-α exposed SGBS cells and abrogated when these CM were preincubated with Enbrel®, although not to baseline levels. For total IκBα the opposite was observed.
Figure 19  Effects of TNF-α-stimulated preadipocytes and adipocytes on microvascular endothelial cell activation
Figure 19

A: TNF-α gene expression of preadipocytes (Pd) and adipocytes (Ad) was assessed after treatment with either medium (Pd, Ad) or 25 ng/ml TNF-α (Pd TNF-α, Ad TNF-α) for 24 hrs. TNF-α gene expression was 46±19- and 66±38-fold in TNF-α-treated Pd and Ad, respectively, compared with untreated Pd. No TNF-α was detectable in untreated Ad. The conditioned media (CM) were used for stimulating HMEC-1 cells. Subsequently, the adhesion of U937 cells to HMEC-1 cells (B-D) and total (t-) and (p-) phosphorylated IκBα in HMEC-1 cells (E) using Western blotting, was assessed. To rule out that the exogenous TNF-α remaining in the CM after TNF-α-stimulation was functional, MCDB-131 medium without (Ctrl) and with 25 ng/ml TNF-α (Ctrl TNF-α) were kept in the incubator at 37 °C for 24 hrs as control medium and then used in the adhesion assay (B and D) or for Western blot analysis (E). For these control media only baseline levels of adhesion and t- and p-IκBα were detected. B: In contrast, monocyte adhesion to HMEC-1 increased 1.7-fold with Pd-CM, 1.2-fold (n.s.) with Ad-CM, 4.1-fold with CM of TNF-α-treated Pd (Pd TNF-α−CM) and 4.7-fold with CM of TNF-α-treated Ad (Ad-TNFα-CM). Fresh TNF-α added to MCBD-131 medium at 25 ng/ml was used as a positive control (TNFα; 5.3-fold increase). The enhancement in monocyte adhesion with CM derived from TNF-α-treated Pd and Ad were 2.5- and 3.9-fold, respectively. C: TNF-α (25 ng/ml) exerted monocyte-endothelial cell-cell adhesion was blocked using several concentrations of the TNF-α receptor blocker Enbrel®. D: For blockade of TNF-α-activity, CM from TNF-α-induced SGBS cells and control media were preincubated with 200 ng/ml Enbrel® (Pd TNFα+E-CM, Ad TNFα+E-CM, Ctrl+E), a soluble TNF-α receptor antagonist, at 37 °C for 10 min before stimulating HMEC-1. Monocyte adhesion was induced 4.3- and 2.2-fold after treatment with Pd TNFα-CM and Pd TNFα+E-CM, respectively, when compared with the Ctrl. Monocyte adhesion was induced 4.9- and 1.4-fold after treatment with Ad TNFα-CM and Ad TNFα+E-CM, respectively, compared with the Ctrl. The Enbrel®-treatment of CM from Pd and Ad decreased the monocyte adhesion 2.0- and 3.6-fold, respectively. E: signals for p-IκBα were strong in HMEC-1 cells after treatment with Pd TNFα-CM and Ad TNFα-CM and decreased after treatment with Pd TNFα+E-CM and Ad TNFα+E-CM, respectively. The opposite is shown for t-IκBα. β-actin served as loading control. Results for A, B and D are means ± SE (n=3) and for C and E (n=2). n.s. means statistically not significant.*P<0.05, **P<0.01, ***P<0.001.
3.3.6 Impact of hypoxia on adipokine gene expression and secretion of SGBS cells and analysis of their CM on endothelial cell activation

Since WAT mass expansion can generate a hypoxic environment (see chapter 1.2.6) it is of interest to find out how the different cell types in WAT react to hypoxia and how this influences their cross-talk. *In vitro* experiments are important to get valuable insights into these complex interactions. Thus, CM of SGBS cells exposed to normoxia, moderate (4% O\(_2\)) and/or severe hypoxia (1% O\(_2\)) were used for comparative studies applying endothelial cell proliferation and monocyte-endothelial cell-cell adhesion assays and adipokines measurements. CM of hypoxia-treated preadipocytes and adipocytes increased the cell proliferation of HMEC-1 at similar rates (Figure 20A). Monocyte-endothelial cell-cell adhesion was enhanced with preadipocyte-CM and significantly induced with adipocyte-CM compared with CM of normoxic SGBS cells (Figure 20B). Importantly, CM of hypoxic adipocytes significantly induced the endothelial ICAM-1 cell surface expression, whereas CM of hypoxic preadipocytes did not further reinforce the already high endothelial ICAM-1 expression induced by CM of normoxic preadipocytes (Figure 20C). A gradual and pronounced upregulation of the hypoxia-sensitive transcription factor HIF-1\(\alpha\) (see chapter 1.2.6) was measured to similar amount for preadipocytes and adipocytes under hypoxia demonstrating the impact of hypoxia on SGBS cells (Figure 20D). Adipokine gene expression and secretion of preadipocytes and adipocytes under 4% and 1% hypoxia were analysed using qRT-PCR and specific ELISAs or bead-based Luminex® assays. An overview of the results shown in Figure 21 is given in Table 2. For adipokine secretion of preadipocytes and adipocytes under 4% hypoxia, an increase of VEGF, leptin, PAI-1 and IL-6 in CM was measured which was strongly enhanced under 1% hypoxia (Figure 21A). The corresponding gene expression data for VEGF and leptin are in agreement with the protein secretion data. In contrast, gene expression for PAI-1 and IL-6 increased in SGBS cells under hypoxia but did not change with the degree of hypoxia (Figure 21A). Adiponectin secretion in adipocytes was not altered by hypoxia, although at mRNA level a decrease was observed (Figure 21A). For MCP-1, an upregulation of secretion was observed in preadipocytes whereas at mRNA level no significant changes were found (Figure 21B). In adipocytes a decrease of MCP-1 secretion was measured under 1% hypoxia (Figure 21B) which is in agreement with the gene expression data. A similar secretion pattern was observed for IL-8, although the IL-8 increase in preadipocytes and the decrease in adipocytes under 1% hypoxia was not statistically significant (Figure 21B). Moreover, RANTES secretion of preadipocytes and adipocytes
increased under 4 % hypoxia and was strongly enhanced in preadipocytes under 1 % hypoxia whereas in adipocytes only baseline levels were observed (Figure 21B). At gene expression level no significant differences were observed (Figure 21B). IL-4 known as endothelial cell co-activator together with IL-1 and TNF-α (Petzelbauer et al., 1993) was significantly increased in preadipocyte-CM and adipocyte-CM under 1 % hypoxia (Figure 21B). Interestingly, SDF1-α showed a trend towards enhancement in CM of preadipocytes exposed to hypoxia (Figure 21B) however, these results were not statistically significant. The proteins of the cytokines IL-1, TNF-α and IFN-γ were measured at detection limit (Figure 21C). In contrast, at mRNA level Il-1α increased in adipocytes exposed to 1 % hypoxia. TNF-α gene expression was elevated in preadipocytes under 4 % hypoxia whereas under 1 % hypoxia no change was observed.
Figure 20  Effects of hypoxia on HIF-1α protein in preadipocytes and adipocytes and their CM on microvascular endothelial cell activation

Preadipocytes (Pd) and adipocytes (Ad) were exposed to normoxia and hypoxia. Subsequently, HMEC-1 cells were treated with control medium (Ctrl), conditioned media (CM) of normoxic cells (Pd-CM, Ad-CM), and reoxygenated CM derived from SGBS cells under 4 % hypoxia (Pd 4%-CM, Ad 4%-CM). Endothelial cell proliferation (A), adhesion of U937 monocytes to HMEC-1 (B) and ICAM-1 cell surface expression (C) on HMEC-1 and HIF-1α protein in SGBS cells (D) was assessed. A: proliferation was increased after treatment with Pd-CM of normoxic vs. hypoxic cells 4- and 9.3-fold and after treatment with Ad-CM of normoxic vs. hypoxic cells 5.5- and 10-fold, respectively, when compared with the Ctrl. The proliferation difference between the treatments with CM of normoxic and hypoxic Pd and Ad was 2.3- and 1.8-fold, respectively. As positive control HMEC-1 were treated with MCDB 131 medium containing 10 % FCS. B: measured monocyte adhesion of TNF-α-treatment (25 ng/ml) of HMEC-1 was considered as 100 % monocyte adhesion (positive control). Correspondingly, monocyte adhesion for Pd-CM of normoxic and hypoxic cells was 28 % and 43 % and for Ad-CM of normoxic and hypoxic cells 20 % and 33 %, respectively. Monocyte adhesion of the control medium was 13 %. Monocyte adhesion differences between the treatment with CM of normoxic and hypoxic Pd and Ad were 1.5- vs. 1.7-fold. C: the increase of ICAM-1 expression after treatment with Pd-CM of normoxic and hypoxic cells was 1.4-fold. Ad-CM of normoxic and hypoxic cells increased ICAM-1 1.1- (n.s.) and 1.3-fold, respectively. ICAM-1 levels differed between the treatments with Ad-CM of normoxic and hypoxic cells by 15 %. (D): the response of Pd and Ad to hypoxia was confirmed by measuring HIF-1α protein. SGBS cells were kept under normoxia and 4 % (Pd 4%, Ad 4%) and 1 % hypoxia (Pd 1%, Ad 1%) for 4 hrs. Western blot analysis was performed using antibodies against HIF-1α and β-actin. For the analysis of the experiments, the protein HIF-1α/β-actin ratio of Pd was considered as 1. HIF-1α/β-actin ratio was 2.8-fold higher in Pd compared with Ad under normoxia. Under 4 % and 1 % hypoxia, the ratio was 2.2- and 4.4-fold increased in Pd and 1.8- and 4.8-fold in Ad, respectively, when compared with baseline levels of Pd. Results are means ± SE (n=4). n.s. means statistically not significant. *P<0.05, **P<0.01, ***P<0.001. Representative blots are shown.
Table 2  Impact of hypoxia on adipokine gene expression and protein secretion of preadipocytes and adipocytes

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<tr>
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<tr>
<td>VEGF</td>
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Preadipocytes (Pd) and adipocytes (Ad) were kept under normoxia, 4 % hypoxia (4%) and 1 % hypoxia (1%) for 24 hrs. Their adipokine mRNA expressions and protein concentrations in the corresponding conditioned media were quantified by qRT-PCR and specific ELISAs or bead-based Luminex assays, respectively. The analysed molecules were: VEGF, leptin, PAI-1, IL-6, adiponectin, MCP-1, IL-8, RANTES, IL-4, SDF1-α, IL-1α, IL-1β, TNF-α and IFN-γ. The values for SGBS cells under 4 % and 1 % hypoxia are presented as relative RNA and protein release in x-fold (x) compared with values of corresponding Pd and Ad under normoxia which were set to the value of 1. Results are means ± SE (n=4). n.s. means statistically not significant. **P<0.01, ***P<0.001.
Figure 21 A  Impact of hypoxia on adipokine mRNA expression and protein secretion of preadipocytes and adipocytes

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Figure 21 B  Impact of hypoxia on adipokine mRNA expression and protein secretion of preadipocytes and adipocytes

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|         |  |  |  |  |  |  |  |  |
| RNA     |  |  |  |  |  |  |  |  |
| MCP-1   |  |  |  |  |  |  |  |  |
| Protein |  |  |  |  |  |  |  |  |
| IL-8    |  |  |  |  |  |  |  |  |
| RANTES  |  |  |  |  |  |  |  |  |
| IL-4    |  |  |  |  |  |  |  |  |
| SDF1-α  |  |  |  |  |  |  |  |  |

RANTES values at detection limit (below 10 pg/ml) not measured not measured not measured not measured
Figure 21 C Impact of hypoxia on adipokine mRNA expression and protein secretion of preadipocytes and adipocytes

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*p<0.05, **p<0.01, ***p<0.001.

Figure 21

Preadipocytes (Pd) and adipocytes (Ad) were kept under normoxia, 4% hypoxia (4%) and 1% hypoxia (1%) for 24 hrs. Their adipokine mRNA expressions and protein concentrations in the corresponding conditioned media were quantified by qRT-PCR and specific ELISAs or bead-based Luminex assays, respectively. The analysed molecules were: VEGF, leptin, PAI-1, IL-6, adiponectin, MCP-1, IL-8, RANTES, IL-4, SDF1-α, IL-1α, IL-1β, TNF-α and IFN-γ. The values for SGBS cells under 4% and 1% hypoxia are presented as relative RNA and protein release in x-fold (x) compared with values of corresponding Pd and Ad under normoxia which were set to the value of 1. Results are means ± SE (n=4). n.s. means statistically not significant. *p<0.05, **p<0.01, ***p<0.001.
3.3.7 Impact of SGBS preadipocyte- and adipocyte-CM on endothelial cell signalling pathways

To further evaluate the signalling pathways activated upon CM-treatment of HMEC-1 cells and involved in CM-induced expression of MCP-1, IL-8 and ICAM-1, different signalling molecules were evaluated. On the basis of their promoters, the regulation of these genes can occur through the stimulation of multiple pathways such as the NF-κB, AP-1 or the JAK/STAT pathway (Roebuck et al., 1999; Roebuck and Finnegan, 1999). Therefore, total and specifically phosphorylated forms of IκBα, RelA, p38MAPK, ERK 1/2, SAPK/JNK, c-Jun, STAT-3 and STAT-1 were analysed at appropriate time intervals by Western blot analysis (Figs. 22 to 24). These time intervals were determined with control and stimulated HMEC-1 ahead of the shown experiments (Figures 22A-D; Figures 23A, C, E and G; Figures 24A and C) Interestingly, the phosphorylation status of IκBα (Figure 22E), RelA (Figure 22F) and the level of total IκBα (Figure 22E) did not change after CM-stimulation in HMEC-1 cells. In contrast, the phosphorylations of RelA and IκBα increased and total IκBα decreased in HMEC-1 cells treated with TNF-α, serving as positive control (Figure 22G). The ratios of phosphorylated forms to total specific protein of endothelial SAP/JNK (Figure 23B), p38 MAPK (Figure 23D), ERK1/2 (Figure 23F), c-Jun (Figure 23H), STAT-1 (Figure 24B) and STAT-3 (Figure 24D) were substantially elevated after stimulation with preadipocyte-CM and adipocyte-CM. Note, the measured increase of SAP/JNK, ERK1/2 and c-Jun after stimulation with adipocyte-CM was not statistically significant. For SAPK/JNK, STAT-1 and STAT-3, the differences in phosphorylation between preadipocyte and adipocyte-CM treated HMEC-1 cells was statistically significant.
Figure 22  Impact of CM from SGBS cells on microvascular endothelial cell NFκB signalling pathway

HMEC-1 cells were treated with control medium (Ctrl), TNF-α at indicated concentrations, preadipocyte-conditioned medium (Pd-CM) and adipocyte-CM (Ad-CM) for different time-points. Western blot analysis was performed using antibodies against the total (t-) and/or phosphorylated (p-) forms of IκBα (A, C, E, G) and RelA (B, D, F and G) and β-actin (B, C and D). A and B: a time course was conducted for HMEC-1 cells after treatment with 25 ng/ml TNF-α was performed. C and D: a time course was conducted for HMEC-1 cells after treatment with Pd-CM was conducted. E and F: HMEC-1 cells were treated for 5 min with Pd-CM and Ad-CM. G: HMEC-1 cells were treated for 5 min with different concentrations of TNF-α. For the analysis of these experiments, protein levels under Ctrl-conditions were considered as 1. Results are means ± SE (n=3).
Figure 23  Impact of CM from SGBS cells on microvascular endothelial cell MAPK and c-Jun signalling
Figure 23
HMEC-1 cells were treated with control medium (Ctrl), preadipocyte-conditioned medium (Pd-CM) and adipocyte-CM (Ad-CM) for different intervals. Western blot analysis was performed using antibodies against β-actin (A, C, E and G) serving as loading control, the phosphorylated (p-) and total (t-) forms of SAPK/JNK (A and B), p38MAPK (C and D), ERK1/2 (E and F) and c-Jun (G and H). For all p-molecules a time course in HMEC-1 cells after treatment with Pd-CM was performed (A, C, E and G). For the quantitative detection of SAPK/JNK (B) p38MAPK (D) and ERK1/2 (F) HMEC-1 were treated with CM for 10 min and for detection of c-Jun (H) for 30 min. B, D, F and H: protein levels under Ctrl-conditions were considered as 1. Phospho/total protein increased 6.8- and 3-fold for SAPK/JNK (B), 2.4- and 2-fold for p38MAPK (D), 3- and 2-fold (n.s.) for ERK1/2 (F) and 2.6- and 2.1-fold (n.s.) for c-Jun (H) after treatment with Pd-CM and Ad-CM when compared with the Ctrl. The differences for the phospho/total protein ratio between the treatments with CM were 2.3-fold for SAPK/JNK, 1.2-fold for p38MAPK (n.s.), 1.5-fold for ERK1/2 (n.s.) and 1.2-fold for c-Jun (n.s.). Results are means ± SE (n=3). n.s. means statistically not significant. *P<0.05 Representative blots are shown.

Figure 24 Impact of CM from SGBS cells on microvascular endothelial cell STAT signalling

Figure 24
HMEC-1 cells were treated with control medium (Ctrl), preadipocyte-conditioned medium (Pd-CM) and adipocyte-CM (Ad-CM) for different time-points. Western blot analysis was performed using antibodies against β-actin (A and C) serving as loading control, the phosphorylated (p-) and total (t-) forms of STAT-1 (A and B) and STAT-3 (C and D). For both p-molecules a time course in HMEC-1 cells after treatment with Pd-CM was performed (A and C). For the quantitative detection of STAT-1 (B) and STAT-3 (D) HMEC-1 were treated with CM for 30 min (arbitary units shown). The differences for the phospho/total protein ratio between the treatments with CM were 6.7-fold for STAT-3 and 3.1-fold for STAT-1. Results are means ± SE (n=3). *P<0.05, **P<0.01, ***P<0.001. Representative blots are shown.
3.3.8 The role of endothelial signalling pathways on monocyte endothelial cell-cell adhesion and ICAM-1 protein expression

To determine the role of the above mentioned signalling pathways in the monocyte endothelial cell-cell adhesion and ICAM-1 protein expression, HMEC-1 cells pre-treated with JNK inhibitor II, SB203580 (p38 MAPK inhibitor), PD98059 (MEK1 inhibitor) or JAK inhibitor I and stimulated with preadipocyte-CM in the presence of the corresponding inhibitors were examined. A low and a high dose of each inhibitor was applied. Preadipocyte-CM significantly increased monocyte adhesion and ICAM-1 cell surface protein expression of HMEC-1 cells whereas in the presence of JNK inhibitor II these effects were suppressed to baseline levels (Figures 25A and B). Concordantly, the CM mediated SAPK/JNK phosphorylation decreased dose dependently, supporting the efficacy of the inhibitor (Figure 25C). In contrast, application of PD98059 (Figures 25D and E) and SB203580 (Figures 25G and H) had no significant effect on the increase of monocyte adhesion or ICAM-1 cell surface protein of preadipocyte-CM treated HMEC-1 cells. The efficacy of the inhibitor PD98059 was confirmed by its ability to decrease preadipocyte-CM mediated ERK1/2 phosphorylation (Figure 25F). SB203580 hinders the activity but not the activation of p38 MAPK and therefore, no phosphorylation experiments are shown (Kumar et al., 1999). The JAK inhibitor I (Figures 25I and J) significantly inhibited the increase of monocyte adhesion and ICAM-1 cell surface expression by preadipocyte-CM to the same extent as the JNK inhibitor II (Figures 25A and B). Since STAT-1 and STAT-3 are mediators of JAK-signalling (Murray, 2007) and activated upon stimulation of HMEC-1 cells with preadipocyte-CM, the effect of JAK inhibitor I on STAT-1 and STAT-3 activation by Western blot analysis was assessed. Stimulation of HMEC-1 cells with CM resulted in a robust phosphorylation of STAT-1 and STAT-3, whereas the blockade with JAK inhibitor I effectively abrogated the phosphorylation of STAT-1 and STAT-3 (Figure 25K).
HMEC-1 cells were preincubated with indicated concentrations of JNK inhibitor II (JNK), SB203580 a p38MAPK inhibitor (SB), PD98059 a MEK1 inhibitor (PD) and JAK inhibitor I (P6) for 45 min. After stimulation with control medium (Ctrl), preadipocyte-conditioned medium (Pd-CM) and Pd-CM supplemented with the corresponding inhibitors at indicated concentrations, monocyte adhesion assay (A, D, G and I), ICAM-1 cell surface ELISA (B, E, H and J) and Western blot analysis for β-actin (C, F and K) and the phosphorylated forms (p-) of SAPK/JNK (C), ERK1/2 (F), STAT1 and STAT3 (G) were performed. Results are means ± SE (n\geq3) *P<0.05, ***P<0.001. Representative blots are shown.
3.4 Functional analysis of factors in CM mediating endothelial cell activation

3.4.1 Introduction

To get initial insights into the mediators in preadipocyte-CM and adipocyte-CM which induce the STAT-1/3 dependent endothelial ICAM-1 expression and monocyte-endothelial cell-cell adhesion, the functional analysis was focused on the adipokines IL-6, leptin and VEGF known to signal via the STAT-pathways (Bartoli et al., 2000; Jin et al., 2003; Korpelainen et al., 1999; Ni et al., 2004; Pan et al., 2007; Wincewicz et al., 2007; Yahata et al., 2003). First, the gene expression of the receptors which mainly signal for these cytokines in HMEC-1 cells were analysed and the expression of the IL-6 receptor at protein level was investigated. Next, function-neutralizing antibodies specific for the above mentioned adipokines and corresponding isotype-matched control antibodies were used in proliferation, ICAM-1 cell surface ELISA, monocyte-endothelial cell-cell adhesion and STAT-1/3 phosphorylations assays. Note, corresponding information about the experiments and fold-changes are presented in the figure legends.

3.4.2 Gene and protein expression of the obese receptor (Ob-R) b, VEGFR-2, gp130 and IL-6 receptor (IL-6R) α in HMEC-1 cells

3.4.2.1 Ob-Rb gene expression

The leptin receptor gene encodes five alternatively spliced variants. They are named Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re. Only the Ob-Rb has a long cytoplasmic region which contains several motifs which are required for signal transduction. Upon leptin binding, the Ob-Rb signals e.g. via the JAK/STAT pathway (Friedman and Halaas, 1998; Schwartz et al., 2000). The Ob-Rb is found on many cell types including endothelial cells (Quehenberger et al., 2002). By RT-PCR experiments with total RNA, a PCR product specific for Ob-Rb in HMEC-1 cells was observed after 35 and less amplification cycles (185-bp; Figure 26A).
3.4.2.2 VEGFR-2 gene expression

The VEGF ligand and VEGF receptor biology is complex (see chapter 1.2.2.4). However, VEGF\textsubscript{165} belonging to the VEGF-A family is the most abundant isoform and according to \textit{in vitro} studies the most biologically active form. Additionally, VEGF\textsubscript{121} and VEGF\textsubscript{145} also belonging to the VEGF-A family are biologically active in endothelial cells. The VEGF proteins measured in the SGBS-CM were VEGF\textsubscript{121} and VEGF\textsubscript{165}. The members of the VEGF-A family bind to VEGFR-1 and VEGFR-2 which both belong to the family of tyrosine kinase receptors. VEGFR-2 (Oh \textit{et al.}, 2002) is described to be the most functional mediator of endothelial VEGF signalling (Zachary and Gliki, 2001). In contrast, it is uncertain if VEGFR-1 has a biological important function in endothelial cells. VEGFR-2 gene expression was detected in HMEC-1 cells by RT-PCR experiments using total RNA, specific primers for VEGFR-2 and 35 and less amplification cycles (793-bp; Figure 26B).

3.4.2.3 gp130 and IL-6 R\alpha gene and protein expression

The cellular IL-6R complex basically consists of the 80 kDa ligand (IL-6) binding glycoprotein IL-6R\alpha and the 130 kDa glycoprotein (gp130) involved in signal transduction. The gp130 protein is ubiquitously expressed on most cell-types including endothelial cells, whereas the IL-6R\alpha subunit is cell type-specifically expressed. A membrane-bound form of IL-6R\alpha of approx. 80 kDa and soluble isoforms of IL-6R\alpha (sIL-6R\alpha) of approximately 55 kDa can be generated due to differential mRNA-splicing and/or limited proteolysis (Rose-John \textit{et al.}, 2006). Since there is a discrepancy in the literature about the presence of the IL-6R\alpha on endothelial cells, especially on HUVECs (Modur \textit{et al.}, 1997; Romano \textit{et al.}, 1997; Watson \textit{et al.}, 1996; Waxman \textit{et al.}, 2003), the expression of the IL-6 receptor subunits in the microvascular endothelial cells, HMEC-1, used in this study was assessed. PBMC, known to express IL-6R\alpha (Jablonska \textit{et al.}, 1999) were used as positive control. By RT-PCR experiments with total RNA, robust amounts of PCR-products specific for the gp130-mRNA (253-bp; Figure 26A) and the 80 kDa IL-6R\alpha-mRNA (350 bp; Figure 26B) in both PBMC and HMEC-1 cells were observed. In contrast, rather small amounts of PCR-product specific for the soluble IL-6R\alpha mRNA-splice variant in PBMC and HMEC-1 cells (256-bp; Figure 26B) were detected. \beta-actin served as housekeeper gene (281 bp, Fig 26C).
For immunoblot experiments, total protein lysates from HMEC-1 cells and PBMC and human IL-6Rα-specific antibody recognizing the membrane-bound (80 kDa) and soluble isoforms of IL-6Rα (50 to 60 kDa) were used. For the 80 kDa IL-6Rα protein, the signal in PBMC was stronger compared with the signals for the 80 kDa IL-6Rα in HMEC-1 cells and the 55 kDa soluble IL-6Rα protein in PBMC and HMEC-1 cells (Figure 26D). It is known that the soluble IL-6Rα after binding IL-6 is able to bind to the gp130 subunit on the cell surface forming a functional IL-6 receptor complex (Rose-John et al., 2006). Therefore, our gene expression data for the gp130 and IL-6α subunits (Figures 26A and B) and the membrane-bound and soluble isoforms of the IL-6Rα protein (Figure 26D) indicate the presence of functional IL-6 receptor complexes on HMEC-1 cells and the generation of IL-6α isoforms by alternative splicing and/or shedding of the membrane-bound IL-6Rα.
Figure 26  Expression analysis of leptin receptor b (Ob-Rb), VEGF receptor 2 (VEGFR-2), IL-6 receptor-α (IL-6Rα) and gp130 in HMEC-1 cells and/or peripheral blood mononuclear cells (PBMC)

A, B, C and D: robust PCR-products are shown for mRNAs encoding for Ob-Rb (arrow 185 bp; A), VEGFR-2 (arrow 793 bp; B), gp130 (arrow 253 bp; C) and membrane bound IL-6Rα (arrow 350 bp; D). The PCR-product for the IL-6Rα splice-variant coding for the soluble IL-6Rα (arrow 256 bp; D) in HMEC-1 cells and PBMC (positive control) is weak. E: as reference gene for C and D β-actin (arrow 281 bp) is shown. 50 bp-DNA ladder (ladder) was used to determine the size of the PCR products. F: immunoblot analysis of IL-6Rα (80 kDa) and soluble IL-6R (sIL-6Rα, 55kDa) in protein lysates of HMEC-1 cells and PBMCs (positive control). The applied IL-6Rα detecting antibody specific for both forms of IL-6Rα generates signals with the expected sizes for the 80 kDa IL-6Rα and 55 kDa sIL-6Rα protein. The signals for the 80 kDa IL-6Rα are stronger compared with the faint signals for the 55 kDa sIL-6Rα. β-actin served as loading control. Representative immunoblots are shown (n=4).
3.4.3 Functional analysis of factors in CM mediating endothelial ICAM-1 expression and monocyte endothelial cell-cell adhesion

First, the effects of recombinant proteins for IL-6, leptin and VEGF$_{165}$ added separately to serum-free HMEC-1 cell cultures were assessed. For IL-6, a robust and significant increase in monocyte endothelial cell-cell adhesion and ICAM-1 protein cell surface expression was observed at a IL-6 concentration of 1 ng/ml. In further experiments 10 ng/ml of IL-6 was used as positive control since this concentration was sufficient to induce stable and rather high levels of monocyte adhesion (Figure 27A) to HMEC-1 cells and ICAM-1 cell surface protein (Figure 27B) on HMEC-1 cells. In the presence of a function-neutralizing anti-human IL-6 antibody, the IL-6 effect on monocyte adhesion and ICAM-1 expression was significantly blocked (Figures 29A, B, E and F). For VEGF$_{165}$ and leptin, no consistent significant effects on ICAM-1 protein expression and monocyte endothelial cell-cell adhesion were detectable (data not shown). However, in endothelial cell proliferation assays performed in parallel, both adipokines VEGF$_{165}$ and leptin and also IL-6 increased HMEC-1 cell proliferation as expected already at concentrations of 1 ng/ml of the respective cytokine (Figures 28A-C) demonstrating their biofunctionality. Since leptin and VEGF$_{165}$ did not appear to play an important role in triggering HMEC-1 cells for endothelial cell-monocyte adhesion and ICAM-1 expression, further analysis was focused on the function of IL-6 in CM. As illustrated in Figures 29A and B, the increase of monocyte adhesion and ICAM-1 protein of HMEC-1 cells stimulated with CM of normoxic preadipocytes in the presence or absence of isotype-matched control antibody was significantly suppressed in the presence of the IL-6 antagonist. Treatment of HMEC-1 cells with CM of normoxic adipocytes in the presence of IL-6 antagonist had no effect on ICAM-1 protein but slightly increased monocyte adhesion, although this effect is not statistically significant within the adipocyte-CM group. To exclude the possibility that basal IL-6 secretion of HMEC-1 cells already exerted effects on monocyte adhesion (Figure 29C) and ICAM-1 cells surface expression (Figure 29D), untreated HMEC-1 cells were stimulated with the IL-6 antagonist or the corresponding isotype control alone. No significant changes were observed. Treatment of HMEC-1 cells with CM from hypoxic preadipocytes or adipocytes containing the IL-6 antagonist, further decreased monocyte adhesion (Figure 29E) and ICAM-1 expression (Figure 29F) significantly, with the exception that the inhibitory effect of preadipocyte-CM on ICAM-1 expression was slightly less compared with the normoxic situation.
Figure 27 Impact of IL-6 on monocyte adhesion to and ICAM-1 cell surface protein expression of HMEC-1 cells

HMEC-1 cells were treated with control medium (Ctrl) and different concentrations of recombinant IL-6. Subsequently, monocyte adhesion (A) and cell surface expression of ICAM-1 (B) was assessed. A: The increase in monocyte adhesion upon IL-6 treatment was 1.8-fold for 1 ng/ml, 2.3-fold for 10 and 25 ng/ml and 2.2-fold for 50 ng/ml, when compared with the Ctrl-treatment. B: ICAM-1 expression was elevated upon IL-6 exposure by 1.25-fold for 1 ng/ml, 1.5-fold for 10 ng/ml, 1.4-fold for 25 ng/ml and 1.5 fold for 50 ng/ml when compared with the Ctrl-treatment. Results are means ± SE (n=4). *P<0.05, **P<0.01.
Figure 28  Proliferation analysis of HMEC-1 cells after treatment with recombinant proteins for VEGF\textsubscript{165}, leptin and IL-6

HMEC-1 cells were incubated in the presence of control medium (Ctrl) and different concentrations of VEGF\textsubscript{165} (A), leptin (B) and IL-6 (C) for 48 hrs. Additionally, BrdU was added for the next 16 hrs. Representative data for 2 independent experiments are shown.
Figure 29  Functional analysis of IL-6 in CM from normoxic and hypoxic SGBS cells on monocyte adhesion to and ICAM-1 cell surface expression of HMEC-1 cells.
Control media (Ctrl) and conditioned media (CM) from preadipocytes (Pd) and adipocytes (Ad) exposed to normoxia were preincubated either with 5 µg/ml function-neutralizing anti-IL-6 antibody as IL-6 inhibitor (IL-6 Inh) or with 5 µg/ml of the corresponding IgG1 isotype-matched control antibody (Iso) for 15 min and subsequently used to stimulate HMEC-1 cells. HMEC-1 cells were treated with Ctrl, CM of Pd (Pd-CM) and Ad (Ad-CM), Ctrl and CM of cells pre-incubated with IL-6 inhibitor (Ctrl+IL-6 Inh, Pd-CM+IL-6 Inh, Ad-CM+IL-6 Inh) and Ctrl and CM of cells pre-incubated with the isotype-matched control antibody (Ctrl+Iso, Pd-CM+Iso, Ad-CM+Iso). Likewise, HMEC-1 cells were treated with reoxygenated CM derived from 4% hypoxic Pd (Pd 4%-CM), Ad (Ad 4%-CM), CM of hypoxic cells pre-incubated with IL-6 inhibitor (Pd 4% CM+IL-6 Inh, Ad 4%-CM+IL-6 Inh) and CM of hypoxic cells pre-incubated with the isotype-matched control antibody (Pd 4%-CM+Iso, Ad 4%-CM+Iso). HMEC-1 cells treated with 10 ng/ml IL-6 added to Ctrl medium (IL-6) or in combination with 5 µg/ml IL-6 inhibitor (IL-6+IL-6 Inh) were used to control the IL-6 blocking efficiency of the IL-6 inhibitor. Monocyte-endothelial cell-cell adhesion (A, C and E) and ICAM-1 cells surface expression of HMEC-1 cells (B, D and F) were determined. The data are presented as x-fold of the Ctrl-treatments. A: Treatment of HMEC-1 cells with Pd-CM+IL-6 Inh decreased monocyte adhesion by 26 % and 24 % compared with the Pd-CM and Pd-CM+Iso. For Ad-CM+IL6 Inh, a slight increase in monocyte adhesion was observed, although, this effect was not significant between the Ad-CM group members. B: Treatment of HMEC-1 cells with Pd-CM+IL-6 Inh decreased ICAM-1 protein expression by 19 % and 22 % compared with the Pd-CM and Pd-CM+Iso. No differences were observed for the Ad-CM group. C and D: Exposure of IL-6 Inh or Iso alone had no significant effects on monocyte adhesion or ICAM-1 cells surface protein expression. E: Treatment of HMEC-1 cells with Pd 4%-CM+IL-6 Inh further decreased monocyte adhesion by 45 % and 43 % compared with HMEC-1 cells stimulated with Pd 4%-CM and Pd 4%-CM+Iso. For HMEC-1 cells treated with Ad 4%-CM a decrease of monocyte adhesion by 21 % was determined. F: As for normoxic CM, ICAM-1 protein expression on HMEC-1 cells decreased by 11 % and 12 % after treatment with Pd 4%-CM+IL-6 Inh compared with the treatment with Pd 4%-CM (n.s.) and Pd 4%-CM+Iso, respectively. In contrast to normoxic Ad-CM, treatment of HMEC-1 with Ad 4%-CM+IL-6 Inh decreased endothelial ICAM-1 protein expression by 14 % compared with the stimulation of HMEC-1 cells with Ad 4%-CM and Ad 4%-CM+Iso. Results are means ± SE (n=6). n.s. means statistically not significant. *P<0.05, **P<0.01, ***P<0.001.
3.4.4 Impact of IL-6 in SGBS-CM on phosphorylations of STAT1/3 in HMEC-1 cells

The IL-6 blockade strategy was applied in order to determine the functional role of IL-6 on the phosphorylations of STAT-1/3 in HMEC-1 cells. First, a time-course for STAT-1/3 phosphorylations using 10 ng/ml IL-6 was performed in order to choose the right time interval for the experiments (Figure 30A). At 30 minutes post-treatment of HMEC-1 cells with IL-6 the signals for STAT-1/3 phosphorylations were strongest as it was also for the treatment with CM, therefore, in further experiments this time was used for stimulations with IL-6. The phosphorylation intensity of STAT-1 and STAT-3 was found to be IL-6 concentration dependent. For STAT-1 and STAT-3 a phosphorylation increase was detectable at least up to IL-6 concentrations of 310 pg/ml and 39 pg/ml IL-6, respectively (Figure 30B). In comparison, SGBS preadipocytes secrete IL-6 on average 400 pg/ml and adipocytes 40 pg/ml (Figure 11A). Therefore, these IL-6 concentrations in the SGBS-CM are high enough to exert effects on STAT-1/3 phosphorylations. Stimulation of HMEC-1 cells with 10 ng IL-6 for 30 min increased phosphorylations of STAT-1 4.6-fold (Figures 31A and C) and STAT-3 5 to 6-fold (Figures 31B and D) compared to the stimulation of HMEC-1 cells with control medium. In the presence of 5µg/ml IL-6 inhibitor, STAT-1 and STAT-3 phosphorylations were abolished to almost baseline levels (Figures 31A-D). Next, we treated HMEC-1 cells with preadipocyte-CM containing IL-6 inhibitor and noticed a decrease of STAT-1 phosphorylation by 2.4- and 2.3-fold (Figure 31A) and STAT-3 phosphorylation by 2.6-fold (Figure 31B) when compared to HMEC-1 stimulated with preadipocyte-CM in the presence or absence of isotype-matched control antibody, respectively. Treatment of HMEC-1 cells with adipocyte-CM containing IL-6 inhibitor also decreased STAT-1 phosphorylation 1.5- and 1.4-fold (Figure 31A) and STAT-3 phosphorylation 1.6-fold (Figure 31B) compared to HMEC-1 cells stimulated with adipocyte-CM in the presence and absence, respectively, of isotype-matched control antibody.

Similar phosphorylations levels of STAT-1 (Figure 31C) and STAT-3 (Figure 31D) were observed for analogue experiments with CM of hypoxic SGBS cells. For the monocyte adhesion and ICAM-1 cell surface experiments, IL-6 antagonist treatment alone of HMEC-1 cells exerted no effects (Figures 31C and D) and neither did the corresponding isotype control (data not shown).
Figure 30  Impact of IL-6 dependent on incubation time and concentration on the phosphorylations of STAT-1 and STAT-3 in HMEC-1 cells

HMEC-1 cells were treated for indicated time intervals with 10 ng/ml of recombinant IL-6. Subsequently, the proteins were analysed for the phosphorylations of STAT-1, STAT-3 and β-actin, serving as loading reference (A). Next, HMEC-1 cells were treated with indicated concentrations of recombinant IL-6 for 30 min. The protein was analysed as described for A (B). Representative blots are shown (n=2).
Figure 31 Functional analysis of IL-6 in CM from normoxic and hypoxic SGBS cells on STAT-1 and STAT-3 phosphorylations in HMEC-1 cells
Control media (Ctrl) and conditioned media (CM) from preadipocytes (Pd) and adipocytes (Ad) exposed to normoxia were preincubated either with 5 µg/ml function-neutralizing anti-IL-6 antibody as IL-6 inhibitor (IL-6 Inh) or with 5 µg/ml of the corresponding IgG1 isotype-matched control antibody (Iso) for 15 min and subsequently used to stimulate HMEC-1 cells. HMEC-1 cells were treated with Ctrl, CM of Pd (Pd-CM) and Ad (Ad-CM), Ctrl and CM of cells pre-incubated with IL-6 inhibitor (Ctrl+IL-6 Inh, Pd-CM+IL-6 Inh, Ad-CM+IL-6 Inh) and Ctrl and CM of cells pre-incubated with the isotype-matched control antibody (Ctrl+Iso, Pd-CM+Iso, Ad-CM+Iso). Likewise, HMEC-1 cells were treated with reoxygenated CM derived from 4 % hypoxic Pd (Pd 4%-CM) and Ad (Ad 4%-CM), CM of hypoxic cells pre-incubated with IL-6 inhibitor (Pd 4% CM+IL-6 Inh, Ad 4%-CM+IL-6 Inh) and CM of hypoxic cells pre-incubated with the isotype-matched control antibody (Pd 4%-CM+Iso, Ad 4%-CM+Iso). HMEC-1 cells treated with 10 ng/ml IL-6 added to Ctrl medium (IL-6) or in combination with 5 µg/ml IL-6 inhibitor (IL-6+IL-6 Inh) were used to control the IL-6 blocking efficiency of the IL-6 inhibitor. Total (t-) and phosphorylated (p-) STAT-1 and STAT-3 in HMEC-1 cells were determined by Western blot analysis. The data are presented as x-fold of the Ctrl-treatments. A and B: Stimulation of HMEC-1 cells with 10 ng IL-6 for 30 min (IL-6) increased phosphorylations of STAT-1 4.6-fold and STAT-3 6.1-fold compared with the stimulation of HMEC-1 cells with control medium (Ctrl). In the presence of 5µg/ml IL-6 inhibitor (IL-6+IL-6 Inh) STAT-1 and STAT-3 phosphorylations were abolished to almost baseline levels. Treatment of HMEC-1 cells with Pd-CM+IL-6 Inh decreased STAT-1 phosphorylation by 2.4- and 2.3-fold and STAT-3 phosphorylation by 2.6-fold when compared with the stimulation of HMEC-1 cells with Pd-CM and Pd-CM+Iso, respectively. Treatment of HMEC-1 cells with Ad CM+IL-6 Inh also decreased STAT-1 phosphorylation 1.5- and 1.4-fold and STAT-3 phosphorylation 1.6-fold compared with HMEC-1 cells stimulated with Ad-CM and Ad-CM+Iso, respectively. C and D: Stimulation of HMEC-1 cells with 10 ng IL-6 for 30 min (IL-6) increased phosphorylations of STAT-1 4.6-fold and STAT-3 5.2-fold compared with the stimulation of HMEC-1 cells with control medium (Ctrl). In the presence of 5 µg/ml IL-6 inhibitor (IL-6+IL-6 Inh) STAT-1 and STAT-3 phosphorylations were abolished to almost baseline levels. Treatment of HMEC-1 cells with Pd 4%-CM+IL-6 Inh decreased STAT-1 phosphorylation by 2.5- and 2.9-fold and STAT-3 phosphorylation by 1.8-fold when compared with the stimulation of HMEC-1 cells with Pd-CM and Pd-CM+Iso, respectively. Treatment of HMEC-1 cells with Ad CM+IL-6 Inh also decreased STAT-1 phosphorylation 1.5- and 1.4-fold (both n.s.) and STAT-3 phosphorylation 1.3-fold compared with HMEC-1 cells stimulated with Ad-CM and Ad-CM+Iso, respectively. Results are means ± SE (n=3). n.s. means statistically not significant. *P<0.05, **P<0.01, ***P<0.001.
3.5 Impact of CM derived from different-sized human primary mature adipocytes on HMEC-1 cell activation

In WAT adipocytes of various cell sizes exist. In obesity the size and/or number of adipocytes increases (see chapter 1.2.4.2). A positive correlation of secretion has been shown for several cytokines, including leptin, IL-6, IL-8 and MCP-1 with increasing fat cell size (Skurk et al., 2007). In contrast, another study found that the fraction of small adipocytes was consistently associated with inflammatory gene expression, independently of BMI and insulin resistance (McLaughlin et al., 2009). Therefore, the role of adipocyte size on endothelial cell activation in the context of adipocyte/endothelial cross-talk in WAT was analysed. Mature adipocytes, consisting of various cell sizes (entirety of adipocytes), were isolated from human WAT by collagenase digestion. These adipocytes were then separated by flotation in order to obtain a fraction with small and a fraction with large adipocytes. The fraction containing adipocytes with medium size was discarded due to technical reasons. HMEC-1 cells were treated with CM derived from the entirety of adipocytes (average diameter: 113 µm), small adipocytes (average diameter: 95 µm) and large adipocytes (average diameter: 130 µm), respectively. Subsequently, monocyte-endothelial cell-cell adhesion (Figures 32A, C and E) and ICAM-1 cell surface expression (Figures 32B, D and F) assays were performed. The corresponding data were normalized to adipocyte cell volume (Figures 32A and B), adipocyte cell surface area (Figures 32C and D) and adipocyte cell count (Figures 32E and F). The absolute cell count for the entirety of adipocytes was \(1.2 \times 10^6 \pm 2.8 \times 10^5\) per ml, for small adipocytes \(1.9 \times 10^6 \pm 4.1 \times 10^5\) per ml and for large adipocytes \(8.5 \times 10^5 \pm 1.7 \times 10^5\) per ml. The data normalized to adipocyte cell volume are equal to the non-normalized data since for CM generation 1 ml of the entirety of adipocytes or of the respective adipocyte fraction was cultured in 5 ml medium, respectively. The data normalized to adipocyte cell volume reveal that monocyte adhesion and ICAM-1 cell surface expression increased after treatment with CM of the entirety of adipocytes, small and large adipocytes (Figures 32A and B). However, there was a tendency towards a greater increase in the assays when HMEC-1 cells were treated with the CM of the entirety of adipocytes. Normalization of the data to adipocyte cell surface area yielded similar results, although the effect exerted on monocyte-endothelial cell-cell adhesion by CM of small adipocytes was not statistically significant anymore (Figures 32C and D). When the data were normalized to adipocyte cell count, neither monocyte adhesion (Figure 32E) to nor ICAM-1 expression (Figure 32F) of HMEC-1 cells was significant after treatment with CM of small adipocytes, in contrast to treatment with the
entirety of adipocytes and large adipocytes. Moreover, the differences between the treatments with CM of all and small adipocytes were for both assays statistically significant. Additionally, monocyte-endothelial cell-cell adhesion differences between treatments with CM of small and large adipocytes were significant.

Figure 32 Impact of human primary mature adipocytes of different size on endothelial cell activation

A

B

C

D

E

F

Figure 32
The conditioned media (CM) of the entirety of human primary mature adipocytes (all adipo), small mature adipocytes (small adipo) and large mature adipocytes (large adipo) were used to stimulate HMEC-1 cells. The CM was generated by culturing 1 ml of cells in 5 ml MCDB 131 medium for 16h. Subsequently, monocyte adhesion (A, C and E) to and ICAM-1 cell surface expression (B, D and F) of HMEC-1 cells was assessed. Data are presented as % of the positive control TNF-α. A and B: Monocyte adhesion was 24 %, 18 % and 19 % for all, small and large adipo, respectively. ICAM-1 expression was 38 %, 28 % and 22 % for all, small and large adipo, respectively. C and D: The values for monocyte adhesion and ICAM-1 shown in A and B, were normalized to the cell surface area. Monocyte adhesion was 28 %, 18 % (n.s.) and 24 % and for ICAM-1 45 %, 28 % and 28 % for all, small and large adipo, respectively. E and F: The values shown in A and B were normalized to the adipocyte cell count. Monocyte adhesion was 40 %, 19 % (n.s.) and 44 % and for ICAM-1 61 %, 28 % (n.s.) and 50 % for all, small and large adipo, respectively. Results are means ± SE (n=4). n.s. means statistically not significant. *P<0.05, **P<0.01.
3.6 Peptidomics analysis of SGBS cell supernatants

3.6.1 Introduction

In order to generate and analyse the peptide secretion profile of SGBS cells and possibly find new secretion products which might be involved in the molecular and cellular cross-talk between preadipocytes, adipocytes and endothelial cells, the peptidomic technology was applied. The CM of normoxic and 1% hypoxic SGBS preadipocytes and adipocytes were collected for analysis. The peptides were extracted and separated by liquid chromatography into 96 fractions by hydrophility which were then subjected to mass spectrometry. The data were visualized as a 2D peptide display. For detailed information refer to chapter 2.10.

3.6.2 Analysis of markers for cell lyses in CM of preadipocytes and adipocytes

As one of the quality controls of the peptidomic-technology used, the supernatants were tested for peptides from intracellular structures. Their amounts reflect the degree of cell lysis before or during supernatant sampling. If the amounts are high, the data evaluated may not be representative and may generate false-positive and false-negative results. In this setting shotgun sequenced peptides from the supernatant of human adipose tissue as cell lysis marker were used. These contain peptide fragments from the cytoplasm, nucleus, lipid droplets, caveolae and membrane proteins. Table 3 gives an overview of the used cell lyses marker and the number of peptide fractions analysed. In figure 33 the data for these markers in SGBS preadipocyte- and adipocyte-CM were visualized. Mean signal intensities between 10 and 20 are considered near the baseline noise according to the company Digilab BioVisioN (Hannover, Germany). It indicates that the amount of the measured markers for cell lyses is extremely low and mitigates in favour of the cell culture procedure. There were no significant differences in cell leakage observed for any of the marker-groups between SGBS preadipocyte- and adipocyte-CM.

In contrast, the values of the peptide markers for cell lyses in supernatants from primary cells, especially from mature adipocytes cultured in DMEM/F12 medium, had signal intensities above 20 and were significantly higher when compared with SGBS-CM (data not shown).
3.6.3 Putative peptides with different expressions in SGBS preadipocytes and adipocytes

The data shown in this section are limited, since the data were generated by the company Digilab BioVisioN (Hannover, Germany) which went insolvent. However, at least it was possible to obtain the data for eight in silico annotated peptides which are visualized in figure 34. An overview of these peptides is shown in table 4.

The samples were blinded before sending to the company with the following coding (#): #1: adipocyte-CM derived of cells under normoxia, #3: adipocyte-CM derived of cells under 1 % hypoxia, #4: preadipocyte-CM derived of cells under normoxia, #6: preadipocyte-CM derived of cells under 1 % hypoxia. It appeared that in the 2D peptide display strong signal differences occurred between the #1/3 (n=4) and #4/6 (n=6) for perilipin (PLIN), chemokine, CC motif, ligand 14 (CCL14), GRO protein, alpha (GROA), IL-8, Secretogranin II (SCG2), Secretogranin V (7B2) and Progranulin (GRN). Since perilipin is an important protein in fat cell biology it was not unexpected that the signals for two perilipin fragments were stronger in the CM of adipocytes (#1/3) compared with preadipocytes (#4/6). However, the signal intensities for perilipin in adipocyte-CM were below critical values which would have indicated increased cell lysis. Signals for a fragment of IL-8 were more pronounced in the CM of preadipocytes (#4/6) versus adipocytes (#1/3). This is in concordance with the data which had been generated before using qRT-PCR and ELISA techniques (see chapter 3.2.2). The band intensities for the fragments of GROA, CCL14, SCG2 and 7B2 were also stronger in preadipocyte-CM (#4/6) compared with adipocyte-CM (#1/3). In contrast, signals for fragments of GRN were more pronounced in adipocyte-CM (#1/3) versus preadipocyte-CM (#4/6). Due to a limited number of analysed samples, the #1 (n=2) and #3 (n=2) and the #4 (n=3) and #6 (n=3) were combined for data analysis. Differences within the groups (#1 versus #3 and #4 versus #6) were not investigated.
Table 3  Overview of markers used for assessing the extent of cell lyses in CM of SGBS cells

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Localization</th>
<th>Number of estimated peptide fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase I transcript release factor</td>
<td>Caveolae</td>
<td>4</td>
</tr>
<tr>
<td>Actin, cytoplasmic 1</td>
<td>Cytoplasm</td>
<td>2</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Lipid droplet</td>
<td>11</td>
</tr>
<tr>
<td>Perilipin</td>
<td>Lipid droplet</td>
<td>6</td>
</tr>
<tr>
<td>Transmembrane BAX inhibitor motif-containing protein 1</td>
<td>Membrane protein</td>
<td>1</td>
</tr>
<tr>
<td>Cytochrom b5</td>
<td>Mitochondrion</td>
<td>1</td>
</tr>
<tr>
<td>La-related protein 1</td>
<td>Nucleus</td>
<td>6</td>
</tr>
</tbody>
</table>

The list gives an overview of the markers used, their general location within the cell and the corresponding number of estimated peptides.

Figure 33  Evaluation of the extent of cell lysis before and during supernatant sampling

![Graph showing mean signal intensity for different locations and conditions.]

Figure 33  Markers for cell leakage were analysed in the conditioned media (CM) of SGBS preadipocytes and adipocytes. Therefore, sequenced peptides which were evaluated before from the supernatant of human adipose tissue were used. A mean signal intensity between 10 and 20 is near the baseline noise. Values around this level infer that the content of intracellular peptides in the CM is very low. Results are means ± SE (n=6 for preadipocytes and n=4 for adipocytes).
Figure 34  Visualization of putative peptides identified by the peptidomic-technology

In the background a 2D peptide display is shown. The x-axis is highlighted in green and represents the mass to charge ratio of peptides. The y-axis is highlighted in orange and represents the 96 chromatographic fractions where the hydrophobicity increases from the top to the bottom. The regions for the presented “in silico” annotated peptides from the 2D peptide display are shown separately enlarged in boxes. The name and the detected amino acid region of the peptide candidates are presented in boxes with arrows pointing to the respective regions on the 2D peptide display. The conditions 1 and 3 (adipocyte-conditioned media) were compared with the conditions 4 and 6 (preadipocyte-conditioned media). The picture was taken from the Digilab BioVisioN report from 18 Nov 2008 and slightly modified.
Table 4  Overview of putative peptide candidates identified by the peptidomic-technology

<table>
<thead>
<tr>
<th>Names</th>
<th>Fragment/fraction</th>
<th>Upregulated under condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perilipin; PLIN</td>
<td>PLIN (11-23)/F039.1512.5 PLIN (458-493) F050.3577.5</td>
<td>1 and 3 (adipocyte-CM)</td>
</tr>
<tr>
<td>Chemokine, CC motif, ligand 14; CCL14</td>
<td>CCL14 (20-93)/F063.8673.5</td>
<td>4 and 6 (preadipocyte-CM)</td>
</tr>
<tr>
<td>GRO protein, alpha; GROA</td>
<td>GROA (35-107)/F063.7861.5</td>
<td>4 and 6 (preadipocyte-CM)</td>
</tr>
<tr>
<td>Interleukin-8; IL-8</td>
<td>IL-8 (80-99)/F076.2457.5</td>
<td>4 and 6 (preadipocyte-CM)</td>
</tr>
<tr>
<td>Secretogranin II; SCG2</td>
<td>SCG2 (529-566)/F078. 155.5</td>
<td>4 and 6 (preadipocyte-CM)</td>
</tr>
<tr>
<td>Secretogranin V</td>
<td>7B2 (181-211)/F050.3594.5</td>
<td>4 and 6 (preadipocyte-CM)</td>
</tr>
<tr>
<td>Granulin precursor, GRN</td>
<td>GRN (281-337)/F045.6174.5</td>
<td>1 and 3 (adipocyte-CM)</td>
</tr>
</tbody>
</table>

This table gives an overview of the candidate peptides and their symbols, the analysed fragment-regions (amino-acid region of the candidate peptides) and under which condition the candidate peptides were upregulated. The conditions 1 and 3 (adipocyte-conditioned media) were compared with the conditions 4 and 6 (preadipocyte-conditioned media).
3.6.4 Validation of putative peptide candidates

After the identification of putative peptide candidates (“in silico annotated” peptides) using the peptidomic technology, I focussed on the three peptides progranulin, secretogranin II and secretogranin V which up to that time, to my knowledge had not been described in the literature as adipokines.

3.6.4.1 Progranulin

Progranulin is a 68.5 kDa cysteine-rich protein and secreted in a highly glycosylated, 90 kDa form. It is expressed in many tissues, particularly in epithelial and hematopoietic cells and is found in human plasma. Its function is complex and different in various organs and the central nervous system, e.g. it operates in wound healing responses and modulates inflammatory events. (Eriksen and Mackenzie, 2008; He and Bateman, 2003). For further description refer to chapter 4.7.

In SGBS cells, gene expression of progranulin was slightly higher in normoxic SGBS adipocytes versus preadipocytes (Figure 35A). Interestingly, the gene expression signals appeared very early, around 20 cycles which is in its intensity comparable to adiponectin mRNA, considered to be the most abundant adipokine. Moreover, progranulin was also detected in the CM of preadipocytes and adipocytes using the ELISA technique (Figure 35B). However, in contrast to the prediction of the in silico annotated peptide analysis, progranulin protein was found in greater amounts in the normoxic preadipocyte-CM compared with adipocyte-CM. Notably, the amount of progranulin in the CM of both, preadipocytes and adipocytes was in the ng- range which is comparably higher than many other cytokines.

3.6.4.2 Secretogranin II

Secretogranin II is an extremely hydrophilic and acidic protein with a molecular weight of 67.5 kDa. It is distributed throughout the endocrine and nervous system where it is localized in large dense core vesicles (Fischer-Colbrie et al., 1995). Secretogranin II can be cleaved proteolytically and is found in human plasma (Stridsberg et al., 2008). Processing of this protein yields proteins of intermediate size and small peptides. The best characterized product is secretoneurin which has a molecular weight of 21 kDa. It is a chemoattractant for monocytes, eosinophils and endothelial cells. It promotes proliferation and angiogenesis and inhibits apoptosis in endothelial cells. Its angiogenic potential has been
said to be comparable to that of VEGF (Egger et al., 1994; Fischer-Colbrie et al., 2005; Kahler et al., 2002). For further description refer to chapter 4.7.

Gene expression of secretogranin II was found in normoxic SGBS preadipocytes and adipocytes. The mRNA expression was more pronounced in the preadipocytes compared with adipocytes (Figure 35C). So far, at mRNA level this is in line with the prediction of the in silico annotated peptide analysis.

### 3.6.4.3 Secretogranin V

Secretogranin V also known as pituitary polypeptide 7B2 is a 21 kDa protein. Its expression is restricted to neurons and endocrine cells. Secretorgranin V is found in human plasma (Stridsberg et al., 2008). Analysis of its genetic variations showed no link to obesity and common forms of type 2 diabetes, although, it was concluded, that some genetic variants might worsen glucose intolerance and insulin resistance in the background of severe and early onset obesity (Bouatia-Naji et al., 2007). For further description refer to chapter 4.7.

In SGBS cells, secretogranin V mRNA was found in both, normoxic preadipocytes and adipocytes. Interestingly, gene expression was higher in the preadipocytes versus adipocytes (Figure 35D) which is in concordance with the prediction of the in silico annotated peptide analysis.
The mRNA quantities of the “in silico” annotated peptides progranulin (A), secretogranin II (C) and secretogranin V (D) was assessed in normoxic SGBS preadipocytes (Pd) and adipocytes (Ad) by qRT-PCR. The Ad values are presented as relative expression compared with values of the corresponding Pd (A, C and D). Progranulin protein was measured in the conditioned media of normoxic Ad and Pd using a specific ELISA (B). A and B: Progranulin mRNA expression was 40 % higher in Ad vs. Pd, whereas the protein was 3.7 fold higher in Pd compared with Ad. C: Secretogranin II mRNA expression was 2.6 fold higher in Pd vs. Ad. D: Secretogranin V gene expression was elevated 5.7 fold in Pd compared with Ad. Results are means ± SE (n=5). **P<0.01, ***P<0.001.
4 Discussion

4.1 Introduction

Since the discovery of leptin (Zhang et al., 1994) the perspectives on the role of WAT have changed dramatically. Besides its role as important energy reservoir it is a complex secretory and endocrine organ (Hauner, 2005). When WAT mass expands, several inflammatory molecules are found at higher amounts within the tissue (Wellen and Hotamisligil, 2005). It is suggested that some of these adipokines contribute to the state of chronic low-grade inflammation which contributes to insulin resistance and endothelial cell activation, the latter if dysregulated leading to micro- and macrovascular endothelial cell dysfunction (Kuldo et al., 2005; Molavi et al., 2006; Trayhurn, 2005). WAT consists of many cell types including adipocytes, preadipocytes, fibroblasts, endothelial cells and immune cells which differentially contribute to the pool of adipokines and are involved in autocrine, paracrine and endocrine processes and cross-talks (Antuna-Puente et al., 2008; Fain et al., 2008; Fain et al., 2004; Hauner, 2005; Weisberg et al., 2003). Obesity is connected with an increased number of macrophages and other immune cells in WAT (Weisberg et al., 2003; Wu et al., 2007). The endothelium as the interface between circulating blood immune cells and adipose tissue plays an interactive role in these infiltration and inflammatory processes (Pober and Sessa, 2007).

The overall aim of this study was to identify factors and elucidate the cellular interactions and the molecular mechanisms involved in the cross-talk of preadipocytes, adipocytes and endothelial cells. In order to mimic these processes in vitro I

1. established a coculture system between SGBS cells and HMEC-1 cells,
2. analysed adipokine expression and secretion of SGBS preadipocytes and adipocytes under normoxia, hypoxia and TNF-α- stimulation by qRT-PCR, ELISA and multiplex bead-based Luminex® assay,
3. analysed the impact of CM from stimulated SGBS cells on microvascular endothelial cell function by investigating monocyte-endothelial cell-cell adhesion, endothelial cell proliferation and signalling pathways,
4. performed functional analysis of mediators in the CM of preadipocytes and adipocytes responsible for the increased monocyte-endothelial cell-cell adhesion,
5. analysed the impact of CM from different-sized primary mature adipocytes on monocyte-endothelial cell-cell adhesion and
6. detected new candidates in SGBS cells which might be involved in the molecular cross-talk between preadipocytes, adipocytes and microvascular endothelial cells by using the peptidomics technology, quantitative qRT-PCR and ELISA.

Major findings mentioned in this study have been presented at congresses (see curriculum vitae) and were published in American Journal of Physiology – Endocrinology and Metabolism (Mack et al., 2009) and in Hormone and Metabolic Research (Skurk et al., 2009).

The results of this study show that preadipocytes produced higher levels of pro-inflammatory cytokines compared with adipocytes leading to endothelial activation in coculture as demonstrated in the functional assays. Exposure of SGBS preadipocytes and adipocytes to hypoxia and TNF-α enhanced and induced, respectively, endothelial cell activation in coculture. Additionally, endothelial cells were also activated by CM of primary mature adipocytes independently of their size. The characterization of the underlying molecular mechanisms of the observed endothelial cell activation found the MAPK pathway members, c-Jun as important element of the AP-1 complex and STAT-1/-3 to be involved, whereas the NF-κB pathway was not activated. It was shown that the preadipocyte-CM-induced monocyte-endothelial cell-cell adhesion and ICAM-1 cell surface protein expression on HMEC-1 was JNK and JAK-1/STAT-1/3 pathway dependent. Furthermore, the functional studies of CM-derived factors revealed IL-6 as a mediator in CM from normoxic and hypoxic preadipocytes and hypoxic adipocytes. It was demonstrated that IL-6 operates on microvascular endothelial cells by increasing monocyte-endothelial cell interactions via the STAT-1/3 pathway. Finally, the characterization of the IL-6 receptor signalling complex on HMEC-1 cells supported the finding that IL-6 acts as an important mediator of the molecular cross-talk between preadipocytes, adipocytes and microvascular endothelial cells. Using the peptidomics technology, qRT-PCR and ELISA three new candidates in SGBS cells were revealed, progranulin and secretogranin II and secretogranin V. Progranulin and secretogranin II could be both involved in the cross-talk between preadipocytes, adipocytes and microvascular endothelial cells and are possibly new adipokines.
4.2 Coculture between SGBS cells and HMEC-1 cells

In the first part of the study an *in vitro* coculture system was established using primary SGBS-CM to stimulate HMEC-1 cells. This was a valuable tool for investigating the molecular and cellular cross-talk between human microvascular endothelial cells, preadipocytes and adipocytes. As yet, only two reports are published analyzing the effects of CM from mature adipocytes isolated from adipose tissue on endothelial cells (Curat *et al.*, 2004; Kralisch *et al.*, 2007). Important to note, the impact of adipocytes differentiated from preadipocytes *in vitro* has not been analysed in this context and the studies mentioned (Curat *et al.*, 2004; Kralisch *et al.*, 2007) neglected the role of preadipocytes in the context of cross-talk with endothelial cells. Considering the importance of the specificity of microvascular endothelial cells in the trafficking of monocytes and T-lymphocytes in tissues, the use of HMEC-1 cells as microvascular endothelial cells is more suitable for studying this cross-talk than using macrovascular endothelial cells like HUVECS as applied by Kralisch *et al.*, 2007. Using SGBS and HMEC-1 cells allows high reproducibility of results and makes the scheduling of experiments easier which is very difficult using primary cells due to the following reasons:

1. primary cells are derived from different donors, each having a unique genetic background and individual life style,
2. the ratios between adipocyte cell size in WAT is heterogeneous in each donor,
3. WAT sampling is not uniformly conducted, the collected WAT region is generally not consistent and different techniques are applied and
4. the scheduling of experiments is difficult, especially if coculture experiments are performed since all involved cell types have to be ready for use immediately.

The analysis of the impact of fresh CM from cultured SGBS cells exposed to different treatments such as TNF-α and hypoxia on HMEC-1 cells in the study presented here avoids confounding variables possibly caused by

1. secondary effects if defrosted CM are used and activity changes may have occurred due to freeze-thaw cycles,
2. serum, antibiotics, high glucose levels or other medium components and
3. other cell types as in the tissue or as they may remain with isolated primary cells.

In contrast, in the studies by Curat *et al.*, 2004 and Kralisch *et al.*, 2007 defrosted CM were used in the coculture experiments. In the report by Curat *et al.*, 2004 the CM were derived from mature adipocytes cultured in the presence of 25 units per ml α-thrombin. Thrombin exerts essential effects in the coagulation cascade and can bind to protease-activated receptors on various cells where the corresponding intracellular signalling leads to increased production...
of pro-inflammatory cytokines and chemokines (Levi, 2010; Tsopanoglou and Maragoudakis, 2009). In the study by Kralisch et al., 2007 the CM were derived from mature adipocytes cultured in the presence of 10 % FCS and 15 mM glucose.

Importantly, SGBS and HMEC-1 cells are particularly suitable for transfection (BelAiba et al., 2007; Laumen et al., 2008) which for example would be helpful in analysing the importance of signalling pathways. The results obtained by this coculture system with CM give valuable directions for future experiments with primary cells and experiments in vivo.

4.3 Adipokine expression of SGBS preadipocytes and adipocytes under normoxia and hypoxia

The reports on the differential contribution of cell types in WAT to the pool of adipokines are heterogeneous (Antuna-Puente et al., 2008; Fain et al., 2008; Fain et al., 2004; Weisberg et al., 2003). In this study, initially the gene expression and secretion of several adipokines in SGBS preadipocytes and adipocytes under normoxic, serum-, hormone- and antibiotic-free conditions with 5 mM glucose in the medium were analysed by qRT-PCRs, ELISAs and bead-based Luminex® assays.

Adiponectin as a late marker for adipocyte differentiation (Scherer et al., 1995) was not expressed by preadipocytes, supporting their preadipocyte status. In contrast, the adipokines IL-4, IL-6, IL-8, MCP-1, VEGF, PAI-1, RANTES and SDF-1α were significantly stronger expressed by preadipocytes compared with adipocytes. These findings are overall in line with other studies demonstrating a pro-inflammatory expression pattern for preadipocytes mostly derived from stromal-vascular fractions of adipose tissues (Bruun et al., 2004; Chung et al., 2006; Fain and Madan, 2005; Fain et al., 2004; Fain et al., 2009) but also for cell lines like 3T3-L1 cells (Harkins et al., 2004; Poulain-Godefroy and Froguel, 2007) and human primary cells (Crandall et al., 2000; Kintscher et al., 2008; Wang et al., 2005). The results for VEGF differ slightly from observations in the literature where its secretion by human WAT has been accredited mainly to the non-fat cells (Fain et al., 2004) but no significant changes between VEGF secretion of preadipocytes and adipocytes were observed in human (Fain et al., 2004) and rat (Mick et al., 2002) primary cell cultures which could be due to different applied cell culture protocols. The strong pro-inflammatory character of preadipocytes versus adipocytes may reflect the close relationship between preadipocytes and macrophages (Charriere et al., 2003; Cousin et al., 1999). The conversion of preadipocytes into macrophages has been
demonstrated to be efficient and rapid and transcriptome profiling also showed a closer relationship between preadipocytes and macrophages than between preadipocytes and adipocytes (Charriere et al., 2003). Interestingly, proliferating preadipocytes also develop phagocytotic activity towards microorganisms (Cousin et al., 1999; Villena et al., 2001).

With regard to the coculture experiments, the typical pro-inflammatory endothelial cell activators TNF-α, IL-1 and IFN-γ were analysed (Hubbard and Rothlein, 2000; Madge and Pober, 2001; Petzelbauer et al., 1993). TNF-α mRNA expression was detectable in preadipocytes but not in adipocytes whereas, at protein level it was not found in either. The literature on TNF-α expression by adipocytes is controversial (Fain et al., 2004; Poulain-Godefroy and Froguel, 2007; Schlesinger et al., 2006). Interestingly, isolation of primary adipocytes from canine and mouse fat pads was associated with an induction of gene expression (Eisele et al., 2005; Ruan et al., 2003) and protein secretion (Ruan et al., 2003) of inflammatory molecules such as TNF-α due to the collagenase treatment of the cells. This might explain the finding of TNF-α in some primary cell culture systems.

IFN-γ was not secreted by SGBS cells and was also only barely detectable in untreated human primary differentiated adipocytes (Schlesinger et al., 2006). IL-1α and IL-1β gene expression but not protein secretion was measured in SGBS cells. In human WAT IL-1α protein was not found whereas protein of IL-1β has been found in amounts around 6 pg/mg (Juge-Aubry et al., 2003) with the secretion mainly attributed to the non-fat cells (Fain et al., 2004). In our laboratory IL-1 was hardly detectable in mature adipocytes and differentiated preadipocytes (Thomas Skurk, Department of Nutritional Medicine, Technische Universität München, Freising, Germany; unpublished data).

In order to allow a comparison between preadipocytes and adipocytes regarding gene expression, cytokine secretion and the effect of their CM on endothelial cell activation, the confluent preadipocytes (Pd0) were cultured for another 16 days (Pd16) in parallel to differentiating adipocytes in the same media, except for the induction to differentiate. The preadipocytes kept the image of fibroblastic-like cells over the culture period but PAI-1 and IL-6 protein release was higher in the Pd0 in comparison with the Pd16 whereas the MCP-1 release remained unchanged. Thus, it can be outruled that the 16 days of preadipocyte culture period was the reason for their observed strong pro-inflammatory character since the opposite was shown. Secondly, the cell culture protocol itself with the present hormones might decrease the expression of some inflammatory adipokines possibly also in adipocytes.
However, these hormones are necessary to maintain and promote lipid accumulation in adipocytes after they have been induced to differentiate.

Since increased adipose tissue mass is associated with reduced tissue perfusion (see chapter 1.2.6) in humans (Adams et al., 2005) and in mice (Hosogai et al., 2007; Rausch et al., 2007; Ye et al., 2007) and tissue function is critically dependent on sufficient tissue perfusion it is of interest how expression changes under hypoxia and what the consequences might be in the cross-talk with endothelial cells. The oxygen partial pressure in air is 159 mm Hg which is equivalent to 21 % oxygen. Studies have shown, that normal tissues are perfused with 20 to 70 mm Hg oxygen, equivalent to 2.5 % to 9.2 % oxygen whereas for example solid tumours, joints with rheumatoid arthritis and ischemic peripheral limbs of patients with diabetes are perfused with less then 10 mm Hg oxygen, equivalent to less then 1.3 % oxygen (Lewis et al., 1999). Cell culture experiments are normally performed with 21 % oxygen in the gas mixture which does not reflect the real biological situation and therefore should be ideally conducted under “cell type specific” oxygen conditions. WAT of lean mice is perfused by 4.6-6.5% oxygen and has been shown to be decreased to only 2 % to 3.3 % in WAT of obese mice (Hosogai et al., 2007; Rausch et al., 2007; Ye et al., 2007). Severe hypoxia might occur particularly in the tip regions as has been shown for mouse epididimal WAT (Cho et al., 2007). In that study several tissues, including, WAT, WAT tip, heart, kidney, brain, liver, lung and hypoxic and non-hypoxic areas of lung carcinoma were stained with Hydroxyprobe-1. WAT showed 30% less staining than the hypoxic area of the tumour. In contrast the staining of the tip WAT region was increased 3- fold when compared with the hypoxic area of the tumour. These data allow speculation that in some areas of WAT, the oxygen supply might be even lower than 2 %. Therefore, experiments with SGBS cells under moderate and severe hypoxia, with 4 % and 1 % oxygen in the gas mixture, respectively, were conducted and compared with the standard normoxic situation (21 % oxygen).

HIF-1 (see chapter 1.2.6) is the key transcription factor involved in the transmission of the hypoxic response (Brahimi-Horn and Pouyssegur, 2009). It is composed of one of the HIF-α-subunits and HIF-1β. Hypoxia stabilizes HIF-1α protein, which is otherwise degraded by an ubiquitin-dependent proteasome. In this study, HIF-1α was upregulated upon hypoxia exposure in a dose-dependent manner to comparable levels in preadipocytes and adipocytes, serving as quality control that the hypoxia experiments were carried out appropriately.
Upon 4% hypoxia mainly modest changes in secretion were examined for both preadipocytes and adipocytes. In contrast, 1% hypoxia strongly enhanced the release of VEGF, leptin, PAI-1, IL-6 and IL-4 in preadipocytes. In comparison, the secretion from hypoxic adipocytes was less significant achieving approximately one-half of the enhancements. Interestingly, it has been found for 3T3-L1 and human primary cells that preadipocytes have a stronger pro-inflammatory response to LPS treatment than adipocytes (Chung et al., 2006; Poulain-Godefroy and Froguel, 2007). In the literature VEGF gene expression in mouse 3T3-F442A adipocytes was not effected by exposure to 5% hypoxia (Lolmede et al., 2003) whereas at 1% hypoxia treatment VEGF gene expression and protein secretion was increased in mouse and human systems in preadipocytes (Wang et al., 2008), stromal vascular cells (Rehman et al., 2004) and differentiated adipocytes (Wang et al., 2007; Ye et al., 2007). These data point towards an important role of VEGF under hypoxia in expanding WAT to promote neovascularization as described for other tissues and tumours (Bishop-Bailey, 2009). Leptin gene expression did not change in 3T3-F442A adipocytes exposed to 5% hypoxia, (Lolmede et al., 2003) whereas protein release by human PAZ-6 adipocytes increased 3-fold after treatment with 6% hypoxia exposure (Grosfeld et al., 2002) and in primary human differentiated adipocyte elevation of gene expression was 27-fold and of protein expression 4-fold with 1% hypoxia exposure (Wang et al., 2007). A substantial increase in leptin gene and protein expression by preadipocytes upon hypoxia stimulation was also found by another study using human primary preadipocytes (Wang et al., 2008). Interestingly, one study also reported an increased leptin secretion by preadipocytes upon treatment with TNF-α and IL-1β (Simons et al., 2005). Therefore, it appears that preadipocytes can also contribute considerably to the pool of leptin in WAT, given circumstances like hypoxia and/or TNF-α and IL-1 secreting macrophages in the neighbourhood. This additional leptin secretion by preadipocytes may be necessary in order to stimulate the development of new blood vessels during WAT expansion in concert with VEGF to allow appropriate oxygenation of the tissue. In this study, IL-4 secretion increased upon hypoxia treatment in preadipocytes and adipocytes. This has been also observed for human peripheral mononuclear cells (Naldini and Carraro, 1999; Naldini et al., 1997). IL-4 acts proangiogenic in the lung under hypoxic conditions (Yamaji-Kegan et al., 2009), has the ability to suppress macrophage cytotoxic activity and exerts inhibitory effects on the expression and/or release of proinflammatory cytokines, including TNF-α, IL-1, IL-6 and IL-8 (Opal and DePalo, 2000). Beneficial effects were found with the administration of human recombinant IL-4 in an experimental model on
hypoxia-induced lung injury (Ozturk et al., 2006) and also hypoxia-induced gastric and intestinal injury (Ozturk et al., 2005). Therefore, IL-4 in the context of WAT hypoxia may protect the cells from hypoxia-inducing adverse effects and contribute to WAT angiogenesis.

In this study, PAI-1 and IL-6 gene expression and cytokine secretion increased in adipocytes after hypoxia treatment. This is in line with other studies, where PAI-1 gene and protein expression was elevated 7-fold and 6-fold, respectively, in mouse 3T3-L1 cells (Chen et al., 2006) and gene expression was elevated in primary human differentiated adipocytes upon 1 % hypoxia treatment (Wang et al., 2007). IL-6 gene expression and protein secretion increased after 1 % hypoxia treatment in mouse (Regazzetti et al., 2009; Ye et al., 2007) and human (Wang et al., 2007) cell culture. PAI-1 and IL-6 elevation upon hypoxia may not primarily be an inflammatory response, but rather of adaptive nature since both molecules are known to be also involved in the development of the vascular system by promoting angiogenesis (Fan et al., 2008; Zagorska and Dulak, 2004). Interestingly, whereas measurements in the study showed a significant hypoxia-sensitive upregulation of IL-6 and PAI-1 in preadipocytes Wang et al., 2008 did not. In most cell types PAI-1 gene regulation is under the control of HIF-1α and elevated upon hypoxic stimulus (Zagorska and Dulak, 2004). IL-6 is also increased under hypoxia in several cell types but its dependency on HIF-1α is not clear (Semenza, 2003). The differences in this expression study compared with the study by Wang et al., 2008, could be due to the fact that different sources of human preadipocytes and adipocytes were used and that these may react differently under hypoxia. However, it may be more likely that the obvious clear differences in the cell culture protocols applied are the explanation. In chapter 3.2.3 where adipokine secretion of SGBS cells cultured in the presence and in the absence of hormones and antibiotics had been compared, it was demonstrated that the cell culture protocol itself affected the cells and changed IL-6 secretion in preadipocytes and MCP-1 secretion in adipocytes. In the report by Wang et al., 2008 preadipocytes were cultured in DMEM/F12, containing 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin. In contrast, in this study serum-free medium without antibiotics was used. In addition, MCDB 131 medium served as basal medium, which contains 5 mM glucose compared with DMEM/F12 which contains 25 mM glucose used in the study by Wang et al., 2008. Moreover, observations in this study indicated that the HIF-1α protein level under normoxia was already elevated in preadipocytes if 10% FCS was present in the medium. After FCS-depletion, the HIF-1α protein content decreased
It is tempting to speculate, that preadipocytes with low basal HIF-1α level might be more responsive to hypoxia, leading to differential cytokine secretion.

In this context, it is interesting that in the hypoxia experiments by Wang et al., 2007, normoxic adipocytes showed a lower level of HIF-1α compared with the preadipocytes cultured in 10% FCS. This might be an explanation why Wang et al., 2007 measured an increase of IL-6 and PAI-1 in adipocytes under hypoxia, but not in preadipocytes. Therefore, using the same cell culture medium would be the first step to resolve this issue in the future.

It is important to note that hypoxia was not always accompanied by a strong upregulation of adipokines, but for example the chemokines MCP-1 and IL-8 decreased in hypoxic adipocytes and only moderately increased in preadipocytes. RANTES secretion was enhanced in preadipocytes after hypoxia exposure, whereas in adipocytes a small increase was only observed with 4% hypoxia but not with 1% hypoxia treatment. In the literature MCP-1 gene expression was increased 2.8-fold in mouse 3T3-L1 adipocytes (Okada et al., 2008) whereas in human primary differentiated adipocytes no alteration after exposure to 1% hypoxia (Wang et al., 2007) was observed.

The secretion of chemotactractants such as MCP-1 by other hypoxic cell types is also controversial (Bosco et al., 2004; Galindo et al., 2001; Hohensinner et al., 2006). IL-8 expression is generally elevated upon hypoxic stimuli in macrophages and other cell types (Hirani et al., 2001; Karakurum et al., 1994; Rydberg et al., 2003; Scannell, 1996) whereas RANTES expression was either reduced or not regulated in human macrophages exposed to hypoxia (Bosco et al., 2004; Hirani et al., 2001; Turner et al., 1999).

In the following, a possible explanation why adipocytes do not change or even decrease their chemoattractant secretion upon hypoxia exposure in vitro is given considering reports that pre-exposure of human epithelial cells and human endothelial cells to hypoxia increased transmigration of human blood polymorphonuclear leukocytes (Colgan et al., 1996) and human monocytes (Kalra et al., 1996), respectively, allowing for recruitment of immune cells. On the other hand, macrophage chemotactic migration during hypoxia within tissues has been reported to be inhibited (Bosco et al., 2008; Grimshaw and Balkwill, 2001; Turner et al., 1999). These observations together may explain how macrophages accumulate in areas of necrosis or inflammation. For human and mouse WAT it has been demonstrated that over 90% of the WAT macrophages are localized to necrotic adipocytes (Cinti et al., 2005). Macrophages may migrate along a chemotactic gradient until an area of hypoxia is reached where they are rapidly inhibited from progressing further in order to phagocyte cell debris and
also have a role in extracellular matrix remodelling and angiogenesis (Grimshaw and Balkwill, 2001). It has been shown in vitro, that chemotaxis via chemoattractants such as MCP-1, RANTES and macrophage inflammatory protein 1 alpha (MIP-1α) (Turner et al., 1999) is abrogated under hypoxic conditions and therefore may explain why adipocytes even decrease the secretion of MCP-1 and IL-8 under severe hypoxia. Since preadipocytes and adipocytes react completely different in chemoattractant secretion upon hypoxia exposure and intensity this may indicate a highly sensitive and regulated cell type-specific response and cell-cell cross-talk within the tissue in order to orchestrate differential immune cell infiltration and distribution.

Since adiponectin is reduced in plasma of obese individuals and is an antidiabetic protein (Spranger et al., 2003), it was of interest to analyse the influence of hypoxia on adiponectin secretion by adipocytes. Interestingly, the robust adipocyte-specific secretion of adiponectin did not significantly change under hypoxia. This is in contrast to five studies, all performed in mouse 3T3-L1 adipocytes (Chen et al., 2006; Hosogai et al., 2007; Magalang et al., 2009; Nakagawa et al., 2008; Ye et al., 2007) where a decrease of adiponectin in response to hypoxia was observed. In this respect, it appears that adipocytes differentiated from mouse 3T3-L1 preadipocytes, which have been derived from embryonic mesenchymal cells, are more responsive than human SGBS adipocytes as used in this study and primary human adipocytes in the report by Wang et al., 2007 which were both differentiated from preadipocytes of postnatal adipose tissues. In this study, a small but significant decrease of adiponectin mRNA under hypoxia was measured but no changes for adiponectin secretion were observed. The results by Wang et al., 2007 indicate similar results: only a slight decrease of 15 % of adiponectin secretion under 1% hypoxia compared with normoxia was reported. Altogether, the effect of hypoxia on the level of human adiponectin protein in cell culture seems to be rather small in comparison to the data generated in mouse 3T3-L1 cells which show a substantial decrease. It remains to be elucidated whether hypoxia results in a reduction of adiponectin secretion by human adipocytes in vivo and if so, whether the reduction would be sufficient to decrease adiponectin plasma levels and thus impact on insulin sensitivity.

Regarding the endothelial cell activators IL-1, TNF-α and IFN-γ no induction of secretion upon hypoxia exposure was measured in this study. At gene expression level, TNF-α increased in preadipocytes under only 4 % hypoxia exposure whereas no TNF-α was
detectable in adipocytes. This is in concordance with the literature in human differentiated adipocytes (Wang et al., 2007) and in contrast to a 1.6-fold increase in mouse primary adipocytes (Ye et al., 2007). In other cell types TNF-α regulation upon hypoxic stimulus is discussed controversially (Gerlach et al., 1993; Hirani et al., 2001; Leeper-Woodford and Detmer, 1999; Scannell, 1996). IL-1α gene expression was elevated around 2-fold in adipocytes upon 1 % hypoxia but not upon 4 % hypoxia exposure and was unchanged in preadipocytes, whereas IL-1β gene expression was not changed at all. In mouse primary adipocytes, IL-1 gene expression increased 2-fold after they had been exposed to 1 % hypoxia (Ye et al., 2007) whereas in 3T3-L1 cells IL-1β secretion was unchanged (Regazzetti et al., 2009). In macrophages IL-1 expression upon hypoxic stimuli is discussed controversially (Gerlach et al., 1993; Koga et al., 1992; Scannell, 1996) as is also IFN-γ expression (Acosta-Iborra et al., 2009; Bosco et al., 2004).

In summary, the strong pro-inflammatory endothelial cell activators IL-1, TNF-α and IFN-γ are not or in negligible amounts produced by preadipocytes and adipocytes under normoxia and hypoxia and their secretion may under these biological situations be limited to immune cells in WAT. This may be important since macrophages can promote neovascularization in a hypoxic environment by producing VEGF, FGF, PDGF and also TNF-α which is both proinflammatory and either pro-or anti-angiogenic and critically depending on the dose applied (Lewis et al., 1999).

### 4.4 Endothelial cell activation by CM of normoxic, hypoxic and TNF-α treated SGBS cells and different-sized primary mature adipocytes

In this study the overall strongly pro-inflammatory observed character of CM derived from normoxic preadipocytes was confirmed by the increase of the monocyte-endothelial cell-cell adhesion, whereas adipocyte-CM showed baseline levels. Furthermore, the endothelial cell expression analysis clearly showed that preadipocyte-CM and adipocyte-CM significantly induced the mRNA for the chemokines IL-8 and MCP-1, whereas the upregulation of ICAM-1 cell surface expression occurred only with preadipocyte-CM. This suggests that adipocyte-CM can increase the expression of these chemokines, but other functionally important molecules during endothelial cell activation, such as ICAM-1 (see chapter 1.2.7.1) are not upregulated and may explain the weak monocyte-endothelial cell-cell adhesion observed for adipocyte-CM. However, the expression pattern of adipocytes changed upon hypoxic...
conditions leading to a significant increase of secreted pro-inflammatory adipokines and monocyte adhesion accompanied by upregulated ICAM-1 expression. Monocyte adhesion also further increased upon treatment with CM of hypoxic preadipocytes but was not followed by ICAM-1 expression, suggesting another molecular mechanism involved, e.g. triggering the presentation of chemoattractants at the endothelial cell surface. For isoprotanes it has been shown that they upregulate monocyte-endothelial cell-cell adhesion independent of the classical adhesion proteins (Kumar et al., 2005). The increase of monocyte-endothelial cell-cell adhesion after treatment with CM of hypoxic SGBS cells matches with findings in the literature where pre-exposure of human epithelial cells and human endothelial cells to hypoxia increased transmigration of human blood polymorphonuclear leukocytes (Colgan et al., 1996) and human monocytes (Kalra et al., 1996), respectively. Thus, the recruitment of immune cells during hypoxia could be increased and together with observations in macrophages where chemotactic migration during hypoxia within tissues was inhibited (Bosco et al., 2008; Grimshaw and Balkwill, 2001; Turner et al., 1999) explain how macrophages accumulate in areas of necrosis or inflammation (see also chapter 4.3).

In addition, the capacity of preadipocyte-CM and adipocyte-CM for endothelial cell activation was characterized additionally by their strong effect on endothelial cell proliferation. Treatment of HMEC-1 cells with CM of SGBS cells exposed to 4 % hypoxia achieved endothelial proliferation values comparable with the positive control and they were not further enhanced or decreased with CM of SGBS cells exposed to 1 % hypoxia (data not shown). This suggests that moderate hypoxia is sufficient for preadipocytes and adipocytes to release a potent mixture of activators for endothelial cell proliferation, possibly VEGF and leptin as shown in chapter 3.4.3 and reported in the literature (Ferrara et al., 2003; Park et al., 2001), in order to achieve a maximum in endothelial cell proliferation as necessary step for neovascularization.

Moreover, it was found that TNF-α-stimulated preadipocytes and adipocytes upregulated the endogenous TNF-α mRNA expression and TNF-α-activity in CM which exerted a marked increase in monocyte adhesion. Since the specific blockade of TNF-α activity by the antagonist Enbrel® (Tracey et al., 2008) reduced the adhesion more than 65%, but not completely, it can be concluded that TNF-α-dependent and TNF-α-independent cell-cell interactions occurred and, besides the TNF-α activity, other adipokines that may act in
concert have been induced. Potential candidates may be among the molecules identified in the
gene expression analysis of TNF-α- treated SGBS cells by (Do et al., 2006).

Overall, the strong impact of preadipocyte-CM compared with the adipocyte-CM on HMEC-1
cells can be explained by the generally less pro-inflammatory secretion activity of adipocytes.
Further, it is proposed that adiponectin in adipocyte-CM decreases monocyte adhesion and
endothelial ICAM-1 as described for TNF-α-stimulated HUVEC (Ouchi et al., 1999) since
gene expressions of the adiponectin receptors AdipoR1 and AdipoR2 was also detected in
HMEC-1 cells (data not shown).

The interesting and still open questions remain:

1. can the observed strong potency of preadipocytes to secrete proinflammatory
cytokines be translated to WAT, and if so
2. how is this proinflammatory potency of preadipocytes in WAT, where preadipocytes
   and adipocytes exist in the neighbourhood of other cell types, controlled in
   physiological and pathological situations?

In order to dissect the differential influence of different-sized primary mature adipocytes
isolated from human WAT on endothelial cell activation, mature adipocytes were separated
by size and their CM were tested in the monocyte-endothelial cell-cell adhesion and ICAM-1
cell surface expression assays. In the literature there is some evidence that a positive
correlation exists between proinflammatory adipokines and increasing adipocyte cell size
(Skurk et al., 2007) whereas there is also reported the opposite (McLaughlin et al., 2009).
This study found that CM of the entirety of primary human mature adipocytes increased
monocyte-endothelial cell-cell adhesion and ICAM-1 expression as described in other reports
(Curat et al., 2004; Kralisch et al., 2007). Additionally, the CM of large adipocytes enhanced
monocyte-endothelial cell-cell adhesion and ICAM-1 expression. These results and those
obtained from the CM of the entirety of adipocytes remained from the statistical point of view
throughout unchanged when the data of the assays were normalized to adipocyte cell volume,
adipocyte cell count and adipocyte cell surface area. In contrast, whereas the CM of small
adipocytes normalized to adipocyte cell volume increased monocyte-endothelial cell-cell
adhesion, no significant elevation was found when the data of the assays were normalized to
adipocyte cell count and adipocyte cell surface area. Additionally, ICAM-1 expression on
HMEC-1 cells after treatment with CM of small adipocytes was enhanced when the data were
normalized to adipocyte cell volume and adipocyte cell surface area but not if normalized to adipocyte cell count. The results show, that the CM of mature adipocytes independently of their cell size can activate endothelial cells. Nevertheless, depending on the data normalization the CM derived from small adipocytes may have the least potential to activate endothelial cells which would support the results by Skurk et al., 2007 who showed that the fraction containing the small adipocytes secreted less proinflammatory adipokines than large adipocytes. These conclusions are limited since the normalized data may not be real. This technical problem could be solved in future experiments by generating the CM in three different ways: firstly, incubate equal volumes of adipocytes per defined volumes of medium; secondly, incubate equal cell numbers of adipocytes per defined volumes of medium; and thirdly, incubate adipocytes with equal cell surface area per defined volumes of medium. Overall, it appears as if the CM of the entirety of adipocytes exerted the strongest effects on endothelial cell activation independently of the normalization, although not statistically significant. Possibly, the secretion products of the entirety of different-sized adipocytes together generate the most potent CM for endothelial cell activation by contributing with differential molecule secretion. Since the medium-sized adipocytes were not analysed separately due to technical reasons but were present in the entirety of adipocytes, these cells may also have contributed to these observations.

These results raise the question why CM of untreated mature adipocytes from WAT, depending on data normalization, increase monocyte-endothelial cell-cell adhesion and ICAM-1 cell surface expression whereas untreated differentiated adipocytes derived from SGBS preadipocytes do not. Most likely these are due to technical reasons. Firstly, the CM of mature adipocytes can be easily “concentrated” by using small volumes of cell culture media and rather large volumes of mature adipocytes in contrast to differentiated adipocytes where the concentration procedure is time and biological material consuming (see chapter 2.1.2). Secondly, the mature adipocytes may secrete more pro-inflammatory and other stress molecules than the differentiated adipocytes due to the collagenase treatment as reported elsewhere (Eisele et al., 2005; Ruan et al., 2003). Thirdly, it should be taken in account that the primary adipocytes in WAT are in continuous cellular cross-talk including immune cells, and in expanding WAT, these are exposed to hypoxia. Therefore, primary adipocytes may be preactivated independently of the collagenase procedure.
In this study, a direct comparison between the results generated from coculture experiments with CM of SGBS adipocytes and those obtained from coculture experiments using CM of primary mature adipocytes can not be made. To investigate the differences between coculture experiments using CM of SGBS cells and primary mature adipocytes in more detail, future experiments have to be performed at the same day and cell culture plate in order to get a direct data comparison.

A further question is whether the results obtained from coculture experiments using CM of SGBS preadipocytes and adipocytes would change if their data were normalized to either the protein content or the cell count of preadipocytes and adipocytes, respectively, or the protein content of the CM. Therefore, at least four independent experiments were performed where the CM of SGBS cells were used to test for endothelial cell activation and the corresponding SGBS cells were analysed for their protein content by the Modified Bradford assay and their cell count by staining the cells with Hoechst 33342, trihydrochloride, trihydrate (data not shown). No significant changes were observed after normalizing the results of the monocyte-endothelial cell-cell adhesion assay or ICAM-1 expression ELISA to the protein content or cell count of SGBS preadipocytes and adipocytes, respectively (data not shown). The protein content of preadipocyte-CM and adipocyte-CM was also similar as analysed by the company Digilab BioVisioN (Hannover, Germany; data not shown). Therefore, it can be ruled out that a different cell count or protein content of preadipocytes or adipocytes, respectively, created unequal conditions for the generation of CM or a different protein content of the CM caused the significant differences observed between CM of preadipocytes and adipocytes.
4.5 Molecular mechanisms of endothelial cells activated by CM of SGBS cells

To understand the underlying molecular mechanisms of endothelial cell activation, different signalling pathways in endothelial cells involved in the regulation of ICAM-1, IL-8 and MCP-1 were investigated. The results indicate that preadipocyte-CM and adipocyte-CM activate p38MAPK, SAPK/JNK and ERK1/2 in endothelial cells. Overall, less activity for adipocyte-CM was observed. Additionally, the transcription factor c-Jun was more strongly activated in HMEC-1 cells treated with preadipocyte-CM compared to adipocyte-CM. Moreover, a strong activation of STAT-1 and STAT-3 was detected for preadipocyte-CM which exerted stronger phosphorylations compared to adipocyte-CM.

The performed inhibitor experiments suggest, that JNK, which phosphorylates members of the AP-1 transcription family including c-Jun, JunD and ATF-2 (Hess et al., 2004) is indispensable for monocyte adhesion and ICAM-1 cell surface protein expression induced by preadipocyte-CM. Furthermore, the blockade of JAK-signalling in HMEC-1 with the specific JAK inhibitor I and subsequent inhibition of the STAT1/3 phosphorylation revealed that this pathway and factors are functionally important for the induction of ICAM-1 cell surface protein expression and monocyte-endothelial cell-cell adhesion of HMEC-1 cells treated with preadipocyte-CM.

Surprisingly, the NF-κB pathway was not involved in the induced endothelial cell activation upon treatment with CM of normoxic and hypoxic (data not shown) SGBS cells. In contrast, stimulation of HMEC-1 cells with TNF-α at low concentration activated the NF-κB pathway in the positive control suggesting that this pathway is inducible in HMEC-1 cells, but not involved in the induction of ICAM-1 by CM, except when CM of TNF-α stimulated SGBS cells was used. It is likely that the NFκB pathway was then involved in the endothelial cell activation, since the IκBα phosphorylation was increased in HMEC-1 cells after treatment with CM of TNF-α exposed SGBS cells and abrogated when these CM were preincubated with Enbrel® whereas, for total IκBα the opposite was shown. This concept is supported by the literature where TNF-α mediates endothelial cell activation via the NFκB pathway (Weber et al., 1995). Further experiments with inhibitors would be necessary for clarification.
STAT-1 which is classically activated in response to IFN-γ (Jaruga et al., 2004) was strongly phosphorylated in HMEC-1 cells stimulated by preadipocyte-CM. However, a substantial functional involvement of IFN-γ in CM as a pro-inflammatory key-activator of endothelium (Ozaki et al., 1999) can be ruled out because IFN-γ was not detectable. Instead of, IL-6, VEGF and leptin which are known to stimulate the JAK/STAT pathways and are present in considerable amounts in CM, are good candidates for transducing signals important for endothelial cell activation (Jin et al., 2003; Ni et al., 2004; Pan et al., 2007; Wincewicz et al., 2007; Yahata et al., 2003). With respect to the apparently uninduced NF-κB pathway in HMEC-1 cells stimulated by CM of normoxic and hypoxic (data not shown) SGBS cells, it is reasonable to speculate that the upregulation of the genes for ICAM-1, MCP-1 and IL-8 might have occurred through increased c-Jun-mediated AP-1 and STAT-binding activities at functional sites of their promoter regions (Roebuck and Finnegan, 1999). Since the separate blockade of the JNK and JAK pathways with specific inhibitors resulted in the downregulation of ICAM-1 cell surface expression and monocyte-endothelial cell-cell adhesion, it is possible that both pathways are important to coordinate ICAM-1 cell surface expression and functional monocyte-endothelial cell-cell adhesion in this coculture system. Future experiments with electrophoretic mobility shift assays (EMSA) (Fried and Crothers, 1981; Garner and Revzin, 1981) would be useful for confirmation of the transcription factor/DNA interactions.
4.6 Functional analysis of mediators in the CM of SGBS cells responsible for endothelial cell activation

In this study, functional studies with function-neutralizing antibodies were performed in order to get first insights into CM-derived factors which mediate the observed increase in ICAM-1 expression and monocyte-endothelial cell-cell adhesion. For leptin and VEGF, no experimental support could be found that they are important in this process, which is also controversially discussed in the literature (Curat et al., 2004; Kim et al., 2001; Skilton et al., 2005; Zhang and Issekutz, 2002). Interestingly, IL-6 was identified as an important mediator in CM inducing increased endothelial STAT-1/3 phosphorylations, ICAM-1 expression and monocyte adhesion to HMEC-1 cells treated with CM. IL-6 blockade in CM substantially suppressed STAT-1/3 phosphorylations, monocyte endothelial cell-cell adhesion and ICAM-1 expression. STAT-1/3-blockade in HMEC-1 cells also significantly decreased monocyte endothelial cell-cell adhesion and ICAM-1 expression. This study consequently shows that the IL-6 effect is primarily transduced by the endothelial STAT-1/3 signalling pathway as described in other systems (Jin et al., 2003; Ni et al., 2004; Pan et al., 2007; Wincewicz et al., 2007). However, the effect of IL-6 blockade had stronger effects on STAT1/3 phosphorylations compared with the smaller effects on monocyte adhesion and ICAM-1 cell surface expression. The results suggest that the preadipocyte-CM induced increase in monocyte adhesion and ICAM-1 expression in HMEC-1 cells is not only mediated by IL-6 but also by other factors present in the CM. Although the CM-mediated phosphorylations of STAT1/3 are largely IL-6 dependent as shown by IL-6 inhibitor suppression, there are other mediators than IL-6 and STAT1/3 which can increase ICAM-1 expression and monocyte endothelial cell adhesion. Since also the JNK-pathway was also identified to operate in the CM-induced monocyte adhesion and ICAM-1 cell surface expression in these inhibitor experiments (see chapter 3.3.8), the JNK-pathway could be a further functional pathway in this cross-talk.

Finally, the important finding of this study that the IL-6Rα/gp130 receptor signalling complex is expressed on HMEC-1 cells, suggests that IL-6 bound to IL-6 receptor complexes on microvascular endothelial cells allows IL-6 signalling and transsignalling in an autocrine and paracrine fashion (Rose-John et al., 2006). This finding integrates IL-6 derived from perivascular preadipocytes and adipocyte as a major mediator in the complex molecular and cellular cross-talk of endothelial cells in adipose tissue especially in the context of IL-6.
signalling and transsignalling of soluble IL-6 receptors from lymphocytes and monocytes as described in other biological situations (Marin et al., 2001; Rose-John et al., 2006). In this context, the differential activation of inducible transcription factors by adipokines may provide an important mechanism which can lead to the expression of particular cell-cell adhesion molecules and chemokines in a cell type-specific and stimulus-specific fashion. These regulatory mechanisms could critically influence the site-specific recruitment of distinct leukocyte subsets during the inflammatory response.

4.7 Peptidomics analysis of SGBS cell supernatants

One of the major aims of this study was to establish an experimental basis for the characterisation of the secretion profile of SGBS cells using the described coculture system and the peptidomic technology in order to find interesting and new candidate peptides possibly involved in the molecular and cellular cross-talk between preadipocytes, adipocytes and endothelial cells. The described data are limited since the analyses could be not completed by the company Digilab Biovision due to insolvency. Therefore, the data which were available are restricted to the analysis with “in silico annotation” algorithm, in order to match the peptides in the samples to already identified and sequenced peptides. This means that these putative candidate peptides could not be isolated and sequenced. Furthermore, new hits of the mass spectrometry analysis could not be matched with already identified peptides and thus, their identity remains unknown. Nevertheless, for the putative candidates perilipin, CCL14, GROA, II-8, secretogranin II and V and progranulin differential signal intensities between preadipocytes and adipocytes were observed. As expected, perilipin fragment signal intensity was found in adipocytes and not in preadipocytes but functions as a quality control, since the samples were “blinded” before sent to the company. II-8 signal intensity was higher in preadipocytes compared with adipocytes. This was confirmed by the data already generated using the qRT-PCR and ELISA techniques, giving confidence in possibly existing differences for the other “in silico annotated candidates”. Focussing on secretogranin II, V and progranulin, their gene expression was confirmed by qRT-PCR and for progranulin, protein secretion in SGBS preadipocytes and adipocytes were detected using ELISA. At that time, to the best of my knowledge, these molecules had not been described as adipokines. Strikingly, a strong progranulin secretion by SGBS preadipocytes and adipocytes was detected in the ng-range in CM which is higher compared with many other cytokines. In the following time of this study, Youn et al., 2009 reported that progranulin mRNA was found in comparable
amounts in human mature adipocytes and stromal vascular cells, with higher amounts of protein being found in visceral versus subcutaneous WAT using the qRT-PCR and Western blot techniques, respectively. Progranulin serum concentrations were found to correlate with the BMI and macrophage infiltration in omental WAT. The degree of chemotaxis mediated by progranulin was comparable to that of MCP-1 (Youn et al., 2009). Overall, progranulin plays important and complex roles in tumorigenesis (Ong et al., 2006), development (Eriksen and Mackenzie, 2008), neurogenesis (Bateman and Bennett, 2009) and several inflammatory events, especially wound repair (He and Bateman, 2003; He et al., 2003), both suppressing inflammation and also being the source of cleavage products named granulins which exert opposing effects allowing for differential regulation (He et al., 2003; Kojima et al., 2009). Importantly, progranulin promotes neovascularization in vivo with similarities to VEGF and induces proliferation, migration and formation of capillary-like tubule structures of human dermal microvascular endothelial cells (He et al., 2003). Therefore, progranulin, like VEGF and leptin (Hausman and Richardson, 2004) may also play an important role in WAT vascularization. Having the capacity to activate endothelial cells with regard to neovascularization it is conceivable that progranulin is also involved in the regulation of endothelial cell adhesion molecules and endothelial cell interactions with immune cells. Experiments with human recombinant progranulin and progranulin inhibition in CM would be useful in gaining new insights into its role in this coculture system and in the context of the molecular and cellular cross-talk between preadipocytes, adipocytes and endothelial cells in WAT.

Another candidate peptide found by the peptidomic technology was secretogranin II and its gene expression was detected in SGBS cells with higher levels having been observed in preadipocytes than adipocytes. This molecule is distributed throughout the endocrine and nervous system. It generates the molecule secretoneurin via proteolytical cleavage (Kahler et al., 1999; Kahler et al., 2002) which can be induced under pathological conditions and hypoxia and has been proposed to play a role in the induction of neo-vascularization in ischemic diseases (Fischer-Colbrie et al., 2005). Secretoneurin is a chemoattractant for monocytes (Kahler et al., 1999; Kahler et al., 2002) and acts as direct angiogenic cytokine in vitro and in vivo (Kirchmair et al., 2004), its potency being comparable to VEGF (Fischer-Colbrie et al., 2005). Secretoneurin impairs endothelial barrier function via the JNK and ERK1/2 pathways (Yan et al., 2006) and could possibly be involved in endothelial cell interactions with immune cells. It is also highly angiogenic and an activator of the JNK
pathway (Fischer-Colbrie et al., 2005) which is besides the JAK/STAT pathway in this coculture system responsible for increased ICAM-1 expression of and monocyte adhesion to HMEC-1 cells. Thus, secretogranin II may be a very interesting candidate to investigate in the cross-talk between the cell types in WAT, provided that protein expression is also detected in SGBS cells and WAT.

Finally, gene expression of the peptide candidate secretogranin V in SGBS cells was similar to that of secretogranin II. Secretogranin V is expressed by neurons and endocrine cells and is a chaperon for proprotein convertase-2 (PC2). PC2 helps to convert large inactive precursor molecules to generate smaller bioactive molecules. It operates within secretory pathways and is essential in the maturation of several types of membrane-bound and secreted molecules (Mbikay et al., 2001). Secretogranin V-null mice had no demonstrable PC2 activity. They were deficient in processing islet hormones and displayed hypoglycemia, hyperproinsulinemia and hypoglucagonemia. The mice had increased circulating levels of adrenocorticotropic hormone (ACTH) and corticosterone with adrenocortical expansion. Secretogranin V-null mice died before 9 weeks of severe Cushing syndrome. Thus, Secretogranin V is required for PC2 activation but also functions in the regulation of pituitary hormone secretion (Westphal et al., 1999). If protein expression of secretogranin V in SGBS cells was confirmed using the ELISA or Western blot techniques this molecule may play an important role in the process and regulation of cytokine secretion.

Overall, the peptidomics analysis was and will be very useful for identifying new and known candidates in biological samples and to dissect the contribution of pro-inflammatory and anti-inflammatory molecules by distinct cell types in WAT. Despite the above mentioned limitations, the analysis with “in silico annotation” algorithm delivered valuable results which when confirmed via gene and protein expression analysis are interesting for future experiments in WAT biology.
4.8 Concluding comments and perspectives

Taken together, this *in vitro* study shows that preadipocytes have the potential to contribute considerably to the total pool of adipokines, and with respect to the close vicinity of preadipocytes to endothelial cells, they may operate as potent activators of endothelial cells in WAT and probably also in an endocrine fashion. These effects can be promoted in preadipocytes and induced in adipocytes by TNF-α and hypoxia and mediated by IL-6 in a manner similar to what may occur in the neighbourhood of macrophages in expanding WAT. STAT1/3 and SAPK/JNK were found to be important players in the activation of endothelial cells.

The finding that IL-6 in CM from preadipocytes or adipocytes can operate as activator of microvascular endothelial cells point to a new path to explaining how IL-6 signalling and/or transsignalling via IL-6/soluble IL-6 receptor complexes may act on the microvasculature in adipose tissue. In the case of pathophysiological levels, this may lead to chronic low-grade inflammation and endothelial cell dysfunction. Therefore, it will be of great importance to study the expression profiles of preadipocytes and adipocytes at the cellular level in WAT *in situ*, and to identify the factors and mechanisms which activate or counteract their pro-inflammatory potency.

In future *in vitro* coculture studies using CM of preadipocytes and adipocytes, the specific blockade of adipokines in CM or the corresponding adipokine receptors on endothelial cells may be useful to further dissect the differential functions of adipokines during leukocyte-endothelial cell-cell adhesion and transmigration. For example, knockdown experiments for AdipoR1 and AdipoR2 on endothelial cells will be important to analyse the role of adiponectin in adipocyte-CM. Adiponectin and its signalling in endothelial cells may be possibly responsible for the observed weak endothelial cell activation by neutralizing the actions of other pro-inflammatory adipokines. For example, it has been reported that TNF-α induced monocyte-endothelial cell-cell adhesion can be attenuated by adiponectin (Ouchi *et al.*, 1999).

Transcriptome and peptidome/secretome analyses will promote the expression and secretion profiling of preadipocytes and adipocytes, with this study contributing by the demonstration
of progranulin, secretogranin II and secretogranin V. Secretogranin II may represent a new adipokine with angiogenic properties and regulated by hypoxia.

In order to gain insights into the full integrated molecular and cellular cross-talk between the different cell types in adipose tissue, in vivo imaging studies if applied in adipose tissue will be very informative. Use of the stimulated emission depletion (STED) microscopy (Lauterbach et al., 2010) in these experiments may allow the observation of real-time events at molecular level. As yet, in vivo imaging showed that leukocyte-endothelial cell interactions were increased in the microcirculation of adipose tissue of obese mice and were normalized by administrating an ICAM-1 antibody (Nishimura et al., 2008). It would be interesting to investigate in this experimental setup whether the leukocyte-endothelial cell interactions are reduced in obese endothelial cell-specific gp130 knock out mice (Yao et al., 2005) compared with control wild-type mice. It would be also important to know whether macrophage clustering in adipose tissue was the same in obese IL-6-deficient mice (Di Gregorio et al., 2004; Savale et al., 2009) compared with control wild-type mice. In this context the analysis of endothelial cell-specific knockout mice for relevant signalling pathway members [e.g. STAT1, STAT3 (Kano et al., 2003), SAPK/JNK] would allow gaining insights into underlying signalling pathways in vivo. Preceding in vitro studies aimed at supporting and complementing the already examined underlying signalling mechanisms in endothelial cells stimulated with CM of preadipocytes and adipocytes exposed for instance to hypoxia would be necessary.

Altogether, this study should contribute to a better understanding of the molecular and cellular cross-talk and underlying mechanisms in WAT regulating the balance between pro-inflammatory and anti-inflammatory factors and insulin sensitivity in the etiology of obesity, the metabolic syndrome and diabetes.
5 Summary

Obesity is associated with a state of chronic low-grade inflammation. Immune cells accumulate in white adipose tissue (WAT) and the vascular endothelium plays an interactive role in these infiltration and inflammatory processes. In humans the underlying molecular and cellular mechanisms are incompletely understood. Mature and hypertrophic adipocytes are considered as the major adipogenic cell type secreting pro-inflammatory cytokines in WAT. In contrast, the investigation of the pro-inflammatory capacity of preadipocytes and their role in endothelial cell activation have been neglected to date.

In this study, the pro-inflammatory expression and secretion of normoxia, hypoxia and TNF-α-treated human preadipocytes and adipocytes (SGBS cells) and their impact and that of different-sized primary mature adipocytes on human microvascular endothelial cell (HMEC-1) activation was examined in order to gain new insights into this molecular and cellular cross-talk. Furthermore, in order to identify new secretion products of SGBS cells possibly involved in this cross-talk, the peptidomic technology was applied.

It was found that HMEC-1 stimulated with conditioned media (CM) from preadipocytes, but not adipocytes, increased endothelial ICAM-1 expression of and monocyte adhesion to HMEC-1 cells. After hypoxia and TNF-α-stimulation of SGBS cells, adipocyte-CM induced and preadipocyte-CM enhanced the monocyte adhesion to HMEC-1 cells. Concordantly, the expression of pro-inflammatory adipokines, measured by qRT-PCR, ELISA and multiplex bead-based Luminex® assays, was considerably higher in preadipocytes than in adipocytes. CM up-regulated the phosphorylation of three MAPK pathways, STAT-1/3 and c-Jun in HMEC-1 cells whereas the NF-κB pathway was not affected. Inhibitor experiments showed that monocyte-endothelial cell-cell adhesion and endothelial ICAM-1 expression was JNK and JAK-1/STAT-1/3 pathway dependent and IL-6 inhibitor experiments revealed IL-6 as a major mediator in CM increasing monocyte-endothelial cell-cell adhesion via the STAT-1/3 pathway. The IL-6Rα/gp130 receptor signalling complex was detected on HMEC-1 cells. CM of mature adipocytes, independently of their size, were able to increase monocyte-endothelial cell-cell adhesion and ICAM-1 expression. Peptidomic analysis using “in silico annotation” algorithm and gene expression analysis identified progranulin, secretogranin II and secretogranin V as new factors being expressed by SGBS cells. Progranulin was found to be a new adipokine as demonstrated in CM of SGBS cells by ELISA. These findings were
supported afterwards by data published by another group describing progranulin protein expression in human WAT by Western blot. Further analyses are necessary to confirm secretogranin II and V as new secretory products of preadipocytes and adipocytes in WAT.

Altogether, this study shows that preadipocytes rather than adipocytes can operate as potent activators of endothelial cells. This can be enhanced in preadipocytes and induced in adipocytes by TNF-α and hypoxia in a manner similar to what may occur in WAT in the etiology of obesity. Secretogranin II may be an interesting candidate in the cross-talk of preadipocytes/adipocytes and endothelial cells due to its angiogenic properties and regulation by hypoxia.
6 References


# Appendix

## 7.1 List of abbreviations

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<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>7B2</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<td>Ad</td>
<td>Adipocyte</td>
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<td>ADD1</td>
<td>Adipocyte determination and differentiation factor 1</td>
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<td>AdipoR</td>
<td>Adiponectin receptor</td>
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<td>ALBP</td>
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<td>AP-1</td>
<td>Activator protein 1</td>
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<td>aP2</td>
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<td>APS</td>
<td>Acetyl peroxide solution</td>
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<td>AT</td>
<td>Annealing temperature</td>
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<td>ATF-2</td>
<td>Activating transcription factor 2</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<td>BSA</td>
<td>Bovine serum albumine</td>
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<tr>
<td>C/EBP</td>
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<td>CaCl₂</td>
<td>Calcium chloride</td>
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<td>CAMs</td>
<td>Cellular adhesion molecules</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>Cluster of Differentiation 99</td>
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<td>CDS</td>
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<td>Cyclooxygenase-2</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>Ctrl</td>
<td>Control</td>
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<tr>
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<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC receptor</td>
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<td>ddH₂O</td>
<td>Double Distilled water</td>
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<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
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<td>DMEM/F12</td>
<td>Dulbecco's Modified Eagle Medium: nutrient mixture F-12</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<td>ds</td>
<td>Double-stranded</td>
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</table>
JAM  Junctional adhesion molecule
KCI  Kalium chloride
KRP  Krebs-Ringer phosphate buffer
LFA-1  Lymphocyte function-associated antigen 1
LPL  Lipoprotein lipase
LPS  Lipopolysaccharide
MAC-1  Macrophage antigen 1
MADCAM1  Mucosal vascular addressin cell-adhesion molecule 1
MAPK  Mitogen-activated protein kinase
MAPKK  MAPK kinases
MCP-1  Monocyte chemoattractant protein-1
MgSO₄  Magnesium sulfate
MgCl₂  Magnesium chloride
MIF  Macrophage migratory inhibitory factor
MIP-1α  Macrophage inflammatory protein 1 alpha
MT  Metallothionein
NaCl  Sodium chloride
NADH+H+/NAD⁺ Nicotinamide adenine dinucleotide
NaF  Sodium fluoride
NaH₂PO₄  Sodium dihydrogen phosphate
NaOH  Sodium hydroxide
NCEP/ATP III  National Cholesterol Education Program/Adult Treatment Panel III
NGF  Nerve growth factor
NFκB  Nuclear factor kappa B
NGF  Nerve growth factor
NO  Nitric oxide
NP-40  Nonyl phenoxypolyethoxyethanol
O₂  Oxygen
Ob-R  Obese receptor or leptin receptor
p38 MAPK  p38 mitogen-activated protein kinase
PAGE  Polyacrylamide gel electrophoresis
PAI-1  Plasminogen activator inhibitor 1
PBMC  Peripheral blood mononuclear cell
PBS  Phosphate buffered saline
PC2  Proprotein convertase-2
PCR  Polymerase chain reaction
Pd  Preadipocyte
PE  Phycoerythrin
PECAM1  Platelet/endothelial-cell adhesion molecule 1
PEPCK  Phosphoenolpyruvate carboxykinase
PFA  Paraformaldehyde
PG  Prostaglandin
PGI2  Prostacyclin
PHD  Prolyl hydroxylase domain-containing enzymes
PI3K  Phosphoinositide 3-kinase
PIGF  Placenta growth factor
PKA  Protein kinase A
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>Vascular endothelial growth factor receptor</td>
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<td>VLA-4</td>
<td>Very late antigen 4</td>
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<td>VLDL</td>
<td>Very low density lipoprotein</td>
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<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
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<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>ZAG</td>
<td>Zinc-α2-glycoprotein</td>
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#### 7.4.1 Chemicals

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<td>Hank's buffered salt solution (HBSS)</td>
<td>Invitrogen, Karlsruhe, Germany</td>
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<tr>
<td>Hydorcortisone</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<tr>
<td>Insulin</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<td>Isobutylmethylxanthine (IBMX)</td>
<td>Serva, Heidelberg, Germany</td>
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<tr>
<td>Isopropanol</td>
<td>Roth, Karlsruhe, Germany</td>
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<tr>
<td>KCL</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<tr>
<td>Mayer's Hematoxylin solution</td>
<td>Merck KGaA, Darmstadt, Germany</td>
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<tr>
<td>MCDB 131</td>
<td>Invitrogen, Karlsruhe, Germany</td>
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<tr>
<td>MCDB 131 without phenol red</td>
<td>PAN Biotech, Aidenbach, Germany</td>
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<td>Mercaptoethanol</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<td>Methanol</td>
<td>Roth, Karlsruhe, Germany</td>
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<tr>
<td>MgCl₂</td>
<td>Merck KGaA, Darmstadt, Germany</td>
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<tr>
<td>MgSO₄</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
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<td>Molecular biology water</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Nicotinamidadenindinucleotide (NADH)</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<tr>
<td>Nile Red</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>N-Nitro-L-Arginine</td>
<td>Merck KGaA, Darmstadt, Germany</td>
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<td>Nonylenoxyl-polyethoxylethanol (NP40)</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Normal goat IgG</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
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<tr>
<td>Nuclease-free water</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Oil Red O</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Page ruler, prestained protein ladder</td>
<td>Fermentas, St. Leon-Rot, Germany</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<tr>
<td>Penicillin/Streptomycin</td>
<td>Invitrogen, Karlsruhe, Germany</td>
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<tr>
<td>Phenyl methyl sulfonyl fluoride (PMSF)</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<tr>
<td>Phorbol 12-myristate 13-acetate</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS) tablets</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>PhosStop phosphatase inhibitor cocktail tablets</td>
<td>Roche, Penzberg, Germany</td>
</tr>
<tr>
<td>Ponceau S</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Precision plus protein standard</td>
<td>Bio-Rad, Munich, Germany</td>
</tr>
<tr>
<td>Primer and Probes</td>
<td>Applied Biosystems, Darmstadt, Germany</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>Cayman, Ann Arbor, USA</td>
</tr>
<tr>
<td>Roswell Park Memorial Institute 1640 medium (RPMI)</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Tetramethylthylendiamin (TEMED)</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Triethanolaminhydrochlorid</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Item</td>
<td>Company</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Triiodothyronine (T₃)</td>
<td>Sigma-Aldrich, Munich, Germany</td>
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<tr>
<td>Trishydroxymethyl aminomethan hydrochlorid (Tris)</td>
<td>Roche, Penzberg, Germany</td>
</tr>
<tr>
<td>Trizol</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Trypsin/EDTA 10 x</td>
<td>PAA, Pasching, Austria</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma-Aldrich, Munich, Germany</td>
</tr>
<tr>
<td>Kits</td>
<td></td>
</tr>
<tr>
<td>Bicinchoninic acid (BCA) Protein Assay Kit</td>
<td>Pierce, Rockford, USA</td>
</tr>
<tr>
<td>Bio-Plex Multiplex Cytokine Assay</td>
<td>Bio-Rad, Munich, Germany</td>
</tr>
<tr>
<td>Cell Proliferation ELISA, BrdU (colorimetric)</td>
<td>Roche, Penzberg, Germany</td>
</tr>
<tr>
<td>DNA-free</td>
<td>Ambion, Darmstadt, Germany</td>
</tr>
<tr>
<td>ECL Advance Western Blot Detection Kit</td>
<td>GE Healthcare, Munich, Germany</td>
</tr>
<tr>
<td>High Capacity cDNA reverse transcriptase kit</td>
<td>Applied Biosystems, Darmstadt, Germany</td>
</tr>
<tr>
<td>HotStarTaq DNA Polymerase kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Human Adiponektin Quantikine® ELISA</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Human IL-6 ELISA</td>
<td>eBioscience, San Diego, USA</td>
</tr>
<tr>
<td>Human Leptin Quantikine® ELISA</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Human MCP-1 ELISA</td>
<td>eBioscience, San Diego, USA</td>
</tr>
<tr>
<td>Human PAI-1 ELISA</td>
<td>Technoclone, Vienna, Austria</td>
</tr>
<tr>
<td>SYBR green PCR Master Mix</td>
<td>Applied Biosystems, Darmstadt, Germany</td>
</tr>
<tr>
<td>TaqMan Universal PCR Master Mix, No Amp Erase UNG</td>
<td>Applied Biosystems, Darmstadt, Germany</td>
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<tr>
<td>Recombinant proteins</td>
<td></td>
</tr>
<tr>
<td>Human interleukin-6 (IL-6)</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Human leptin</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Human tumor necrosis factor alpha (TNF-α)</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Human vascular endothelial growth factor (VEGF)</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Human epidermal growth factor (EGF)</td>
<td>Immunotools, Friesoythe, Germany</td>
</tr>
<tr>
<td>Antibodies</td>
<td></td>
</tr>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
</tr>
<tr>
<td>Goat anti-human intercellular adhesion molecule 1 (ICAM-1)</td>
<td>BBA17</td>
</tr>
<tr>
<td>Goat anti-human vascular cell adhesion molecule 1 (VCAM-1)</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Rabbit anti-human β-actin</td>
<td>BBA19</td>
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<tr>
<td>Mouse anti-human hypoxia inducible factor alpha (HIF-1α)</td>
<td>New England Biolabs GmbH, Frankfurt, Germany</td>
</tr>
<tr>
<td>#4967</td>
<td>BD Biosciences; Heidelberg, Germany</td>
</tr>
<tr>
<td>610958</td>
<td></td>
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</table>
Mouse anti-human IL-6Ra MAB227 R&D Systems, Wiesbaden, Germany
Rabbit anti-human phospho c-Jun (Ser 63) #9164 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human phospho ERK1/2 (Thr202/Tyr204) #9101 New England Biolabs GmbH, Frankfurt, Germany
Mouse anti-human phospho IκBα (Ser 32/Ser 36) #9246 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human phospho p38 MAPK (Thr180/Tyr182) #9211 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human phospho Rel A (Ser536) #3031 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human phospho SAPK/JNK (Thr183/Tyr185) #9251 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human phospho STAT1 (Tyr 701) #9167 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human phospho STAT3 (Tyr 705) #9131 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total c-Jun #9165 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total ERK1/2 #9102 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total IκBα #9242 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total p38 MAPK #9212 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total Rel A #3034 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total SAPK/JNK #9252 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total STAT1 #9175 New England Biolabs GmbH, Frankfurt, Germany
Rabbit anti-human total STAT3 #4904 New England Biolabs GmbH, Frankfurt, Germany

Secondary antibodies

<table>
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<tr>
<th>Catalog number</th>
<th>Company</th>
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</thead>
<tbody>
<tr>
<td>Anti-mouse IgG, horseradish peroxidase (HRP)-linked antibody #7076</td>
<td>New England Biolabs</td>
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<tr>
<td>Anti-rabbit IgG, HRP-linked antibody #7074</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>Donkey anti-goat IgG, HRP-linked antibody 705-035-003</td>
<td>Dianova, Hamburg, Germany</td>
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</table>

Antibodies used for inhibitor experiments

<table>
<thead>
<tr>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enbrel®, soluble TNF-α receptor antibody</td>
</tr>
<tr>
<td>Monoclonal anti human IL-6 antibody MAB2061</td>
</tr>
<tr>
<td>Mouse IgG2α isotype control MAB004</td>
</tr>
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### Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Company</th>
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<tbody>
<tr>
<td>JNK Inhibitor II</td>
<td>Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>SB203580</td>
<td>Merck KGaA, Darmstadt, Germany</td>
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<tr>
<td>JAC Inhibitor I</td>
<td>Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>PD98059</td>
<td>Merck KGaA, Darmstadt, Germany</td>
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### Primer and Probes

#### Primer for RT-PCR

Primer pairs for RT-PCR were produced by Eurofins MWG, Ebersberg, Germany

<table>
<thead>
<tr>
<th>Human target gene</th>
<th>Primer</th>
<th>T_A (°C)</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>forward</td>
<td>57</td>
<td>5' GTGGCATCCACGAAACTACCTT 3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>reverse</td>
<td></td>
<td>5' GGAATTCCTTACACTCTCGCTT 3'</td>
</tr>
<tr>
<td>gp130</td>
<td>forward</td>
<td>60</td>
<td>5' AGCCTAATCGCATCCACATAA 3'</td>
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<tr>
<td>gp130</td>
<td>reverse</td>
<td></td>
<td>5' TCTTCTCCATACGCGATCT 3'</td>
</tr>
<tr>
<td>IL-6Rα</td>
<td>forward</td>
<td>60</td>
<td>5' CAGGACGTTACGACCAAGG 3'</td>
</tr>
<tr>
<td>IL-6Rα</td>
<td>reverse</td>
<td></td>
<td>5' CCCGAGTACGCGGATGT 3'</td>
</tr>
<tr>
<td>Ob-Rb</td>
<td>forward</td>
<td>55</td>
<td>5' AGACGAAAGCCAGAGACAA 3'</td>
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<tr>
<td>Ob-Rb</td>
<td>reverse</td>
<td></td>
<td>5' AATATTCCTGCGCTCTCT 3'</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>forward</td>
<td>55</td>
<td>5' GAAATTCATTATTCTAGGACAGACG 3'</td>
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<td>VEGFR-2</td>
<td>reverse</td>
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<td>5' CCTGTGGATACACTTTTACG 3'</td>
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TaqMan® Primer and Probes

TaqMan® Primer and Probes were obtained from Applied Biosystems; Darmstadt, Germany

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<tr>
<th>Human target gene</th>
<th>Primer/Probe product number</th>
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<tbody>
<tr>
<td>18S</td>
<td>Hs99999901_sl</td>
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<tr>
<td>GAPDH</td>
<td>Hs99999905_ml</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>Hs00605917_ml</td>
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<tr>
<td>IL-1α</td>
<td>Hs00174092_m1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Hs00174097_m1</td>
</tr>
<tr>
<td>IL-4</td>
<td>Hs00174112_m1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Hs00174131_ml</td>
</tr>
<tr>
<td>IL-8</td>
<td>Hs00174103_ml</td>
</tr>
<tr>
<td>Leptin</td>
<td>Hs00174877_ml</td>
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<tr>
<td>MCP-1</td>
<td>Hs00234140_ml</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Hs_00167155_ml</td>
</tr>
<tr>
<td>RANTES</td>
<td>Hs00174757_m1</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Hs00171022_m1</td>
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<tr>
<td>TNF-α</td>
<td>Hs99999043_m1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Hs_00173626_ml</td>
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Primer for qRT-PCRs with SYBR® Green

Primer pairs for qRT-PCRs with SYBR® Green were produced by Eurofins MWG, Ebersberg, Germany

<table>
<thead>
<tr>
<th>Human target gene</th>
<th>Primer</th>
<th>T_a (°C)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulin</td>
<td>forward</td>
<td>60</td>
<td>5'CACGCCACCTGCTCTCCG 3'</td>
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<tr>
<td>Granulin</td>
<td>reverse</td>
<td></td>
<td>5'GCTTAGTGAGGAGGGTCCGCTGTTAG 3'</td>
</tr>
<tr>
<td>Secretogranin II</td>
<td>forward</td>
<td>60</td>
<td>5'GATCAGTGGAACCGGAGCG 3'</td>
</tr>
<tr>
<td>Secretogranin II</td>
<td>reverse</td>
<td></td>
<td>5'TCCCAGACGACCGAGGTGT 3'</td>
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<tr>
<td>Secretogranin V</td>
<td>forward</td>
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<td>5'CTCCAAATCCCTGCTCTTGTG 3'</td>
</tr>
<tr>
<td>Secretogranin V</td>
<td>reverse</td>
<td></td>
<td>5'ACTCTCGACTGACTCTGAGTGT 3'</td>
</tr>
</tbody>
</table>
7.4.2 Consumables

**Consumables**

- 15 ml and 50 ml tubes
- ABsolute QPCR Seal
- Amicon Ultra-15 Centrifugal Filter Units 5kDA
- Baktolin wash
- Barycidal 36
- Cell culture plates, 96-well, 6-well, 6 cm
- Cell Scraper
- Chromatography paper, 1 mm
- EPTwin PCR plate 96 skirted
- Gloves S, gentle skin classic
- Gloves S, safeskin, purple nitrile-xtra
- Hybond P PVDF transfer membrane
- Hyperfilm ECL chemiluminescence film
- Hypodermic needles
- Korsolex Plus
- Pasteur pipets
- Pipet tips
- Pipet tips
- Pipet tips, filtered
- Plastic disposable serological pipets
- Reaction tubes, 0.2 ml, 0.5 ml, 1.5 ml, 2.0 ml
- Reaction tubes, 1.5 ml, 2.0 ml
- Single use syringe filter, 0.2 µm
- Sterile insulin syringe, micro-fine plus (29 G)
- Sterilium
- Sterivex sterile filter unit 0.22 µm
- Syringes
- Taktosan

**Company**

- BD Biosciences Clontech, Heidelberg, Germany
- AB Gene, Hamburg, Germany
- Millipore, Schwalbach, Germany
- Bode Chemie, Hamburg, Germany
- Biohit Deutschland GmbH, Rosbach, Germany
- BD Biosciences Clontech, Heidelberg, Germany
- TPP, Trasadingen, Switzerland
- Whatman, Dassel, Germany
- Eppendorf, Hamburg, Germany
- Meditrade, Kiefersfelden, Germany
- Kimberly Clark, Koblenz-Rheinlafen, Germany
- GE Healthcare, Munich, Germany
- GE Healthcare, Munich, Germany
- Braun, Melsungen, Germany
- Bode Chemie, Hamburg, Germany
- Corning, Kaiserslautern, Germany
- Brand, Wertheim, Germany
- Gilson, Limburg-Offheim, Germany
- Biozym Scientific GmbH, Hess. Oldendorf, Germany
- Molecular BioProducts, Fisher Scientific GmbH, Schwerte, Germany
- Corning, Kaiserslautern, Germany
- Eppendorf, Hamburg, Germany
- Zefa, Harthausen, Germany
- Sartorius AG, Goettingen, Germany
- BD Biosciences Clontech, Heidelberg, Germany
- Bode Chemie, Hamburg, Germany
- Millipore, Schwalbach, Germany
- BD Biosciences Clontech, Heidelberg, Germany
- Stockhausen, Krefeld, Germany
7.4.3 Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biophotometer</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Bio-Plex Suspension array system</td>
<td>Bio-Rad, Munich, Germany</td>
</tr>
<tr>
<td>Centrifuge 5415R, 5430 and 5810</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>CO2-Incubator CB210</td>
<td>Binder, Tuttlingen, Germany</td>
</tr>
<tr>
<td>CO2-Incubator HERAcell 150</td>
<td>Thermo Scientific, Schwerte, Germany</td>
</tr>
<tr>
<td>Dewar flasks</td>
<td>Nalgene, Hereford, England</td>
</tr>
<tr>
<td>Fastblot, semi-dry blotting unit</td>
<td>Biometra, Goettingen, Germany</td>
</tr>
<tr>
<td>Fluorescence microscope DMIL and camera DC300</td>
<td>Leica Microsystems, Wetzlar, Germany</td>
</tr>
<tr>
<td>Freezer, -86°C ULT</td>
<td>Thermoscientific, Schwerte, Germany</td>
</tr>
<tr>
<td>Freezer, HERAfreeze</td>
<td>Sanyo, Munich, Germany</td>
</tr>
<tr>
<td>Freezer, VIP Series -86°C</td>
<td>Schott GmbH, Mainz, Germany</td>
</tr>
<tr>
<td>Glass ware</td>
<td>Schuett, Göttingen, Germany</td>
</tr>
<tr>
<td>Heidolph Polymax wave platform shaker 1040</td>
<td>Biorad, Munich, Germany</td>
</tr>
<tr>
<td>Horizontal electrophoresis units</td>
<td>IUL Instruments, Koenigswinter, Germany</td>
</tr>
<tr>
<td>Hypoxia workstation, InVivo O2 400</td>
<td>WTW, Weilheim, Germany</td>
</tr>
<tr>
<td>Inolab pH meter</td>
<td>Denver Instruments, Goettingen, Germany</td>
</tr>
<tr>
<td>Laboratory balance</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Mastercycler gradient</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Mastercycler® ep realplex</td>
<td>Zeiss, Jena, Germany</td>
</tr>
<tr>
<td>Microscope, Axiovert 40C</td>
<td>LMS Consult, Brigachtal, Germany</td>
</tr>
<tr>
<td>Mini centrifuge, model GMC-060</td>
<td>Biometra, Goettingen, Germany</td>
</tr>
<tr>
<td>Minigel-Twin, vertical electrophoresis units</td>
<td>Millipore, Schwalbach, Germany</td>
</tr>
<tr>
<td>MultiScreen Separations System 96-well suction</td>
<td>Brand, Wertheim, Germany</td>
</tr>
<tr>
<td>Neubauer Chamber</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Pipettes</td>
<td>Gilson, Limburg-Offheim, Germany</td>
</tr>
<tr>
<td>Pipetting Aid</td>
<td>Biometra, Goettingen, Germany</td>
</tr>
<tr>
<td>Power Pack P25T</td>
<td>VWR, Darmstadt, Germany</td>
</tr>
<tr>
<td>Scissors, forceps</td>
<td>Beckman Coulter, Krefeld, Germany</td>
</tr>
<tr>
<td>Spectrophotometer with cuvette holder, DU 800</td>
<td>Thermoscientific, Schwerte, Germany</td>
</tr>
<tr>
<td>Sterile workbench, HERAsafe</td>
<td>Tecan, Crailsheim, Germany</td>
</tr>
<tr>
<td>Tecan Safire multiwell photo- and fluorometer</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Thermomixer confort for 1.5 ml and 2.0 ml tubes</td>
<td>Brand, Wertheim, Germany</td>
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<td>Transferpette 12 multichannel pipettes</td>
<td>GE Healthcare, Munich, Germany</td>
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<tr>
<td>Typhoon TRIO+, variable mode imager</td>
<td>Bandelin, Berlin, Germany</td>
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<tr>
<td>Ultrasound homogenator Sonopuls HD 2070</td>
<td>Intas Science Imaging Instruments, Götingen, Germany</td>
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<td>UV transilluminator and camera, INTAS UV Systeme</td>
<td>Vacuubrand, Wertheim, Germany</td>
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<td>Vacuu hand control</td>
<td>Vacuubrand, Wertheim, Germany</td>
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<td>Vacuum pump unit, PC 2004 Vario</td>
<td>Thermoscientific, Schwerte, Germany</td>
</tr>
<tr>
<td>Varioskan multiwell photo- and fluorometer</td>
<td>Scientific Industries, Bohemia, USA</td>
</tr>
<tr>
<td>Vortex gene 2</td>
<td>Julio, Seelbach, Germany</td>
</tr>
<tr>
<td>Water bath with heating circulator TW20</td>
<td></td>
</tr>
</tbody>
</table>
7.5  Monocyte-endothelial cell-cell adhesion assay

7.5.1  Quantitation of Calcein green AM labelled U937 cells

U937 cells were labelled with Calcein green AM, counted and diluted to the indicated cell numbers and transferred as 100 µl cell suspension to the designated wells of a 96-well cell culture plate. After cell lysis and centrifugation the fluorescence signal was detected at 494 and 517 nm using the Varioskan multiwell photo- and fluorometer (Thermo Scientific; n=3).
7.5.2 Example for the analysis of the monocyte-endothelial cell-cell adhesion assay data

An example for the analysis of the monocyte-endothelial cell-cell adhesion assay data is given below. HMEC-1 cells were treated with control MCDB 131 medium, preadipocyte-CM (Pd-CM), adipocyte-CM (Ad-CM) and TNF-α as positive control. Next, the monocyte-endothelial cell-cell adhesion assay was carried out as described in chapter 2.1.6. The fluorescence signal was detected at 494 and 517 nm using the Varioskan multiwell photo- and fluorometer (Thermo Scientific).

Raw data (optical density) of one single experiment with at least four replicates:

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pd16-CM</th>
<th>Ad16-CM</th>
<th>TNF alpha 25 ng/ml</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,3325</td>
<td>0,4246</td>
<td>0,3753</td>
<td>1,509</td>
<td>0,09424</td>
<td>0,332</td>
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<td>0,227</td>
<td>0,5197</td>
<td>0,3217</td>
<td>1,27</td>
<td>0,08868</td>
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<tr>
<td>0,3507</td>
<td>0,4436</td>
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<td>0,359</td>
<td>0,458</td>
<td>0,3643</td>
<td>1,104</td>
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<td>0,359</td>
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<tr>
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</table>

Mean of the blank:

- 0,09424
- 0,08868

Mean 0,09146

Raw data minus mean of the blank:

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<th>TNF alpha 25 ng/ml</th>
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<tbody>
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<td>0,24104</td>
<td>0,3314</td>
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<tr>
<td>0,13554</td>
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<td>0,23024</td>
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<tr>
<td>0,25924</td>
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<td>0,21064</td>
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<tr>
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<td>0,27284</td>
<td>1,01254</td>
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<tr>
<td>0,33764</td>
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<tr>
<td>0,41754</td>
<td>0,34374</td>
<td>0,86084</td>
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<td></td>
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</tbody>
</table>

Mean of the replicates of the control:

- Control 0,24104
- 0,13554
- 0,25924
- 0,26754

Mean 0,22584

The mean value of the control replicates was considered as 1 in order to obtain the relative values in fold for the other groups for comparison. 0,22584 = 1
In order to combine the results of independent experiments for analysis, the relative mean values of all replicates of each experiment were used to calculate the final mean values and standard deviations.

<table>
<thead>
<tr>
<th>Independent experiment 1</th>
<th>Treatment</th>
<th>Relative mean values of all replicates</th>
<th>Independent experiment 3</th>
<th>Treatment</th>
<th>Relative mean values of all replicates</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
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<tr>
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<td>Ad16-CM</td>
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<tr>
<td></td>
<td>TNF alpha</td>
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<td>TNF alpha</td>
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<table>
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<th>Treatment</th>
<th>Relative mean values of all replicates</th>
<th>Independent experiment 4</th>
<th>Treatment</th>
<th>Relative mean values of all replicates</th>
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<tbody>
<tr>
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<td>Control</td>
<td>1,00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pd16-CM</td>
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<td>Pd16-CM</td>
<td>1,82</td>
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<tr>
<td></td>
<td>Ad16-CM</td>
<td>1,20</td>
<td>Ad16-CM</td>
<td>1,20</td>
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<tr>
<td></td>
<td>TNF alpha</td>
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<td>TNF alpha</td>
<td>3,74</td>
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<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Control</th>
<th>Pd16-CM</th>
<th>Ad16-CM</th>
<th>TNF alpha</th>
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<tbody>
<tr>
<td>Experiment 2</td>
<td>1,00</td>
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<td>Experiment 3</td>
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<tr>
<td>Experiment 4</td>
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<td>Mean</td>
<td>1</td>
<td>1,69</td>
<td>1,18</td>
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<td>Standard deviation</td>
<td>0,17</td>
<td>0,02</td>
<td>1,85</td>
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7.6 Acknowledgements

I like to thank the following people for their assistance in completing this work:

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\textsuperscript{1}Else Kröner-Fresenius-Center for Nutritional Medicine, Research Center for Nutrition and Food Science, Nutritional Medicine Unit, Technische Universität München, Freising/Weihenstephan, Germany;
\textsuperscript{2}Experimental Pediatric Cardiology, Department of Pediatric Cardiology and Congenital Heart Disease, German Heart-Center Munich, Technische Universität München, Munich, Germany;
\textsuperscript{3}Research Center for Nutrition and Food Science, Biofunctionality Unit, Technische Universität München, Freising/Weihenstephan, Germany;
\textsuperscript{4}Else Kröner-Fresenius-Center for Nutritional Medicine, Research Center for Nutrition and Food Science, Molecular Nutrition Unit, Technische Universität München, Freising/Weihenstephan, Germany;
\textsuperscript{5}Research Center for Nutrition and Food Science, Molecular Nutrition Medicine, Technische Universität München, Freising/Weihenstephan, Germany;
\textsuperscript{6}Institute of Nutrition, Department of Human Nutrition, University of Jena, Jena, Germany;
\textsuperscript{7}Obesity Biology Research Unit, School of Clinical Sciences, University of Liverpool, Liverpool, UK.
## Curriculum Vitae

<table>
<thead>
<tr>
<th>Name</th>
<th>Isabelle Mack geb. Eisele</th>
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<tbody>
<tr>
<td>Geburtsdatum</td>
<td>23.05.1980</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>E-mail</td>
<td><a href="mailto:isabelle.mack@wzw.tum.de">isabelle.mack@wzw.tum.de</a></td>
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<td>Doktorarbeit zur Erlangung des Dr. rer. nat. am Lehrstuhl für Ernährungsmedizin der Technischen Universität München, Freising/Weihenstephan</td>
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<td>05.2004 – 08.2004</td>
<td>Laborpraktikum in der Abteilung für Klinische Ernährung am Deutschen Institut für Ernährungsforschung (DifE), Potsdam-Rehbrücke</td>
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<tr>
<td>01.1988 – 01.1991</td>
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### Publikationen

| Wissenschaftliche Artikel | Mack I, BelAiba RS, Djordjevic T, Gorlach A, Hauner H and Bader BL: Functional analyses reveal the greater potency of preadipocytes compared with adipocytes as endothelial cell activator under normoxia, hypoxia and TNF alpha exposure. Am J Physiol Endocrinol Metab 297(3): E735-748, 2009 |