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CHARACTERIZATION AND REMODELING OF THE VASCULATURE IN HUMAN ADIPOSE TISSUE

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ZUSAMMENFASSUNG

Fettleibigkeit (Adipositas), deren Verbreitung sowohl in den Industrie- als auch in den Entwicklungsländern auf dramatische Weise zunimmt, stellt ein bedeutendes gesundheitliches Problem dar. Adipositas per se wird von gesundheitlichen Störungen begleitet, die von der übermäßigen Fettmasse herrühren. Diese erhöht den gewichtsabhängigen Druck auf die Lunge, die Knochen und die Gelenke. Viel wichtiger: Adipositas stellt ein Risikofaktor für Typ-2-Diabetes und Herz-Kreislauf-Krankheiten dar.

Die Ursache von Adipositas liegt im übermäßigen Wachstum von Fettgewebe, welches hauptsächlich aus Fettzellen, den Adipozyten, besteht. Die Adipozyten sind Sitz des Fettgewebe-Metabolismus, d.h. Sitz der Lipogenese (Speicherung von freien Fettsäuren in Form von Triglyzeriden), und Sitz der Lipolyse (Freisetzung von freien Fettsäuren aus Triglyzeriden). Außerdem sezernieren Adipozyten ein breites Spektrum unterschiedlicher aktiver Moleküle mit lokalen und systemischen Effekten. Diese Moleküle werden unter der Bezeichnung Adipokine zusammengefasst. Leptin zum Beispiel, Produkt des Ob-Gens, übt eine Kontrollfunktion im Hypothalamus auf Appetit und Energieverbrauch aus und hat außerdem sekundäre Effekte auf den Lipid-Metabolismus in der Leber, den Muskeln und dem Fettgewebe ebenso wie auf die Angiogenese und die Immunantwort. Ein anderes Adipokin, Adiponectin, reguliert den Glukose- und Lipid-Metabolismus hauptsächlich in der Leber und in den Muskeln durch eine Erhöhung der Insulinsensitivität und hat einen anti-atherosklerotischen Einfluss auf Blutgefäßwände. Im Laufe der Ausbildung der Adipositas kommt es zu einer Verschiebung in der Produktion bestimmter Adipokine (erhöhte Leptinproduktion in Verbindung mit einer Abnahme von Adiponectin im Plasma). Es wird vermutet, dass diese Verschiebung an der Entstehung Adipositas-assoziierter Pathologien beteiligt ist.

Aufgrund ihrer bedeutenden Rolle im Fettgewebe-Metabolismus wurde bislang Adipozyten größte Aufmerksamkeit geschenkt, um die Mechanismen der Entwicklung des Fettgewebes zu erforschen. Dennoch weist das Fettgewebe eine fettzellfreie Fraktion auf, die sogenannte stroma-vaskuläre Fraktion, welche Vorläuferzellen, Makrophagen und Zellen des lokalen Gefäßnetzwerks enthält. Die Zellen der stroma-vaskulären Fraktion sind außerdem an der Homöostase des Fettgewebes beteiligt. Insbesondere liegen neue Erkenntnisse darüber vor, dass das Gefäßsystem des Fettgewebes in Nagetieren eine wichtige Rolle im Fettgewebewachstum spielt, da die Hemmung der Angiogenese in genetisch- und diät-induzierten fettleibigen Mäusen die Entstehung von Adipositas verhindert. Zudem wurde

dieser Effekt von einer Verbesserung der metabolischen Parameter begleitet. Demnach stellt das Gefäßsystem des Fettgewebes ein attraktives therapeutisches Ziel dar, um das übermäßige Wachstum des Fettgewebes zu kontrollieren und die Adipositas assoziierten Pathologien zu reduzieren. Dennoch wurde das Gefäßsystem des menschlichen Fettgewebes bis heute nicht erforscht.

Das Ziel der vorliegenden Arbeit ist, dass im menschlichen Fettgewebe vorhandene Gefäßsystem zu charakterisieren und die Veränderungen, die es während des Wachstums des Fettgewebes erfährt, zu erforschen.

Durch immuno-histochemische Analysen am subkutanen menschlichen Fettgewebe konnten zwei verschiedene Gefäßsysteme identifiziert werden: das vaskuläre Netzwerk des Bluts und das lymphatische vaskuläre Netzwerk. Während die Endothelzellen von beiden Gefäßsystemen die gemeinsamen Endothelzellmarker von Willebrand factor (vWf) und CD31 (PECAM, Platelet Endothelial Cell Adhesion Molecule) exprimierten, konnten die Endothelzellen der Blutgefäße an der Expression des Markers CD34 (Stamm/Blutgefäß-Endothel-Zell-Marker) und die Endothelzellen der Lymphgefäße an der Expression der beiden lymphatischen Marker Podoplanin und VEGFR3 (Vascular Endothelial Growth Factor Receptor 3) spezifisch erkannt werden. Zellen, die in der Stroma- und nicht in der vaskulären Fraktion vorhanden waren, wurden ebenfalls charakterisiert: Es handelte sich um ausschließlich für den Marker CD34-positive Zellen und in Rosetten angeordnete CD31-positive Zellen, welche als residente Makrophagen charakterisiert wurden.

Um die beiden Gefäßsystemen des menschlichen Fettgewebes weiterhin zu erforschen, wurde ein auf Immunoselektion basiertes Protokoll entwickelt. Es ermöglicht, Blutgefäß- und Lymphgefäß-Endothelzellen spezifisch zu isolieren, indem Antikörper verwendet werden, die an magnetische Microbeads gekoppelt sind. Die Antikörper sind dabei gerichtet gegen CD34, CD31 und den Makrophagenmarker CD14. Aufgrund der gemeinsamen Expression von CD31 durch beide Endothelzelltypen und der spezifischen Expression von CD34 durch Blutgefäß- jedoch nicht durch Lymphgefäßendothelzellen, konnten wir CD34+/CD31+ Blutgefäßendothelzellen (BEC) isolieren. Nach Depletion der Makrophagen (CD34-/CD14+ Zellen) aus der CD34-negativen Zellfraktion konnten CD34-/CD14-/CD31+ Lymphgefäßendothelzellen (LEC) isoliert werden. Die CD34+/CD31- Zellen stellten die Progenitor-Zellen dar. Real time PCR ebenso wie immuno-histochemische Analysen wurden mit frisch isolierten Zellen durchgeführt. Die Expression von gemeinsamen Endothelzellmarkern wie den Vascular Endothelial Growth Factor Receptors 1 und 2 (VEGFR1 und VEGFR2), vWf und dem Transmembran-Rezeptor Notch4 wurde untersucht.

Sowohl BEC als auch LEC exprimierten VEGFR1, VEGFR2, vWf und Notch4 in ähnlichen Mengen wie humane Endothelzellen der Nabelschnurvene (HUVEC). Hingegen konnten die Transkripte der getesteten Gene in mononuklearen Zellen, welche aus menschlichem Blut isoliert worden waren, nicht entdeckt werden. Außerdem nehmen beide Zelltypen acetyliertes LDL auf, was sich in der Expression vom Scavenger Rezeptor für acetyliertes LDL wiederspiegelt, welche auf Endothelzellen und Makrophagen beschränkt ist. Diese Befunde zeigen, dass BEC und LEC dieselben Endothelzell-Charakteristika aufweisen. Darüber hinaus konnte in LEC die Expression von Genen, welche spezifisch für das Lymphgefäßsystem sind, wie Podoplanin, Reelin, VEGFR3, Desmoplakin, LYVE-1 nachgewiesen werden. Zusammengefasst belegen die Ergebnisse, dass die aus dem menschlichen Fettgewebe isolierten BEC und LEC unterschiedliche Genexpressionsmuster bezüglich der Marker von Blut- bzw. -Lymphgefäßen aufweisen.

Wie beeinflusst das Wachstum des Fettgewebes die Anzahl von BEC und LEC in diesem Gewebe? Dieser Frage wurde mit Hilfe von flusscytometrischen Analysen im Fettgewebe von Patienten mit unterschiedlichen *Body Mass Indices* (BMI) nachgegangen. Während die Anzahl von BEC pro Gramm unabhängig vom BMI der Patienten konstant blieb, war die Anzahl von LEC in übergewichtigen/fettleibigen Personen (BMI>25) signifikant geringer als in normalgewichtigen Personen (BMI<25).

Diese Ergebnisse deuten darauf hin, dass das Blutgefäßsystem, nicht aber das Lymphgefäßsystem sich zusammen mit der Fettmasse entwickelt, und, dass die Ausbildung der Adipositas mit einer Erweiterung des Blutgefäßsystems, nicht jedoch des Lymphgefäßsystems, innerhalb des Fettgewebes einhergeht.

Zwei Mechanismen könnten an der Neovaskularisation im adulten Gewebe beteiligt sein: die Vaskulogenese, welche die Differenzierung von Progenitor-Zellen zu Endothelzellen beinhaltet, und die Angiogenese, welche mit der Migration, der Proliferation und der Organisation von ruhenden Endothelzellen aus einem bereits vorhandenem Gefäß einhergeht.

Flusscytometrische Analysen belegen, dass es in der CD34+/CD31- Stroma-Zellpopulation Zellen gibt, die den endothelialen Progenitor-Zellmarker CD133 und den primitiven Stammzellmarker ABCG2 exprimieren. Durch eine hohe Serumkonzentration im Kulturmedium der CD34+/CD31- Zellen, die aus menschlichem Fettgewebe mit Hilfe des Immunoselektionsprotokolls isoliert worden waren, konnte die Bildung einer „cobblestone area“, charakteristisches Kennzeichen adulter Stammzellen, beobachtet werden. Außerdem zeigten die aus dem Fettgewebe stammenden CD34+/CD31- Zellen eine signifikant stärkere Proliferation und Expression von Endothelzellmarkern wie CD31 und vWf, wenn dem

Kulturmedium zuvor die Faktoren Vascular Endothelial Growth Factor A (VEGF A) und Insulin-Like Growth Factor-1 (IGF-1) zugefügt worden waren. VEGF A ebenso wie IGF-1 werden in der Literatur beschrieben als Faktoren, die die Proliferation und Differenzierung von endothelialen Progenitorzellen zu Endothelzellen induzieren. Die Ergebnisse weisen darauf hin, dass es unter den CD34+/CD31-Zellen eine Population von endothelialen Progenitorzellen gibt, die -bei geeigneter Stimulation- zu Endothelzellen differenzieren. Anhand des Modells der Hinterbeinischämie der Maus konnte zudem gezeigt werden, dass diese Zellpopulation auch in vivo die Charakteristika endothelialer Progenitorzellen aufweist. Innerhalb dieser Versuche wurden Mäusen mit Hinterbeinischämie frische, aus menschlichem Fettgewebe isolierte, CD34+/CD31-Zellen in vivo injiziert. Mittels verschiedener Techniken konnte gezeigt werden, dass sich die CD34+/CD31- Zellen an der Neovaskularisation des ischämischen Hinterbeins beteiligen: Eine signifikante Zunahme des Blutflusses im ischämischen Bein war in der Laserdoppler-Analyse erkennbar, gekoppelt an einer erhöhten Kapillardichte im ischämischen Muskel und einer Integration der menschlichen Zellen in die Blutgefäße der Maus, wie immuno-histochemische Analysen zeigen konnten. Diese Ergebnisse belegen, dass die aus menschlichem Fettgewebe isolierten CD34+/CD31-Zellen die Neovaskularisation des ischämischen Gewebe verstärkten. Somit zeigen CD34+/CD31-Zellen sowohl *in vivo* als auch *in vitro* Eigenschaften endothelialer Progenitor-Zellen. Demnach kann die Beteiligung dieser Zellpopulation am Wachstum des Blutgefäßsystems innerhalb des Fettgewebes - im Sinne der Vaskulogenese - angenommen werden.

Parallel dazu wurden die lokalen Faktoren untersucht, die potentiell an der Wachstumskontrolle, der Migration und der Organisation der ruhenden, aus dem Fettgewebe stammenden, BEC und LEC beteiligt waren. Insbesondere wurde die Proliferation der BEC und LEC in Medien getestet, die mit ausdifferenzierten Adipozyten oder Progenitor-Zellen aus menschlichem Fettgewebe konditioniert wurden. Sekrete der Adipozyten, jedoch nicht der CD34+/CD31-Zellen, induzierten eine signifikante BEC- und LEC-Proliferation. Weiterhin wurde untersucht, wie BEC und LEC auf den angiogenetischen Faktor VEGF A, auf die lymphangiogenetischen Faktoren VEGF C und VEGF D sowie auf die Adipokine Leptin und Adiponectin antworten. Dazu wurde einerseits die Expression der Rezeptoren dieser Faktoren (z.B. VEGFR1, VEGFR2, VEGFR3, Leptinrezeptor und Adiponectinrezeptoren 1 und 2) mittels Real Time PCR bestimmt. Die Transkripte aller untersuchten Gene wurden in beiden Zelltypen gefunden. Verglichen mit den anderen Rezeptoren wurde VEGFR2 in BEC am stärksten exprimiert, während in LEC der Adiponectinrezeptor am stärksten exprimiert wurde. Weiterhin exprimierten LEC eine geringere Anzahl von Transkripten des Leptinrezeptors im

Vergleich zu BEC. Andererseits wurde die Proliferationsantwort der BEC und LEC auf zunehmende Konzentrationen der pro-angiogenetischen sowie pro–lymphangiogenetischen Faktoren (VEGF-A, -C and –D), Leptin und Adiponectin mittels Bromodeoxyuridin (BrdU)-Inkorporation gemessen. Keiner der Faktoren alleine konnte die Proliferation der BEC beeinflussen. Allerdings führte die Kombination von Leptin und VEGF A oder des basic Fibroblast Growth Factor zu einer signifikanten Zunahme der BrdU-Inkorporation in BEC. LEC zeigten eine unterschiedliche Proliferationsantwort: Adiponectin, VEGF C und VEGF D induzierten bereits alleine konzentrationsabhängig die Proliferation von LEC, während die Inkubation mit Leptin ohne Effekt blieb. Diese spezifische Reaktion von BEC auf Leptin bzw. von LEC auf Adiponectin konnte am Studium der Effekte beider Adipokine auf die Endothelzell-Migration und -Organisation im Matrigel Assay bestätigt werden: Leptin, und nicht Adiponectin, führte zu signifikant höherer BEC-Migration und Röhrenformung, während Adiponectin, und nicht Leptin, die LEC-Migration und -Organisation förderte.

Nicht zuletzt wurden die durch beide Adipokine (Leptin und Adiponectin) induzierten Transduktionswege mittels Western Blot-Analysen untersucht. Dabei führte Leptin in BEC und Adiponectin in LEC zeitabhängig zu einer signifikanten Zunahme der Phophorylierung der Kinase Akt. Diese Ergebnisse belegen, dass die beiden aus Adipozyten stammenden Adipokine Leptin und Adiponectin eine tragende Rolle in der Umverteilung von BEC bzw. LEC spielen.

Im Rahmen der Adipositas kommt es zu einer Modulation in der Synthese von Leptin und Adiponectin: Während die Plasmakonzentration von Leptin ansteigt, sinkt die Plasmakonzentration von Adiponectin. Demnach könnten Veränderungen, in der Adipositas, der Adipokinfreisetzung durch Adipozyten am Umbau des vaskulären Netzwerks des Bluts und am ausbleibenden Wachstum des lymphatischen vaskulären Systems innerhalb des Fettgewebes beteiligt sein.

Die in der vorliegenden Arbeit beschriebenen Ergebnisse liefern grundlegende Erkenntnisse über die Phänotypen der Endothelzellen des Blut- sowie Lymphgefäßsystems des menschlichen subkutanen Fettgewebes. Außerdem deuten die Ergebnisse daraufhin, dass Adipokine an der Steuerung des Gefäßumbaus innerhalb des menschlichen Fettgewebes im Rahmen der Adipositas beteiligt sein könnten. Dabei konnte Leptin als lokaler pro-angiogenetischer Faktor identifizieren und Adiponectin als neuer lymphangiogenetischer Faktor im menschlichen Fettgewebe beschreiben. Auch wenn weitere Untersuchungen notwendig sind, um die Faktoren und Mechanismen des Blut- und Lymphgefäßumbaus im Fettgewebe noch präziser aufzuklären, eröffnet die vorliegende Studie ein neues

Forschungsfeld, nämlich die Rolle des Lymphgefäßsystems innerhalb des Fettgewebewachstums. Kürzlich konnte gezeigt werden, dass ein Defekt in der Lymphoangiogenese assoziiert ist mit am späten Ausbruch von Adipositas gekoppelt ist. Das Lymphgefäßsystem könnte demzufolge einem übermäßigen Wachstum der Fettmasse entgegenwirken.

Schließlich belegen die vorliegenden Ergebnisse das Vorhandensein einer Progenitor-Zell-Population in der Stroma-Fraktion des menschlichen Fettgewebes. Diese Progenitor-Zellen sind in der Lage sich an der Neovaskularisation ischämischen Gewebes zu beteiligen. Diese Population könnte im Hinblick auf zelltherapeutische Strategien eine interessante Alternative zu Stammzellen aus dem Knochenmark darstellen.

I- INTRODUCTION

Obesity, which is characterized by an excessive accumulation of adipose tissue in the body, represents one of the greatest public health challenges for the 21st century and has now reached epidemic proportions. Indeed, according to the World Health Organization, the prevalence of obesity ranges from 5% to 20% in men and reaches up to 30% in women in Europe. In the United States, one third of the population is considered obese. Even children are affected since 10% to 30% of 7-11-year-old children and 8% to 25% of 14-17-year-old children are overweight or obese in Europe.

Obesity per se is associated with health problems linked to increased weight-dependent pressure overload on lung, joints and bones. More importantly, obesity represents a risk factor for life-threatening diseases such as cardiovascular diseases (CVD), type 2 diabetes and certain forms of cancer (TABLE 1) (101). When one takes into account that CVD are the leading cause of death in the adult population of all ages in the United States and Europe and that obese people have 2- to 3-fold increased risk to develop CVD, it appears urgent to understand the mechanisms responsible for the hyperplasia/hypertrophy of the adipose tissue, as well as those involved in the development of metabolic and cardiovascular complications on the basis of an excess body fat mass.

Obesity and overweight are usually assessed using the body mass index (BMI) which is the ratio of the body weight and the square of the height (kg/m^2). The World Health Organization defines overweight by a BMI over 25 kg/m^2 and obesity by a BMI over 30 kg/m^2. The body fat mass and its repartition are determined by a complex combination of environmental, genetic and psychosocial parameters that influence energy expenditure, such as physical activity, basal metabolism and adaptative thermogenesis, and energy intake (101). The imbalance between energy intake and expenditure is associated with enhanced storage of triglycerides within the adipocytes which are the main cell type present in the adipose tissue, leading to adipocyte hypertrophy. The further imbalance between energy input and output leads to an increased adipocyte cell number within the fat mass. Both adipocytes hypertrophy and hyperplasia participate to the enlargement of the fat mass and thus to the development of obesity. Since the last 10 years, the discovery of a wide range of metabolic active proteins produced by the adipocytes, grouped under the term adipokines, has highlighted the secretory activity of adipose tissue. Moreover, several studies have suggested that adipokines might be directly involved in the growth of the adipose tissue itself but also in the genesis of the obesity-associated pathologies (36,61,94,151). However, the adipose tissue is also composed

of non-adipose cells, contained in the so-called stroma-vascular fraction (SVF), which includes the endothelial cells composing the local microcirculation. Recently, several studies have pointed out the role of the endothelial cells in the development of the fat mass (8,58,83), suggesting that such a population might represent a new original therapeutic target to control the growth of the adipose tissue.

Relative risk of health problems associated with obesity in developed countries.

Greatly increased (relative risk >>3)	Moderately increased (relative risk ca 2-3)	Slightly increased (relative risk ca 1-2)
► Diabetes	► Coronary heart disease	► Cancer (breast cancer in postmenopausal women, endometrial cancer, colon cancer)
► Gall bladder disease	► Osteoarthritis (knees)	► Reproductive hormone abnormalities
► Hypertension	► Hyperuricaemia and gout	► Polycystic ovary syndrome
► Dyslipidaemia		► Impaired fertility
► Insulin resistance		► Low back pain
► Breathlessness		► Increased anaesthetic risk
► Sleep apnoea		► Foetal defects arising from maternal obesity

TABLE 1: Main health problems associated with obesity in developed countries
The excessive accumulation of adipose tissue which leads to the development of obesity is associated with a risk to develop pathologies, as indicated in the table according to the relative risk.

I.1. Physiology of the adipocyte

Adipocytes are the major cell type present in adipose tissue. Adipocytes consist of more than 95% of triglycerides stored in a unique lipid droplet. As terminally differentiated cells, mature lipid-filled adipocytes are not able of cell division (68).

I.1.1. Metabolic activity of adipocytes

Adipocytes are the site of the metabolic activities of human adipose tissue, i.e. lipogenesis or the storage of free fatty acids in the form of TG and lipolysis or the release of TG in the form of free fatty acids and glycerol (148).

The main source of fatty acids incorporated into the triglycerides during lipogenesis is provided by the chylomicrons and the very low density lipoproteins (VLDL). VLDL and chylomicrons are hydrolysed by the lipoprotein lipase, localized at the apical surface of the endothelial cells (122). The hydrolysis of VLDL and chylomicrons by lipoprotein lipase releases fatty acids that are taken up by the adipocytes where they are coupled to glycerol-3-phosphate, mainly originated from the glycolysis (149) (FIGURE 1). The synthesis of fatty acids from carbohydrates, i.e. “de novo” lipogenesis, is considered to be a minor contributor of triglyceride formation in human (47,48,72).

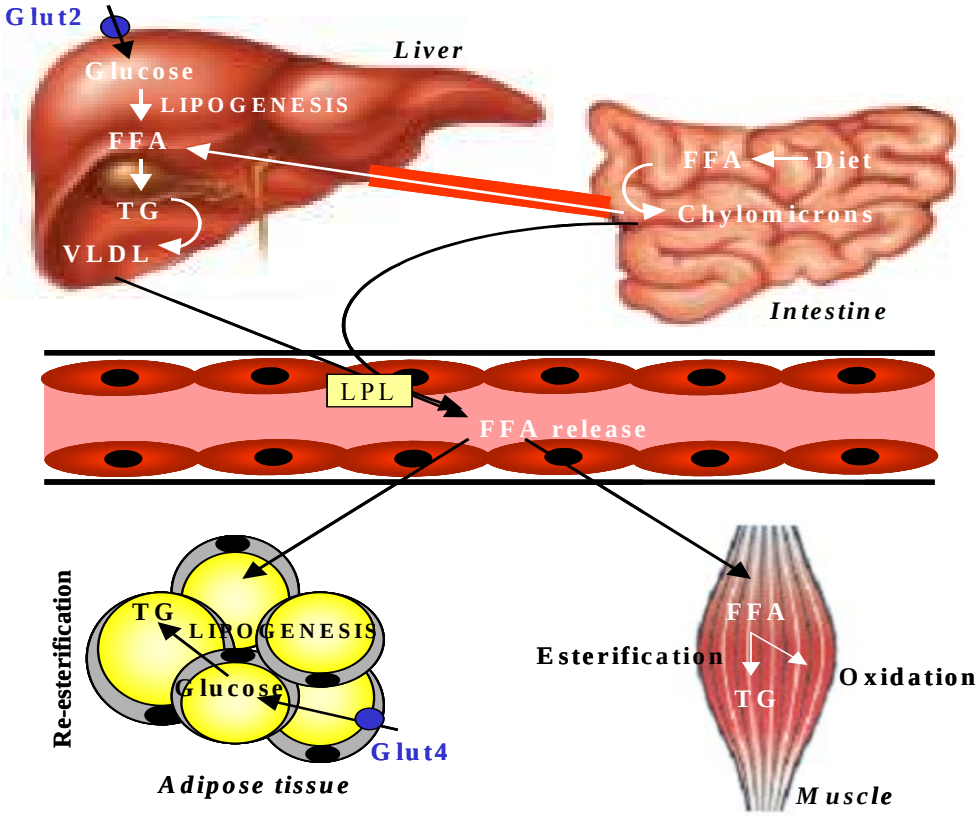

FIGURE 1: Schematic illustration of fatty acids release after a meal (adapted from (113))
Free fatty acids (FFA) present in the intestine after a meal are incorporated into chylomicrons that are released into the blood circulation. Chylomicrons reach the liver via the portal vein where endogenous triglycerides (TG), partly coming from lipogenesis, are incorporated into very low density lipoprotein (VLDL). Both chylomicrons and VLDL are transported to the tissues via the blood circulation, where they are hydrolysed by the lipoprotein lipase (LPL) expressed at the apical surface of endothelial cells. The hydrolysis of chylomicrons and VLDL releases FFA that are taken up by muscle for oxidation and by adipocytes for storage. The association of FFA to glycerol-3-phosphate that originates from the glycolysis (uptake of glucose under the control of the glucose transporter GLUT-4) forms TG.

Adipocytes hydrolyse triglycerides into glycerol and fatty acid by a process called lipolysis (10). Lipolysis depends on the activity of three lipases: the adipose triglyceride lipase (ATGL) that is responsible for the first step of triglyceride catabolism (201) leading to the formation of diglycerides, the hormone-sensitive lipase (75) that hydrolyses diglycerides into monoglycerides (111) and the monoglyceride lipase that catalyses the breakdown of monoglycerides to glycerol and fatty acids (89,182).

Both lipogenesis and lipolysis are under the tight control of hormonal and nervous signals. In humans, the main regulators are insulin (42), catecholamines (106,112) and natriuretic peptides (107,108,163,165).

Catecholamines (norepinephrine and adrenaline), natriuretic peptides and insulin control the phosphorylation state of the hormone-sensitive lipase through the modulation of the protein kinases PKA and PKG activities that depend on the intracellular concentrations of cAMP (increase by the catecholamines and decrease by insulin) and cGMP (increase by ANP), respectively (FIGURE 2). Insulin induces lipogenesis in short-term via the recruitment of glucose transporters at the plasma membrane in adipocytes (95) and in long-term via the induction of lipogenic enzymes such as fatty acids synthase (FAS) and acetyl-coenzymeA carboxylase (ACC) and the lipoprotein lipase (12).

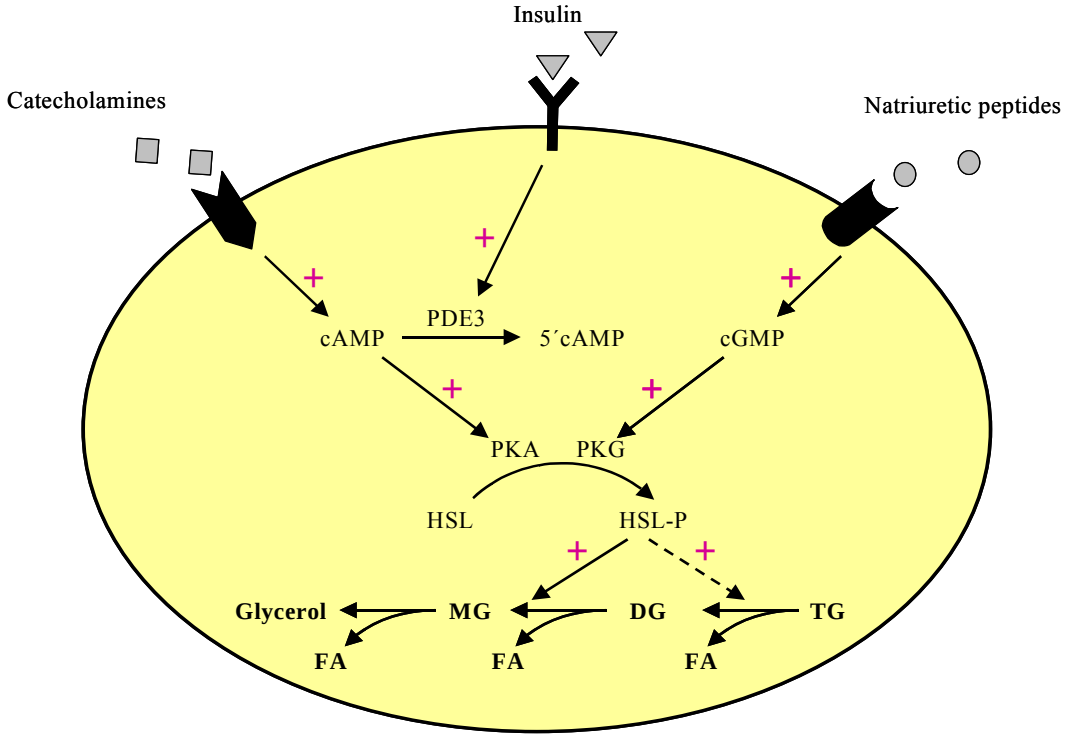

FIGURE 2: Regulation of lipolysis in the adipocyte
Catecholamines (adrenaline and norepinephrine) bind to their receptor and induce the formation of cAMP by the adenylate cyclase. cAMP activates the protein kinase A (PKA) that phosphorylates and activates the hormone-sensitive lipase (HSL). HSL is the key enzyme of triglycerides (TG) hydrolysis, i.e. lipolysis, leading to the release of fatty acids (FA) and glycerol. Insulin activates the phosphodiesterase 3 (PDE3) that degrades cAMP leading to a decrease of cAMP concentration. The subsequent reduction in PKA activity leads to a decreased HSL phosphorylation and thus activation, which in turn leads to the inhibition of lipolysis. Natriuretic peptides activate the formation of cGMP by guanylyl cyclase. cGMP activates PKG that phosphorylates HSL, thus leading to an enhanced lipolysis.

Increased lipogenesis and/or decreased lipolysis are responsible for the adipocyte hypertrophy. The accumulation of triglycerides can lead to a 10-fold increase of the cell diameter, reaching more than 100µm, which means a 1000-fold increase in the cell volume (19). Pharmacological modulations of the metabolic pathways in adipocytes, and more specifically directed toward increased lipolysis have been attempted to reduce adipocyte hypertrophy and thus adipose tissue growth development. However no positive results have been obtained in humans and such approaches have been given up. Indeed, it is now well recognized that the storage of the excess lipid within the fat mass is necessary to protect liver, muscle and pancreas against the adverse effects of free fatty acids (lipotoxicity) (49,116,121,159). This is well illustrated in the syndrome of lipodystrophy where the lack of adipose tissue is associated with insulin resistance and liver steatosis mainly due to the lipotoxic effects of the free fatty acids (59).

I.1.2. Secretory activity of adipocytes

During the last years, the adipose tissue has acquired the status of an endocrine organ. It produces a wide range of hormones, factors and cytokines (TABLE 2), that are grouped under the term adipokines (36,61,94,151). Adipokines have been shown to influence various metabolic processes. This has revealed the existence of new peripheral and central metabolic axes, via effects of adipose tissue-derived secretions on glucose and lipid metabolism in the liver, the skeletal muscle as well as on the hypothalamic centers which are involved in the control of energy homeostasis. Moreover, some adipokines have been involved directly in the genesis of insulin resistance and endothelial dysfunction (36).

Adipokines	Effect on
Leptin	Food intake, reproduction, angiogenesis, immunity
Adiponectin	Inflammation, atherosclerosis, insulin resistance
IL-6	Inflammation, atherosclerosis, insulin resistance
PAI-1	Vascular homeostasis
Apelin	Insulin resistance
Visfatin	Insulin resistance
Resistin	Inflammation, insulin resistance
C-reactive protein	Inflammation, atherosclerosis, insulin resistance
TNF-α	Inflammation, atherosclerosis, insulin resistance
Angiotensinogen	Vascular homeostasis
Adipsin	Immune stress response
IGF-1	Lipid metabolism, insulin resistance
Monobutyrin	Vasodilation of the microvessel
TGF-β	Cell adhesion and migration, growth and differentiation

TABLE 2: Adipokines and their main effects (adapted from (151))

Adipokines are secreted by adipose tissue. Short list of the best studied adipokines (IL-6: interleukin 6, PAI-1: plasminogen activator inhibitor 1, TNFα: tumor necrosis factor α, IGF-1: insulin-like growth factor-1, TGF-β: transforming growth factor β)

I.1.2.1 Adipokines and energy homeostasis

a) Leptin

Leptin was one of the first adipokines identified in 1994 in mice as the product of Ob gene (199), the deficiency of which led to obesity in ob/ob mice due to an enhanced hyperphagia combined with a reduced energy expenditure. Leptin acts directly in the hypothalamus as a satiety signal to regulate food intake (51,52). Besides its central effect, peripheral effects of leptin have been described on the liver, pancreas, muscle and adipose tissue itself (FIGURE 3)(79). Leptin also plays an important role in reproduction and fertility and might thus explain the well-known link between the amount of adipose tissue and the reproductive capacity. Indeed, leptin accelerates puberty in wild-type mice (3) and facilitates reproductive behavior in rodents (188). Moreover, in genetically obese ob/ob female mice that exhibit low fertility, leptin treatment corrects the sterility, thus resulting in ovulation, pregnancy and parturition (38,126). Taken together, these observations has led to the concept that leptin represents the link between adipose tissue and the hypothalamus that regulate reproduction and metabolism according to the amount of energy stored in the fat mass.

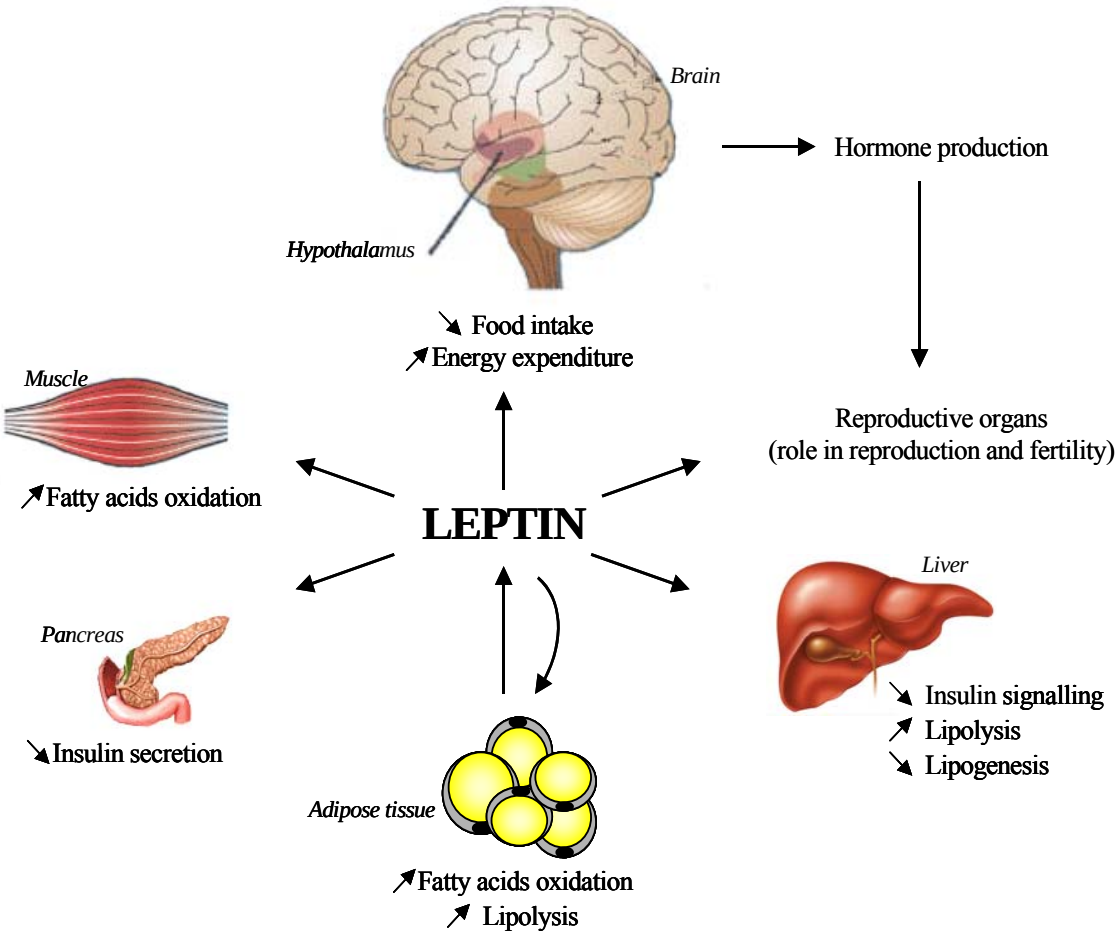

FIGURE 3: Main central and peripheral effects of leptin

Leptin, produced by the adipocytes exert central and peripheral effects involved in the regulation of glucose and lipid metabolism. Leptin acts in the hypothalamus as a satiety signal. Moreover, leptin induces the production of hormones that support the functional activity of the reproductive organs. Leptin also regulates lipid and glucose metabolism via peripheral effects on muscle, pancreas, liver and adipose tissue. Indeed, the insulin secretion by the pancreas is inhibited by leptin while lipolysis and fatty acid oxidation are increased in adipose tissue and muscle and lipogenesis is decreased in the liver.

The further identification of leptin receptor as the product of the db gene in mice, the mutation of which led to obesity in the db/db mice (39), allowed to better characterize the leptin-activated cell signaling pathways (FIGURE 4). Leptin receptor belongs to the class I cytokine receptor family. The product of db gene is spliced in six isoforms: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf. All of these isoforms have transmembrane domains except Ob-Re, which is a soluble receptor. ObRe circulates in the blood and binds leptin with high affinity (110,115). Among the transmembrane receptors, Ob-Rb, the long-form receptor, is considered as the active form linked to the activation of intracellular signaling pathways. Indeed, the cytoplasmic region of Ob-Rb contains several proline-rich domains that are essential for the recruitment and activation of cytoplasmic kinases, such as the Janus tyrosine Kinase 2 (JAK2) (18,190). The activation of JAK2 leads to the activation of the Signal Transducer and Activator of Transcription (STAT3) which then translocates into the nucleus and acts as a transcription factor. JAK2 also activates the mitogen-activated protein kinases (MAPK)-dependent signaling pathway (18). The short-form transmembrane receptors are thought to be mainly involved in leptin internalization and degradation (177).

Due to the central role of leptin, attempts have been made to administrate leptin to obese patients in order to restore an appropriate energy balance. However, no convincing results have been obtained except in the rare obesity-associated genetic defect in leptin gene (114). The lack of effect of leptin on energy intake and expenditure in obese humans has been explained by the development of a leptin-resistant state associated with hyperleptinemia (50,181). One of the potential pathways responsible for the inhibition of leptin signaling in obesity involves the Suppressor-of-Cytokine-Signaling (SOCS3). SOCS3 decreases the activation of the leptin receptor both in vitro and in vivo and is considered as an inhibitory feedback signal that terminates leptin signaling. Indeed its expression is dependent on STAT3 activation (29). Expression levels of SOCS3 have been shown to be increased in several rodent models of obesity associated with a leptin-resistant state (17,128), suggesting that SOCS3 might be involved in the insulin resistance state observed in obesity. The tyrosine phosphatase PTP1B might also be involved in leptin resistance. On one hand overexpression of PTP1B in cultured cells has been shown to attenuate leptin signaling (195) and on the other hand, high fat diet-induced leptin resistance was associated with increased levels of PTP1B in liver (109).

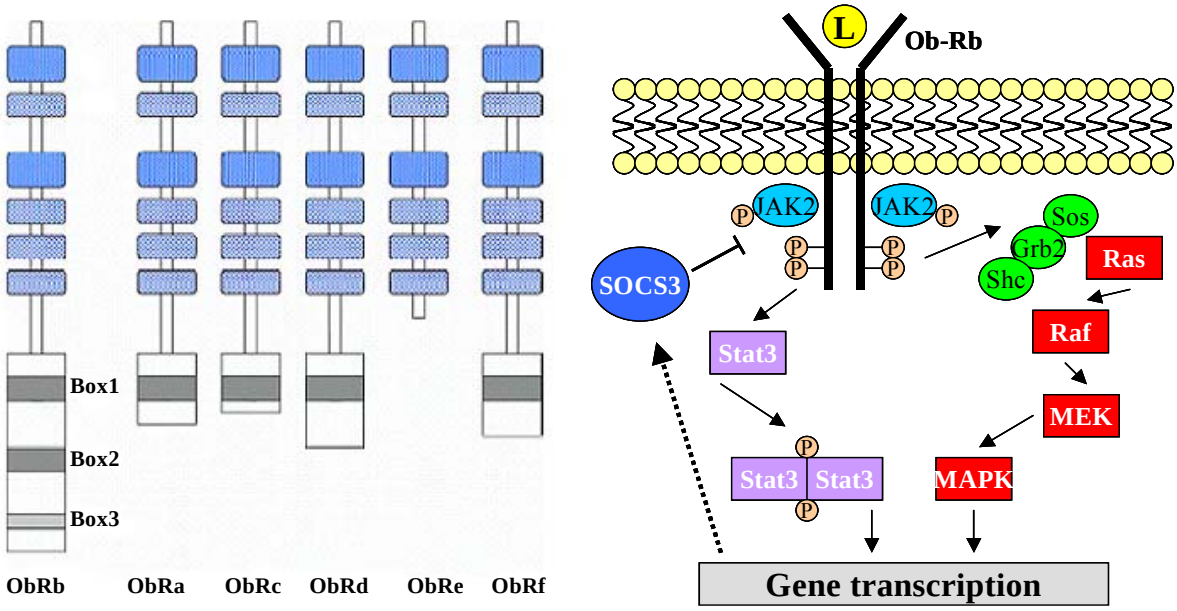

FIGURE 4: Leptin receptors and leptin signaling pathways
(a: from (70); b: adapted from (65))
(a) The 6 isoforms of the leptin receptor: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Rf are the five transmembrane receptors for leptin. Ob-Rb, the long form receptor is the only one able to induce signal transduction. Ob-Re is a soluble receptor
(b) Signaling pathways activated by leptin: The binding of leptin (L) to its receptor leads to the activation of Janus Kinase 2 (JAK2). JAK2 phosphorylates the Signal Transducer and Activator of Transcription 3 (STAT3) that translocates to the nucleus and regulates gene expression. JAK2 can also activate the mitogen-activated protein kinases (MAPK)-dependent signaling pathway. The Suppressor-of-Cytokine-Signaling (SOCS3) can exert a negative feedback by inhibiting JAK2.

b) Adiponectin

Adiponectin (also called adipocyte complement-related protein ACRP, adipose most abundant gene transcript 1 apM1, gelatine-binding protein 28 GBP28 or AdipoQ) has been discovered in 1995 (160). Adiponectin is secreted into the bloodstream where it accounts for approximately 0.01% of all plasma protein (around 5-10 µg/mL in human blood). Adiponectin is a complex protein that oligomerizes via the interaction of disulfide bonds leading to the formation of trimers, hexamers and high molecular weight (HMW) forms circulating in the plasma (37,136,176,189). Each form has a distinct ability to activate intracellular signaling pathways (176), leading to the induction of different physiological responses (134,189). Adiponectin is considered as an insulin-sensitizing agent (24). Indeed, adiponectin increases fatty acids oxidation in muscle and liver through the activation of peroxisome proliferator activated receptor (PPARα) (71). Moreover, adiponectin-induced activation of AMP-activated protein kinase (AMPK) leads to glucose uptake via the activation

of the translocation of the glucose transporter GLUT4 to the cell membrane (34) in the skeletal muscle and to the inhibition of gluconeogenesis in the liver. Taken together, these effects induce activation of the insulin receptor substrates, IRS1 in muscle and IRS2 in liver, improving insulin sensitivity. The major role of adiponectin as an insulin-sensitizing adipokine is further stressed by the observation that PPARγ agonists, such as the thiazolidinediones, well known insulin-sensitizing drugs used in the treatment of type 2 diabetes, up-regulate the expression of adiponectin (15,20). In addition, adiponectin exhibits anti-atherosclerotic effects. Indeed, adiponectin decreases adhesion molecule expression by endothelial cells and inhibits foam cell formation as well as smooth muscle cell migration in the vascular wall, thus inhibiting the development of atherosclerotic plaques (64,80)(FIGURE 5). Two receptors of adiponectin have been described, adipoR1 and adipoR2 (192). In addition, a third receptor, T-cadherin, has recently been described although its tissue distribution and functional significance need further investigations (81). In mice, AdipoR1 was predominantly found to be expressed on muscle cells whereas AdipoR2 was mainly expressed by liver cells (192). In humans, both adiponectin receptors are ubiquitously expressed (99). Both receptors are able to induce signal transduction after binding with adiponectin. In particular, adiponectin is able to activate AMPK as well as the MAPK signaling pathway (192). In addition, a new molecule, APPL1, has recently been described to play the role of an adaptator protein that mediates adiponectin signal transduction (120).

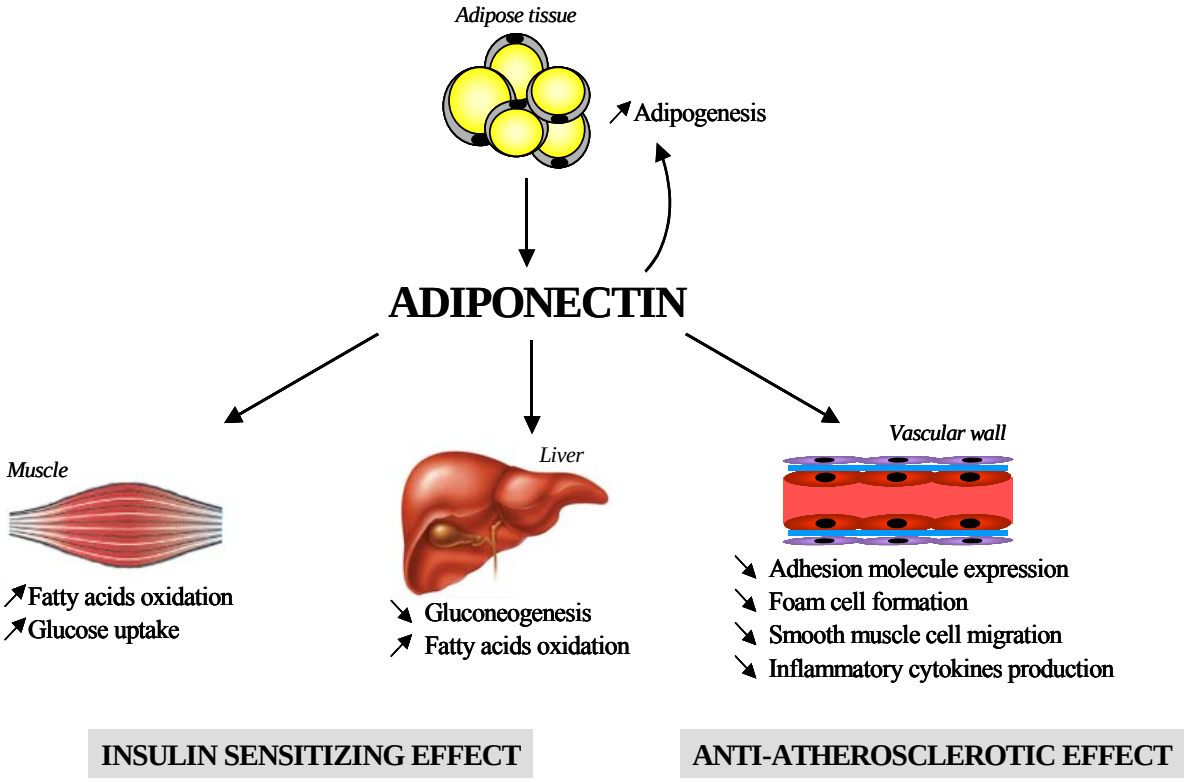

FIGURE 5: Biological actions of adiponectin
Adiponectin, produced by the adipocytes exerts its effects on the liver where it increases fatty acids oxidation and glucose uptake as well as on the liver where fatty acids oxidation is also enhanced while the production of glucose (gluconeogenesis) is inhibited. These effects lead to the activation of the insulin receptor substrate (IRS 1 in the muscle and IRS 2 in the liver) what results in insulin sensitizing. In addition, adiponectin acts on the various cells present in the vascular wall. Indeed, adiponectin decreases the expression of adhesion molecules by endothelial cells, inhibits the activation and production of inflammatory cytokines in immune cells as well as foam cell formation and prevents smooth muscle cell migration. These effects result in the inhibition of atherosclerotic plaque formation.

c) Resistin

Resistin, also called FIZZ3 (found in inflammatory zone) or ADSF (adipocyte secreted factor) was discovered in 2000 and was identified as a target of the PPARγ agonists, the thiazolidinediones (74,96,173). Indeed, in murine adipocytes, thiazolidinedione treatment led to a marked decrease in the expression of resistin together with an improvement of the insulin sensitivity (173). Those observations have led to the hypothesis that resistin is involved in the insulin-resistance associated with obesity in rodents. In agreement with such an hypothesis, resistin levels were shown to be increased with obesity in several rodent models (173). However, in humans, the relevance of resistin in the insulin resistance associated with obesity is questionable. Indeed the human protein exhibits only 59% homology with the murine resistin (99). Moreover, the production of resistin by human adipocytes is very low as compared with murine adipocytes. Finally, only weak relationship between resistin and

insulin sensitivity have been observed in human obese individuals (146). Recent observations suggest that the role of resistin in humans is more likely related to the inflammatory processes. In humans, resistin has been described to be predominantly expressed by the macrophages accumulated in the adipose tissue (45) and to induce the expression of pro-inflammatory cytokines such as tumor necrosis factor TNFα, interleukin IL-12 in monocytes (170) as well as IL-6 (21). Moreover, resistin has been shown to induce the expression of adhesion molecules by endothelial cells, supporting the hypothesis of a role of resistin in the inflammatory reaction (30). To note, the receptor for resistin is still not identified.

I.1.2.2 Adipokines and inflammation

Besides the adipokines involved in the regulation of the metabolic fluxes, adipocytes produce a wide range of factors involved in inflammatory reactions. Various pro-inflammatory markers are increased in the plasma of obese individuals and obesity is now considered as a chronic low-grade inflammatory state (138). This enhanced pro-inflammatory marker expression might originate from the adipose tissue itself. Indeed, adipocytes have been shown to express a wide range of chemokines, such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and IL-8, as well as the major pro-inflammatory cytokines TNFα and IL-6 that are both produced in higher amounts in obese individuals (61,138,175). The production of pro-inflammatory cytokines by adipose tissue is thought to be involved in the genesis of the insulin resistance associated with obesity (138). Indeed, TNFα interferes with the insulin signaling pathway via the activation of serine/threonine kinases such as PKC isoforms that phosphorylate the insulin receptor substrate (IRS) on serine residues (77,91,92). This phosphorylation reduces the association between IRS and the insulin receptor as well as the consecutive tyrosine phosphorylation of IRS, what leads to an inhibition of the insulin signaling. The production of TNFα by adipocytes appears to be a local production since no arterio-venous differences of TNFα plasma concentration through the adipose tissue have been shown (67,123). However a local inhibition of insulin signaling in the adipocyte by TNFα may participate to the systemic insulin resistance through the consecutive increased lipolysis leading to enhanced release of the free fatty acids in the circulation and then to lipotoxicity (55,154). IL-6, in contrast to TNFα, is released in the circulation by the adipose tissue. It has been reported that the plasma concentration of IL-6 found in veins originating from adipose tissue are higher than arterial plasma concentration and the adipose tissue is thought to be responsible for 1/3 of the plasma

IL-6 (194). IL-6 has been described to inhibit insulin signal transduction via the induction of SOCS3. In addition, IL-6 might inhibit insulin signaling directly in the liver and the muscle (166).

Recent approaches that target inflammatory cytokines production have been postulated to improve insulin-sensitivity in obese individuals. It has been demonstrated that in rodent models of obesity, TNFα gene knock-out led to the improvement of insulin sensitivity (179,180). However, in obese human subjects with type 2 diabetes, a 4-week systemic administration of TNFα neutralizing antibody failed to improve insulin sensitivity (130). To note, although anti-IL6 neutralizing antibodies have been developed, their effect on obesity-associated disorders has not yet been evaluated (61).

To summarize this first part, adipocytes are metabolically active cells that store and release free fatty acids as well as adipokines. Obesity is characterized by a modulation of both metabolic and secretory activities and those alterations are likely involved in the genesis of the systemic insulin-resistance and endothelial dysfunction leading to diabetes and CVD. Beside the adipocytes, the adipose tissue is composed of the so-called stroma-vascular fraction. Only few studies have addressed this cell fraction and its potential role in the growth and maintenance of adipose tissue. However, since adipocytes are metabolically active cells, it is evident that the local circulation in the tissue plays a major role in providing the metabolic substrates and clearing the adipose-derived products.

I.2. The vasculature of adipose tissue

Older histological studies performed in rodents have demonstrated that a dense network of capillaries is responsible for the adipose tissue supply and it has been postulated that each adipocyte is in close proximity to a blood capillary (152). The relative richness of blood supply in adipose tissue seems to be related to the size of the fat depot and increasing amount of data strongly suggest that the growth and maintenance of the fat store are dependent on a functional blood capillary network within the fat mass (43). Another vascular system is present in the adipose tissue: the lymphatic vascular system. It has been described for a long time that the rate of the lymphatic drainage in the adipose tissue is inversely related to its growth and that lymphatic vessels tend to be absent where fat deposition is more abundant (157). These observations suggest that in contrast to what is observed in the blood

capillary network, a reduced lymphatic microcirculation supports adipose tissue development. However, no studies have addressed this circulatory system in the adipose tissue. (68,157).

I.2.1. The blood vasculature of adipose tissue

Increasing amount of data shows that the blood vasculature plays an important role in the growth of the adipose tissue and more particularly in the process of adipogenesis as well as in the maintenance of the fat store.

I.2.1.1 Blood vasculature and adipogenesis

Mature adipocytes are differentiated cells that can not proliferate. New adipocytes, that are observed during the development of obesity, originate from the precursor cells, i.e. the preadipocytes that are present in the stroma of the adipose tissue. Preadipocytes differentiate into adipocytes through a process called adipogenesis. This process requires the growth arrest of proliferating preadipocytes and is initiated by activation of several transcription factors, such as the PPARγ and the CCAAT-enhancer binding proteins C/EBP α, β and δ and the sterol responsive element binding protein SREBP1c family that induce the expression of adipose specific genes (56). Adipogenesis in vitro is induced by insulin in combination with IGF-1 as well as by glucocorticoids and PPARγ ligands (153) whereas it is inhibited by cytokines such as TNFα, IL-1 and other pro-inflammatory molecules (131,140).
Several studies have suggested that endothelial cells play an important role in the control of the adipogenesis process. For example, it has been showed that endothelial cells promote the development of preadipocytes in a three-dimensional collagen gel co-culture system of mature adipocytes and endothelial cells (8). Furthermore, an “in vivo” study has demonstrated that the inhibition of blood vessel formation by an anti-angiogenic compound, e.g. an anti-VEGFR2 antibody, led to the strong reduction of the adipogenesis of preadipocytes injected subcutaneously in mice (58). This link between adipogenesis and endothelial cells is further stressed by the observation that secretion derived from adipose tissue-derived endothelial cells induced the differentiation of preadipocytes into mature adipocytes (83). Taken together, these data underline that endothelial cells modulate adipogenesis, i.e. the differentiation of preadipocytes into adipocytes.

I.2.1.2 Blood vasculature and adipose tissue growth in vivo

Recent studies have demonstrated that the vascular network plays an important role in the maintenance and growth of adipose tissue in rodents. Indeed, it has been shown that in ob/ob mice that normally become obese, treatment with angiogenesis inhibitors such as TNP-470 prevented the development of obesity (156). Similar results were obtained with other mice model of obesity (high-fat diet induced obesity in C57BL6 mice (25). The link between the development of the fat mass and the formation of blood vessels has further been demonstrated in nude mice. In these animals, the injection of preadipocytes in dorsal skin-fold chamber resulted in the formation of vascularized fat pads. Moreover, the injection of preadipocytes, transfected with an adenovirus encoding for PPARγ-dominant negative mutant receptor that blocks preadipocyte differentiation, led to the inhibition of the development of blood vessels at the site of injection, demonstrating that adipocytes directly regulate the development of their own vasculature (58). Finally, a recent study demonstrated that the use of an apoptosis-inducing agent specifically targeted to the adipose tissue vasculature, led to the reversion of obesity in mice (100). Interestingly, the regression of obesity was accompanied with an amelioration of the metabolic situation and an improvement of insulin sensitivity. Taken together, these studies show the key role of vasculature in the development of adipose tissue and make it an attractive target to control the fat mass growth. However, the responsiveness of the adipose tissue vasculature to anti-angiogenic compounds is quite surprising. Indeed, only immature vessels such as those in tumors seem to be reactive to such treatment and do regress in response to anti-angiogenic drugs. It is thus suggested that the blood vessels in the adipose tissue might be particular and more sensitive to remodeling than other adult vessels.(156).

To conclude, it appears that a functional blood capillary network is necessary for the differentiation of preadipocytes into adipocytes but also the maintenance of the fat store. Furthermore, the extension of the blood capillary network within the adipose tissue is indispensable for the growth of the tissue itself and appears to be regulated by the adipose tissue itself.

I.2.2. Mechanisms of neovascularization in the adipose tissue

The ability of the adipose tissue to promote neovascularisation is for a long time recognized. In particular, the omentum has been used clinically to promote revascularization and healing of ischemic organs and tissues, including the myocardium (171) (186). However, the processes involved in the formation of new blood vessels promoted by the adipose tissue are still to be defined. Classically, neovascularization in adult tissues can be achieved by two distinct processes: vasculogenesis and angiogenesis.

I.2.2.1. Vasculogenesis

Vasculogenesis was first defined as the process responsible for the formation of the primitive vascular network in the embryo from blood islands due to the aggregation of mesodermal cells in the yolk sac. These cells, called hemangioblasts, might be common precursor cells for the hematopoietic and endothelial cells (150). An important number of studies have demonstrated that vasculogenesis can participate to post-natal neovascularization since cells that exhibit properties of endothelial precursor cells (EPC) have been identified in adults (11). Those cells were characterized by their expression of various cell surface markers, such as the hematopoietic cell surface marker CD34, the vascular endothelial growth factor receptor VEGFR2 (or Flk-1) and the hematopoietic stem cell marker CD133 (or AC133). Such EPC have been first identified in the circulation although at very low amounts. The main source of EPC is considered to be localized in the bone marrow. The bone marrow in adults is constituted by various populations of cells that retain plasticity and the capacity to differentiate into distinct lineages. Those populations are characterized by different cell surface markers (TABLE 3). Hematopoietic stem cells, that express CD34 and CD45, give rise to blood and immune cells. Mesenchymal stem cells (MSC) express Stro-1 but not the stem cell marker CD34 and are characterized by their ability to give rise to bone, cartilage and adipose cells (93). Finally, bone marrow contains endothelial progenitor cells (EPC) that can be mobilized to the peripheral circulation, thus representing about 0,002% of total mononuclear cells (137), in response to factors including vascular endothelial growth factor A (VEGF A), granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage-colony stimulating factor (GM-CSF) as well as in response to ischemia and tissue damage. Their homing to damaged or ischemic tissues is not well understood yet but seems to involve the chemokine stroma-derived factor-1 (SDF-1) and its receptor CXCR4. The role of EPC in neovascularization is mainly mediated by two mechanisms. On one side EPC can promote

angiogenesis via the secretion of angiogenic factors such as VEGF A, IGF-1 and hepatocyte growth factor (HGF) (69,82,178) that induce proliferation and migration of the quiescent mature endothelial cells constituting the vascular network. EPC also produce several chemoattractants of monocytes and macrophages to increase neovascularization (174,178). On the other side, EPC can directly incorporate into the newly formed vessels and differentiate into endothelial cells (11). In vitro, under appropriate culture conditions, EPC are able to differentiate into mature endothelial cells. Indeed, in the presence of VEGF, basic fibroblast growth factor (bFGF) and IGF-1, EPC express markers for mature endothelial cells (145). The migration of EPC from the bone marrow to the peripheral circulation might be the starting point of EPC differentiation. Their homing and adhesion into the monolayer of surrounding endothelial cells might achieve the differentiation process of EPC into mature endothelial cells (78). Recent studies have demonstrated that EPC can be found in particular zones of the human vascular wall called vasculogenic zones (197), suggesting the presence of an inherent source of EPC in the blood vessels to support vasculogenesis in post-natal neovascularization.

Cell surface marker	Expression	Nature	Function	References
CD34	-Hematopoietic stem cells -Progenitor cells -Fibroblasts -Blood capillary endothelial cells	Cell surface glycoprotein (Cell-cell adhesion molecule)	Attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells	Krause, Blood 1996 Fina, Blood 1990
CD133 (AC133, Prominin-1)	-CD34(bright) hematopoietic stem cells -Progenitor cells -Endothelial progenitor cells	Five transmembrane domain cell surface glycoprotein	Not well defined (might be involved in cell polarity, migration and the interaction of stem cells with neighboring cells and/or the extracellular matrix)	Yin, Blood 1997 Peichev Blood 2000 Schmelkov, Int J Biochem Cell biol, 2005
ABCG2	-Side-population of hematopoietic stem cells	ATP-binding cassette transporter	Transport substances accros extra- and intra-cellular membrane	Scharenberg, Blood 2002 Sarkadi, Febs Lett 2004 Zhou, Nat Med 2001
VEGFR2	-Endothelial progenitor cells -Mature endothelial cells	Vascular endothelial growth factor receptor	Essential for neovascularization	Peichev Blood 2000
CD45 (leucocyte common antigen)	-Hematopoietic cells (except thombocytes and erythrocytes) -Hematopoietic stem cells	Cell surface glycoprotein with tyrosine phosphatase activity	Role in T and B cell activation and maturation Role in neutrophil activation	Dahlke, Leuk lymphoma, 2004

TABLE 3: Main cell surface markers expressed on bone-marrow progenitor cells

I.2.2.2. Angiogenesis

Angiogenesis is involved in the adult physiological (wound healing, female reproductive cycle) and pathophysiological (inflammatory- and hypoxia-driven angiogenesis) neovascularization. It is a process regulated by a fine balance between pro- and anti-angiogenic factors which involves the interaction between endothelial cells, basement membrane and periendothelial cells, i.e. smooth muscle cells or pericytes (41). The activation of endothelial cells by pro-angiogenic factors, in particular VEGF A, stimulates their proliferation and migration. Endothelial cells migration requires the secretion of matrix metalloproteinases (MMP) that degrade the basement membrane thus facilitating the migration of endothelial cells on this support (155). Degradation of the basement membrane also allows the release of growth factors such as VEGF A, bFGF and angiopoietin-1 (Ang-1) that are sequestered in the matrix network. This mechanism further supports the proliferation and migration of endothelial cells and maintains the angiogenic process. The stabilization of the newly formed vessels is achieved by the recruitment and proliferation of periendothelial cells, a process that requires the secretion of late angiogenic factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β) by the endothelial cells (9). Indeed, periendothelial cells inhibit endothelial cell proliferation and stimulate their differentiation and survival. Moreover, the inhibition of MMP by tissue inhibitor of metalloproteinases (TIMP) supports the formation of the basement membrane (FIGURE 6).

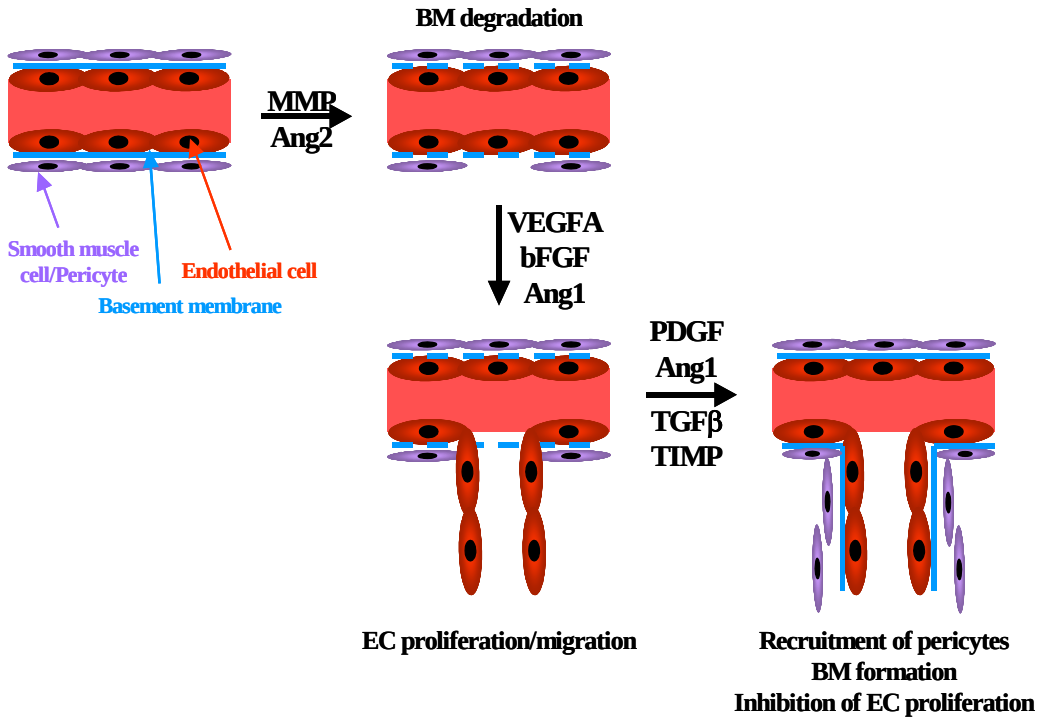

FIGURE 6: Mechanisms of angiogenesis

Angiogenesis is initiated by the activation of endothelial cells that secrete key factors such as matrix metalloproteinases (MMP) and angiopoietin 2 (Ang2) that degrade the basement membrane (BM), allowing the loosening of the vessel and the migration of endothelial cells. The BM degradation releases angiogenic factors that are sequestered in the matrix network (VEGF A, bFGF, Ang-1), thus enhancing endothelial cells proliferation and migration. Once a tube-like structure is formed, endothelial cells produce late angiogenic factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGFβ) and angiopoietin-1 (Ang1) that induce the recruitment and proliferation of pericytes, thus stabilizing the newly formed vessel. Finally, the inhibition of MMP by the tissue inhibitor of metalloproteinases (TIMP) completes the process.

I.2.2.3. Lymphangiogenesis

During embryogenesis, the lymphatic system appears after the development of blood vascular system when a subset of venous endothelial cells becomes committed to the lymphatic lineage. The homeodomain transcription factor Prox-1 appears to be essential in this commitment. Indeed, even in mature blood vascular endothelial cells, ectopic expression of Prox-1 leads to a down-regulation of blood vascular endothelial cell genes and induces the expression of lymphatic specific genes (90). The blood and lymphatic specific genes are indicated in the TABLE 4.

Blood marker	Lymphatic marker	Molecular type	Biological activity	Reference
CD34		Sialomucin glycoprotein	Attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells	Krause, Blood 1996 Fina Blood 1990
	Podoplanin	Glomerular podocyte mucoprotein	Maintaining lamellar permeability and shape of podocyte foot process in kidney	Breiteneder-Geleff, Am J Pathol 1997
	Prox-1	Transcription factor	Development and differentiation of lymphatic vessels	Wigle, Cell 1999
	LYVE-1	Hyaluronan receptor	Transports hyaluronan from extracellular matrix to lymph nodes	Panerji, J Cell Biol 1999
	VEGF R3	Receptor tyrosine kinase	Receptor for VEGF C and VEGF D	Kaipainen, J Exp Med 1993
	Desmoplakin	Endothelial adhering junction	Provide gaps through which macromolecules and cells pass	Schmelz, Differentiation 1994
	Reelin	Glycoprotein	Crucial for cortex lamination and cell positioning	Samama, Anat Rev A Discov Mol Cell Evol Biol 2005

TABLE 4: Main specific blood and lymphatic endothelial cell markers

The development of lymphatic vessels is essentially under the control of the lymphangiogenic factors VEGF C and VEGF D that are ligands for the receptor VEGFR3, principally expressed on lymphatic endothelial cells (LEC) (FIGURE 7) (84,184). VEGF C and VEGF D induce the proliferation, migration and survival of LEC in vitro. VEGF C and VEGF D do not have the same function during embryogenic lymphangiogenesis. Indeed, VEGF C is essential for proper lymphatic development since genetic ablation of this factor leads to failure in the migration and proliferation of LEC in mice (88). On the contrary, VEGF D seems to be dispensable for lymphangiogenesis in vivo (13). VEGFR3 is also required for the maintenance of newly formed lymphatic vessels during embryogenesis and first post-natal weeks since the inhibition of VEGFR3 signaling leads to the regression of lymphatic vessels due to LEC apoptosis (118). However, in adulthood, VEGFR3 signaling is not involved anymore in LEC survival but restricted to the development of new lymphatic vessels since the inhibition of VEGFR3 signaling does not affect established lymphatic vessels. In addition to VEGFR3, VEGF C and VEGF D can bind to neuropilin 2, a semaphorin receptor in the nervous system also expressed in LEC.

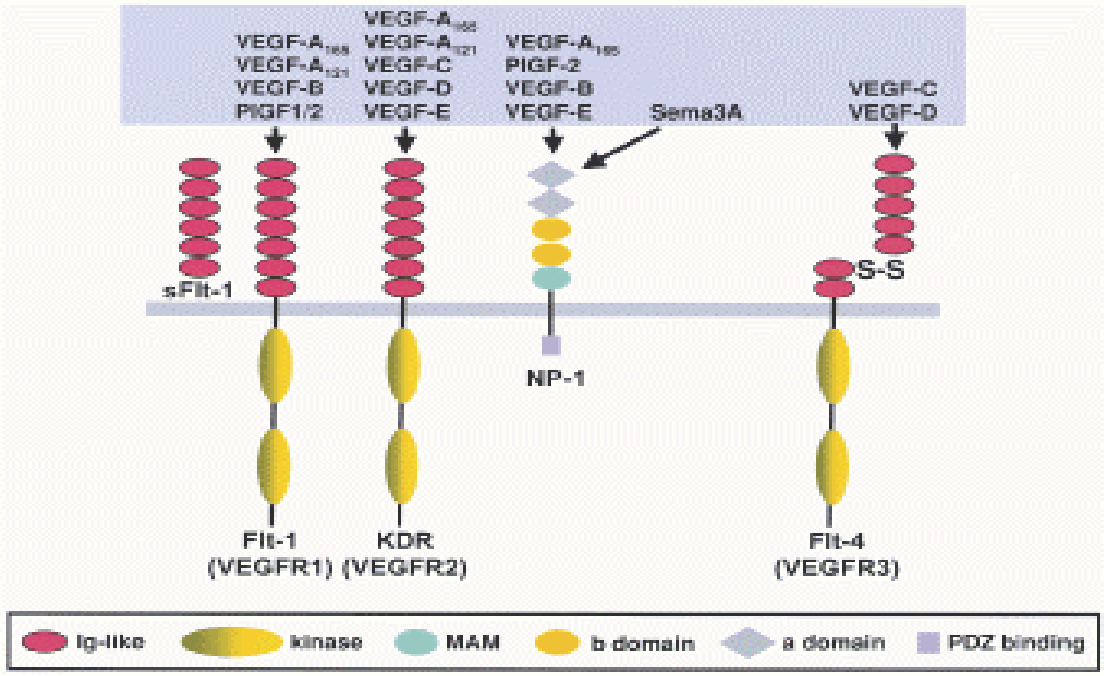

FIGURE 7: VEGF family, ligands and receptors (from (196))
Three receptors for the VEGF ligands family have been described: VEGFR1, VEGFR2 and VEGFR3 that have specific binding ability with the different ligands VEGF A, VEGF C, VEGF D, VEGF E and placental growth factor (PlGF). In addition, neuropilin (NP-1), a semaphorin receptor, is also able to bind ligands from the VEGF family.

Other factors have recently been described as lymphangiogenic molecules. Indeed, IL-7 specifically increases the expression of lymphatic markers in endothelial cells and induces the formation of lymphatic vessels in vivo (6). In addition, HGF has been identified as a lymphangiogenic factor in vitro and in vivo (86). Finally, angiopoietin-1 (Ang1) has also been recently described to promote lymphatic vessel formation since it induces LEC colony formation in vitro and lymphangiogenesis in the model of mouse cornea in vivo (125).

I.2.2.4. Adipose tissue and neovascularization

The vasculogenic and lymphangiogenic processes have not yet been studied in the adipose tissue. However, several studies have reported that adipocytes produce several pro-angiogenic factors such as VEGF A, MMP-2 and -9, bFGF, angiopoietin-like 4 and monobutyrin (23,117,198). Moreover, the classical metabolic adipokines, leptin and adiponectin, have been described to modulate angiogenesis. Indeed, leptin induces the proliferation and migration of human umbilical vein endothelial cells (HUVEC) in vitro. In addition, using in vivo models of chick chorioallantoic membrane (22) and rat cornea (168), it has been demonstrated that leptin induces the formation of new blood vessels. More recent studies have shown that leptin enhances vascular permeability (31) as well as nitric oxide (NO) production in endothelial cells (183). Moreover, the production of MMP as well as TGFβ and collagen type IV by endothelial cells is induced by leptin, what might participate to the stabilization of the newly formed vessels. Finally, it has been demonstrated that leptin stimulates the expression of angiogenic factors such as VEGF and bFGF, suggesting that it could indirectly participate to angiogenesis (31).

Adiponectin has been described to stimulate the organization of endothelial cells into capillary-like structures as well as the activation of nitric oxide synthase (eNOS) in HUVEC. Moreover, adiponectin stimulates blood vessel growth in vivo in mouse matrigel plug implantation and rabbit corneal models of angiogenesis (133). However, the role of adiponectin in angiogenesis is controversial. Indeed, other studies have demonstrated that adiponectin inhibits endothelial cell migration and proliferation and prevents in vivo the growth of new blood vessels in the chick chorioallantoic membrane and mouse corneal assays. The anti-angiogenic effect of adiponectin has been associated with a pro-apoptotic effect of adiponectin on the endothelial cells due to the activation of caspases (26).

The link between adipose tissue and the production of pro-angiogenic factors is further stressed by the observation that the plasma concentrations of various pro-angiogenic factors such as VEGF A, VEGFR2, angiopoietin-2, angiogenin and angiostatin are increased in obese

people (169). This increased pro-angiogenic capacities associated with obesity has been suggested to play a potential role in the association between obesity and risk to develop tumors and metastasis.

The first aim of the present work was to characterize the blood capillary network present in the human adipose tissue and to study the potential mechanisms involved in its remodeling with obesity, i.e. vasculogenesis and angiogenesis.
The second aim was to characterize the lymphatic endothelial cells from human adipose tissue and to study its trophic responsiveness to adipokines and lymphangiogenic factors.

II. MATERIALS AND METHODS

II.1 MATERIALS

Collagenase was purchased from Biochrom AG (Berlin, Germany).

Magnetic microbeads for positive selection of CD34+ cells and CD14+ cells were from CellSystems (Cellsystems, easysep, St Katharinen, Germany) and magnetic microbeads for positive selection of CD31+ cells were obtained from Dynal Biotech (Hamburg, Germany).

Endothelial cell culture media (basal medium (EBM), growth medium (ECGM) and growth medium for microvascular cells (ECGM MV)) were purchased from Promocell (Heidelberg, Germany).

FACS antibodies: the mouse IgG antibodies (FITC, perCP and PE) and the anti-human CD14 (PE), CD34 (perCP) and CD45 (PerCP) antibodies were from BD Bioscience (Heidelberg, Germany). The anti-CD31 antibody (FITC) was purchased from Cymbius Biotechnology (Hofheim, Germany), the anti-CD133 antibody (PE) from Miltenyi Biotech (Germany) and the anti-ABCG2 antibody (FITC) from Chemicon (Hofheim, Germany).

Immunohistochemistry antibodies:

First antibodies: rabbit anti-human von Willebrand factor (vWf) and mouse anti-human CD31 antibodies were from Dako (Glostrup, Denmark), rabbit anti-human VEGFR3 antibody from Chemicon (Temecula, USA), mouse monoclonal anti-human podoplanin and rabbit anti-human Prox1 antibody from Acris (Hiddenhausen, Germany), rabbit anti-human LYVE1 antibody from Upstate biotechnologies (Germany), mouse anti-human desmoplakin from Cymbius biotechnology (Hofheim, Germany), VEGFR2, VEGFR1, mouse anti-human CD34 from Chemicon (Hofheim, Germany).

Second antibodies: anti-rabbit antibody conjugated with Alexa Fluor 568 or anti–mouse antibody conjugated with Alexa Fluor 488 were purchased from Molecular probes (Göttingen, Germany).

Acetylated low density lipoprotein (Ac-LDL) was provided by Harbor Bioproducts (Norwood, MA).

Antibodies for Western blot analysis:

Rabbit anti-phospho serine 473 Akt and rabbit anti-phospho Erk1/2 antibodies as well as antibodies directed against the non phosphorylated forms of Akt and Erk1/2 (rabbit anti-Akt

and rabbit anti-Erk1/2 antibodies) were purchased from Cell signalling (New England Biolabs GmbH, Frankfurt am Main, Germany).
Second antibody coupled with peroxidase (anti-rabbit IgG) was provided by Calbiochem (Darmstadt, Germany).

RNA isolation kit (RNeasy kit) was provided by Qiagen (Hilden, Germany), ribogreen fluorometric assay by Molecular Probes and the thermoScript RT system by Life Technologies (Invitrogen, Carlsbad, CA).

Taqman primers:
Primers for human VEGF A (Hs 00173626_m1), VEGF C (Hs00153458_m1), VEGF D (Hs 00189521_m1), VEGFR1 (Hs00176573_m1), VEGFR2 (Hs00176676_m1), VEGFR3 (Hs00176607_1), leptin receptor (Hs00174497_m1), adiponectin receptor-1 (Hs00360422_m1), adiponectin receptor-2 (Hs00226105_m1), Prox-1 (Hs00160463_m1), LYVE1 (Hs00272659_m1), reelin (Hs00192449_m1), desmoplakin (Hs00189422_m1) and podoplanin (Hs00366764_m1) as well as Taqman universal PCR master mix were provided by Applied Biosystems (Applied Biosystems, Darmstadt, Germany).

Cell proliferation assay kit (Cell proliferation ELISA, BrdU colorimetric) was provided by Roche applied science (Mannheim, Germany).

Human fibronectin and growth factor reduced matrigel were purchased from BD Biosciences (Bedford, MA).

Human recombinant VEGF C, VEGF D, bFGF, leptin and adiponectin were provided by RnDsystems (Wiesbaden, Germany) and human recombinant VEGF A was from Preprotec (Cell concepts GmbH, Imkirch, Germany).

8–10 week old (18–22 g) athymic NMRI nude mice were purchased from Jackson Laboratory (Bar Harbor, Maine).

II.2. METHODS

II.2.1. Isolation of cells from human adipose tissue

II.2.1.1. Isolation of mature adipocytes

Mature adipocytes were isolated from subcutaneous adipose tissue obtained from individuals undergoing plastic surgery by lipoaspiration. The study was approved by the ethical committee of the University Hospital/Frankfurt am Main. After digestion of the adipose tissue in collagenase solution (300 U/mL in PBS, 2% bovine serum albumin; BSA) for 15 min, the resulting suspension was filtered (210-µm polyamid filter) and washed with phosphate buffer saline (PBS) containing 0.5%BSA. Mature adipocytes were used either for preparation of 24h-secretions or for gene expression experiments.

II.2.1.2. Isolation of cells from the stroma-vascular fraction

Cells from the stroma-vascular fraction (SVF) were isolated from adipose tissue obtained from individuals undergoing plastic surgery by lipoaspiration. The adipose tissue was digested using collagenase solution for 1h under constant shaking. Following removal of the floating mature adipocytes, the lower layer containing the SVF was centrifuged (200 g, 10 min) and the pellet resuspended in erythrocyte lysis buffer (155 mmol/L NH_4Cl; 5.7 mmol/L K_2HPO_4; 0.1 mmol/L EDTA, pH 7.3) for 10 min. After successive filtrations through 100, 70 and 40 µm sieves, the cells were suspended in PBS/2% fetal calf serum (FCS).

The SVF (maximal concentration of 20 million cells/mL) suspended in PBS/2%FCS was incubated (24°C, 15 min) with the positive selection cocktail to isolate CD34+ cells (100µL/mL). Following the additional incubation with magnetic nanoparticles (50 µL/mL) for 10 min, cells were collected by successive magnetic sorting steps. The CD34+ cells were suspended in PBS/0.1% BSA and the double positive CD34+/CD31+ cells were isolated using CD31-coupled magnetic microbeads (100µL/mL). After incubation (4°C, 20 min), the cell suspension containing the beads, diluted in 10mL PBS/0.1%BSA, was exposed to the magnet for 1 min. The magnetic bead-free fraction, CD34+/CD31- cells, was collected and centrifuged (200 g, 10 min). Then, the CD34+/CD31- cells were either put in culture or used for in vivo experiments whereas the CD34+/CD31+ cells were suspended in endothelial cell growth medium for microvascular cells(ECGM MV) and put in culture.

The CD34 negative (CD34-) cells, collected after each sorting step, were centrifuged (200 g, 10 min) and suspended in PBS/2%FCS. Cells were incubated (24°C, 15 min) with the positive selection cocktail to isolate CD14+ cells (100µL/mL). Following the incubation with magnetic nanoparticles (50 µL/mL) for additional 10 min, cells were collected by successive magnetic sorting steps. CD34-/CD14+ cells were used for other experiments performed in our group.

The CD34-/CD14- cells, collected after each sorting step were centrifuged (200 g, 10 min) and suspended in PBS/0.1%BSA. CD34-/CD14-/CD31+ cells were isolated using CD31-coupled magnetic microbeads (50µL/mL). After incubation (4°C, 20 min), the cell suspension containing the beads, suspended in 10mL PBS/0.1%BSA, was exposed to the magnet for 1 min. The magnetic bead-free fraction, CD34-/CD14-/CD31- cells was collected, centrifuged (200 g, 10 min) and used for in vivo experiments. The CD34-/CD14-/CD31+ cells were suspended in endothelial cell growth medium (ECGM) and put in culture.

II.2.2. Isolation of bone marrow mononuclear cells

Bone marrow aspirate (50mL) was obtained from healthy individuals and bone marrow-derived mononuclear cells (BM-MNC) were isolated by density gradient centrifugation. After washing steps, cells were suspended in 10mL X-vivo 10 medium (Cambrex, East Rutherford, New Jersey).

II.2.3. Flow cytometry analysis

FACS analysis was performed using freshly harvested SVF or CD34+/CD31- cells from human adipose tissue. At least 50,000 cells (in 100µL PBS/0.5% BSA/2mmol/L ethylenediaminetetraacetic acid (EDTA)) were incubated with fluorescent labeled monoclonal antibodies or the respective isotype control (1/20 diluted, 4°C, 30 min). After washing steps in PBS/0.5%BSA/2mmol/L EDTA, the labeled cells were suspended in 500µL PBS and analyzed by flow cytometry using a FACSCalibur flow cytometer and the CellQuest Pro software (BD Biosciences, Heidelberg, Germany).

II.2.4 Gene expression analysis

II.2.4.1. RNA isolation and reverse transcriptase reaction

RNA was extracted from human mature adipocytes, CD34+/CD31- cells, CD34+/CD31+ cells, CD34-/CD14-/CD31+ cells, human umbilical vein endothelial cells as well as blood monocytes isolated from buffy coats obtained from healthy donors (Transfusion Center of Frankfurt Hospital) using a Biocoll gradient solution (density 1.077). Total RNA was isolated using a RNeasy kit and its concentration determined using a Ribogreen fluorometric assay. RNA (1 µg) was reverse transcribed using the thermoScript RT system under the following conditions: 25°C for 10 min, 55°C for 50 min and 85°C for 5 min. Reactions were also performed without reverse transcriptase to provide a control for contamination of samples with genomic DNA. The products of reverse transcriptase reactions were stored at –80°C for later utilization.

II.2.4.2. Real time PCR analysis

Taqman probes (assays-on-demand) for VEGF A (Hs 00173626_m1), VEGF C (Hs 00153458_m1), VEGF D (Hs 00189521_m1), VEGFR1 (Hs00176573_m1), VEGFR2 (Hs00176676_m1), VEGFR3 (Hs00176607_1), leptin receptor (Hs00174497_m1), adiponectin receptor-1 (Hs00360422_m1), adiponectin receptor-2 (Hs00226105_m1), Prox-1 (Hs00160463_m1), LYVE1 (Hs00272659_m1), reelin (Hs00192449_m1), desmoplakin (Hs00189422_m1) and podoplanin (Hs 00366764_m1) were used to perform real time PCR analysis on RNA derived from mature adipocytes, progenitor cells as well as blood and lymphatic endothelial cells (BEC and LEC, respectively) from human adipose tissue. The PCR mixtures were prepared in TaqMan Universal PCR Master Mix. All amplification reactions were performed in duplicate from 20ng cDNA using the Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA) using the following conditions: 50°C for 2min, 95°C for 10min, followed by 40 cycles at 95°C for 15s and 60°C for 1min. Results were analyzed with Stratagene Mx4000 software and all values were normalized to the levels of the ribosomal RNA (18S).

II.2.5 Protein analysis

II.2.5.1. Immunohistochemistry analysis

Immunohistochemistry analyses were performed on freshly harvested human subcutaneous adipose tissue as well as on cultured CD34+/CD31- cells, CD34+/CD31+ cells and CD34-/CD14-/CD31+ cells. After 20 min fixation in PBS/4%paraformaldehyde PFA, tissue or cells were incubated for 1h at room temperature in the blocking solution (PBS/2%BSA) followed by a 1h incubation with the relevant primary antibodies diluted in PBS/2%BSA or PBS/2%BSA containing 0,1%Triton for membrane permeabilization depending on the localization of the tested marker. After washing 3 times in PBS/0.2%Tween, tissue or cells were incubated for 1h with the corresponding fluorescence-labeled second antibodies diluted in PBS/2%BSA. After washing 3 times with PBS/0.2%Tween, tissue or cells were incubated 10 minutes with 0.4µg/mL DAPI (4',6-diamino-2-phenylindole, dihydrochloride) and washed in PBS. Cells were directly observed under fluorescence microscopy whereas tissue was first included between two mounting slides.

II.2.5.2. Acetylated-LDL uptake assay

Acetylated-LDL uptake assay was performed on the isolated CD34+/CD31+ cells and CD34-/CD14-/CD31+ cells cultured on fibronectin in ECGM MV and ECGM, respectively. At confluence, 10µg/mL of ac-LDL (Harbor Bioproducts, Norwood, MA) was added to the cells and incubated 4h at 37°C. The cells were then washed 3 times in ECGM MV or ECGM and the fluorescence was directly visualized by microscopy using a standard rhodamine excitation/emission filter.

II.2.5.3. Western-blot analysis

Experiments were performed on CD34+/CD31+ cells and CD34-/CD14-/CD31+ cells cultured on 12-well plates coated with fibronectin in ECGM MV and ECGM, respectively. At confluence, cells were cultured in serum-free medium (EBM/0.1%BSA) for 24h. Then, cells were incubated with 2 ng/mL leptin or 2 ng/mL adiponectin for 2, 5 and 15 min. Cells were washed in cold PBS and incubated with 50µL per well of lysis buffer for 15min. Cells were scraped, collected in 1.5mL tubes and centrifuged 10 min at 1000rpm at 4°C. The protein

concentration in the supernatant was measured using the Bradford method. 20µg of proteins were loaded in 8% polyacrylamide gel. After electrophoresis and transfer, the nitrocellulose membrane was rinsed in water and colored with Ponceau solution to verify equal loading of the lanes. Then, it was rinsed in washing buffer and incubated 1 hour in the blocking buffer containing 3%BSA at room temperature. The nitrocellulose membrane was then incubated overnight with the first antibody (rabbit anti-human phosphorylated Erk1/2 dilution 1/500 in the blocking buffer or rabbit anti-phosphorylated Akt dilution 1/1000) at 4°C. After 5 washes (within 50 min), the nitrocellulose was again incubated with the blocking buffer during 40 min at room temperature followed by an incubation of 1 hour at room temperature with the second antibody coupled with peroxidase (anti-rabbit IgG, dilution 1/10000 in the blocking buffer). Then, it was rinsed 5 times (within 1h) in washing buffer. The revelation was then performed by ECL.

Then, nitrocellulose membrane was washed several times in washing buffer for 1h and the amount of total Erk1/2 and Akt was assessed after stripping. Nitrocellulose membrane was incubated 30min in stripping buffer (2%SDS/62.5µL/mL Tris/Cl pH 6,8/70µL/10mLβ-mercaptoethanol in bidest water) at 50°C and washed for 1h in washing buffer. Antibody reaction (Erk1/2 and Akt) was performed using the protocol described above.

II.2.6. Cell culture

II.2.6.1. Proliferation assay (BrdU incorporation)

Proliferation assays were performed on CD34+/CD31+ cells and CD34-/CD14-/CD31+ cells. Cells were plated in 96-well plates coated with fibronectin at the density of 5000 cells/well/100µL of EBM/2%FCS for one night at 37°C. Then, cells were treated with recombinant VEGF A, VEGF C, VEGF D, bFGF, leptin and adiponectin at increasing concentrations (0.2, 2 or 20 ng/mL for VEGF A, bFGF, leptin and adiponectin and 20, 200, 1000 ng/mL for VEGF C and VEGF D) or with 24h-conditioned medium from mature adipocytes and progenitor cells from human subcutaneous adipose tissue for 29h at 37°C. BrdU (10µM/well) was added to the culture medium for additional 3h. Cells were washed 3 times in washing buffer (Roche applied science) and fixed with cell fix solution (Roche applied science). BrdU incorporation was measured following the BrdU proliferation assay kit protocol (Roche applied science).

II.2.6.2. Tube-like formation assay (matrigel)

Tube-like formation ability was assessed in CD34+/CD31+ cells and CD34-/CD14-/CD31+ cells. 200µL/well growth factor reduced matrigel was poured in 48-well plates and polymerized for 30min at 37°C. 15 000 cells/well were then plated on the polymerized matrigel and treated with VEGF A (0,2ng/mL), VEGF C (20ng/mL), VEGF D (20ng/mL), leptin (2ng/mL) and adiponectin (2ng/mL) diluted in EBM/0.1%BSA for 24h at 37°C. The branching formation was observed by phase microscopy and the number of branching points as well as the length of cytoplasmic extensions was measured using LUCIA image software.

II.2.6.3. Endothelial cell differentiation assay

This assay was performed on the CD34+/CD31- cells from human subcutaneous adipose tissue. Cells were plated at high density (20 000 cells/cm^2) on fibronectin-coated 48-well plates for 1 to 10 days in different culture media (EBM/10%FCS, ECGM or ECGM containing VEGF (0.5ng/mL) and IGF (20ng/mL)). Cells were fixed with PBS/4%PFA, blocked with PBS/2%BSA (1 h, 24°C) and incubated with the primary antibody (anti-CD31, 1/10 and anti-vWf, 1/50, 1h, 24°C). After washing steps and 1 h-incubation time with the corresponding secondary antibody (1/200 diluted), DAPI (4',6-diamino-2-phenylindole, dihydrochloride) (Vectashield, Vector Laboratories, Burlingame, USA) staining was performed and the cells observed under fluorescence microscopy.

II.2.6.4. Preparation of adipose tissue cells-derived conditioned medium

CD34+/CD31- cells isolated from human subcutaneous adipose tissue were cultured in EBM/10%FCS in 10cm^2 cultured dishes. At confluence, cells were rinsed with PBS and cultured in 2mL endothelial cell basal medium (EBM)/0.1%BSA for 24h. The 24h-conditioned medium was collected, centrifuged and the supernatant was frozen at -80°C.
Mature adipocytes (~400 000) were included in fibrin gels (1.5 mg fibrinogen/mL EBM) supplemented with 25 units/mL α-thrombin) and cultured in EBM/0.1% BSA. Control gels were prepared without adipocytes. After 24 h, the adipocytes-conditioned media were collected and frozen at -80°C.

II.2.7. In vivo studies

II.2.7.1. Ligature of deep and superficial femoral arteries and laser Doppler imaging

The neovascularization capacity of human adipose tissue-derived CD34+/CD31- cells was investigated in a murine model of hindlimb ischemia, using 8–10 week old (18–22 g) athymic NMRI nude mice (The Jackson Laboratory, Bar Harbor, Maine). The proximal portion of the femoral artery including the superficial and the deep branch and the distal portion of the saphenous artery were ligated with 7.0 silk suture. All arterial branches between the ligation were obliterated using an electrical coagulator. The overlying skin was closed using three surgical staples. After 24 hours, 200 µL of PBS/2%FCS containing 500 000 freshly isolated human adipose tissue-derived CD34+/CD31- cells were injected intravenously. Identical number of freshly harvested bone marrow mononuclear cells (BM-MNC) and human adipose tissue-derived CD34-/CD14-/CD31- cells were used as positive and negative controls, respectively.

After two weeks, ischemic (right)/ normal (left) limb blood flow ratio was measured using a laser Doppler blood flow meter (Laser Doppler Perfusion Imager System, moorLDI™-Mark 2, Moor Instruments, Wilmington, Delaware). Before initiating scanning, mice were placed on a heating plate at 37°C. After recording laser Doppler color images twice, the average perfusion of the ischemic and non-ischemic limbs was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature, perfusion is expressed as the ratio of the ischemic to the non-ischemic hindlimb.

II.2.7.2. Immunohistochemistry

Tissue vascularization was determined in 5-µm frozen sections of the adductor and semi-membranous muscles from the ischemic and the non-ischemic limb. Endothelial cells were stained with FITC-labeled monoclonal antibody directed against CD146 (Chemicon, Temecula, California). Capillary density is expressed as the number of capillaries/myocyte relative to the individual non-ischemic limb. Incorporation of injected human cells was verified by co-staining for HLA class I-APC (BD Pharmingen, Heidelberg, Germany) and CD146-FITC.

II.2.8. Statistical analysis

Values are given as mean ± sem of *n* independent experiments. Comparisons between groups were analyzed using *t* test, when appropriate (two-sided) or ANOVA for experiments with more than two subgroups followed by Kruskal analysis and the non-parametric Mann-Whitney test. Post-hoc range tests and pair-wise multiple comparisons were performed with the *t* test (two-sided) with Bonferroni adjustment *P* values <0.05 were considered statistically significant.

III. RESULTS

III.1. Characterization of the vascular network in the human adipose tissue

III.1.1 The stroma-vascular fraction of the human adipose tissue

III.1.1.1 Immunohistochemistry analyses

Fluorescence immunohistochemistry was performed using human subcutaneous adipose tissue and antibodies directed against the endothelial cell markers, CD31 and vWf. As shown in **figure 8A** (upper panels), numerous capillaries stained with CD31 and vWF antibodies surrounded almost each adipocyte. Further analysis of the CD31 and vWF-positive structures showed the presence of blind-ended capillaries, which are a hallmark of the lymphatic vascular system (**Figure 8A**, lower panels). Thus, blood and lymphatic capillaries co-exist within the fat mass and both express the endothelial cell markers CD31 and vWF. Other cell structures, namely cells rosettes, were also found in the stroma of the tissue to be positive for CD31 (**figure 8A**, lower left panel). Since CD31 is also expressed by monocytes/macrophages, it is highly probable that these structures in fact represent clusters of monocytes/macrophages (**figure 8A**).

Vascular structure in the human adipose tissue was further studied by co-staining with antibodies directed against endothelial cell-specific markers such as CD34, the expression of which is restricted to blood capillary endothelial cells (BEC), as well as LYVE-1, VEGFR3, desmoplakin, Prox-1 and podoplanin, that are expressed by lymphatic endothelial cells (LEC). The CD34 staining allowed us to identify two distinct cell structures (**Figure 8B**, upper panels). Some capillaries positive for vWF were also stained with the antibody directed against CD34 (**Figure 8B**, upper left panel). Moreover, sparse cells within the stroma distinct from capillary structures were identified with the anti-CD34 antibody (**Figure 8B**, upper right panel). Although associated with strong non-specific staining of the adipocytes, probably due to interactions with lipids (data not shown), the co-staining using VEGFR3 and podoplanin antibodies identified capillaries that were positive for both markers (**figure 8B**, lower left panel). The LYVE-1 antibody identified cells, localized at the periphery of larger vessels (**Figure 8B**, lower right panel). Desmoplakin as well as Prox-1 antibodies did not allow the identification of specific structures.

Thus a dense CD31- and vWF-positive capillary structure is present within the human adipose tissue. This structure is heterogeneous and is composed of blood capillaries positive for CD34 and lymphatic capillaries positive for VEGFR3 and podoplanin. In addition to the vascular components, distinct cell types were also identified in the stroma: rosettes of cells positive for CD31, probably monocyte/macrophage groups as well as single CD34 positive cells which will be further characterized in this study.

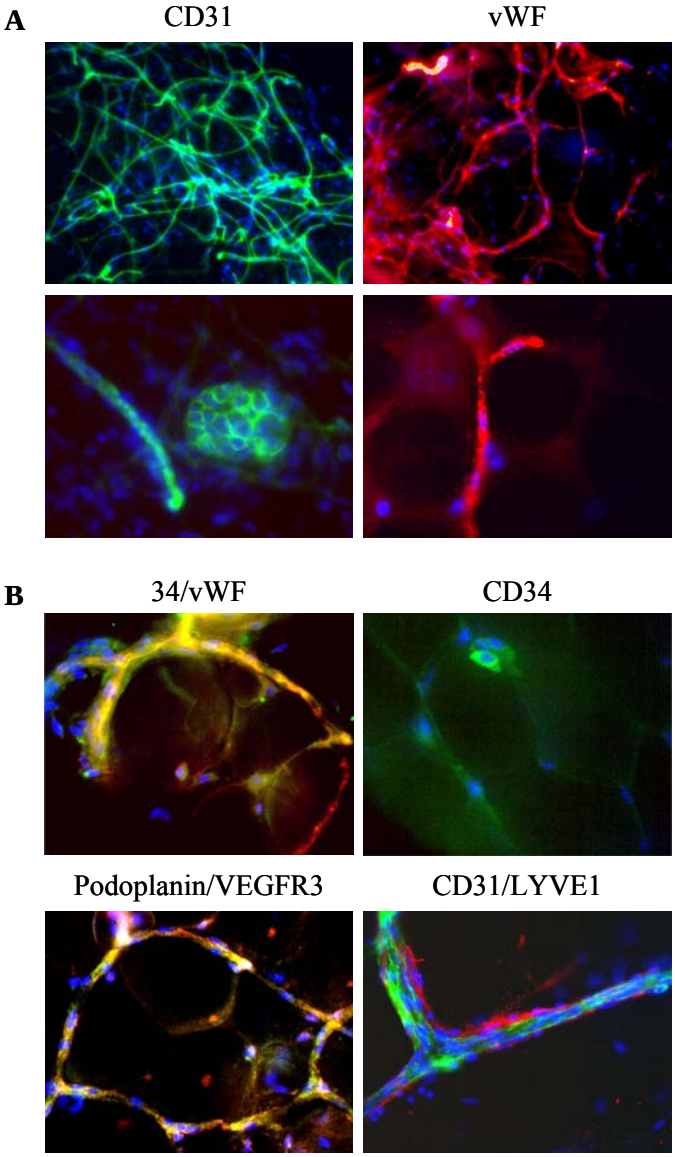

Figure 8: Characterization of the vascular network from human subcutaneous adipose tissue

Freshly harvested adipose tissue was stained with antibodies directed against the common endothelial cell markers CD31 (green) and von Willebrand factor (vWf, red) (A) as well as specific blood (CD34, green) and lymphatic endothelial (podoplanin (green), VEGFR3 (red) and LYVE-1 (red)) cells markers (B). Representative photomicrographs obtained by fluorescent microscopy are shown (n=6).

III.1.1.2 Flow cytometry analyses

The SVF of human subcutaneous adipose tissue was separated from the mature adipocytes after collagenase digestion. Cells were then analyzed by multiparameter flow cytometry and identified by their size (FSC, Forward Scatter) and granularity (SSC, side scatter), as well as their expression of cell surface markers assessed through the use of a combination of antibodies coupled to distinct fluorochromes. We used antibodies against CD31 coupled to FITC in combination with anti-CD34 antibodies coupled to perCP and the monocyte/macrophage cell marker CD14 coupled to PE. On the basis of differences in granularity and marker expression, distinct cellular populations that express CD31 could be identified. As shown in **figure 9A**, approximately 1/3 of the cells of the SVF of the human adipose tissue expressed CD31 (upper right panel of the dot blot analysis).

When selecting the cells that expressed CD34 in the SVF (CD34+ gating), 2 distinct cellular populations were apparent and differing in the expression of CD31 (**figure 9B**): one population co-expressed both CD34 and CD31 (upper right panel of the dot blot analysis, 11.1±1.4% of the total SVF, n=19), whereas the other cell type expressed only CD34 (upper left panel of the dot blot analysis, 16.6±2.3% of the total SVF, n=19).

When selecting the cells expressing CD14 in the SVF (CD14+ gating), one single CD14+ cell population that co-expressed CD31 was identified (**figure 9C,** upper right panel, 10.2±1.7% of the total SVF, n=19).

Finally, when selecting the cells negative for CD34 and CD14 in the SVF, distinct cell populations could be shown depending on their expression of CD31. Indeed, CD31 positive but CD34 and CD14 negative cells were present (**Figure 9D,** upper right panel, 3±0.3 % of the SVF, n=19) together with cells negative for the three markers (**Figure 9D**, upper left panel).

Thus, the results obtained by flow cytometry analyses together with the immunohistochemical data allowed us to identify distinct cellular populations present in the SVF of human adipose tissue. The three distinct cellular populations identified were endothelial cells: both BEC (CD34+/CD14-/CD31+) and LEC (CD34-/CD14-/CD31+) as well as a monocyte/macrophage population (CD34-/CD14+/CD31+). In addition, a population of cells that express only the stem/blood capillary endothelial cell marker CD34 (CD34+/CD31-/CD14-) as well as cells negative for the three markers (CD34-/CD31-/CD14-) were present in the stroma of human adipose tissue.

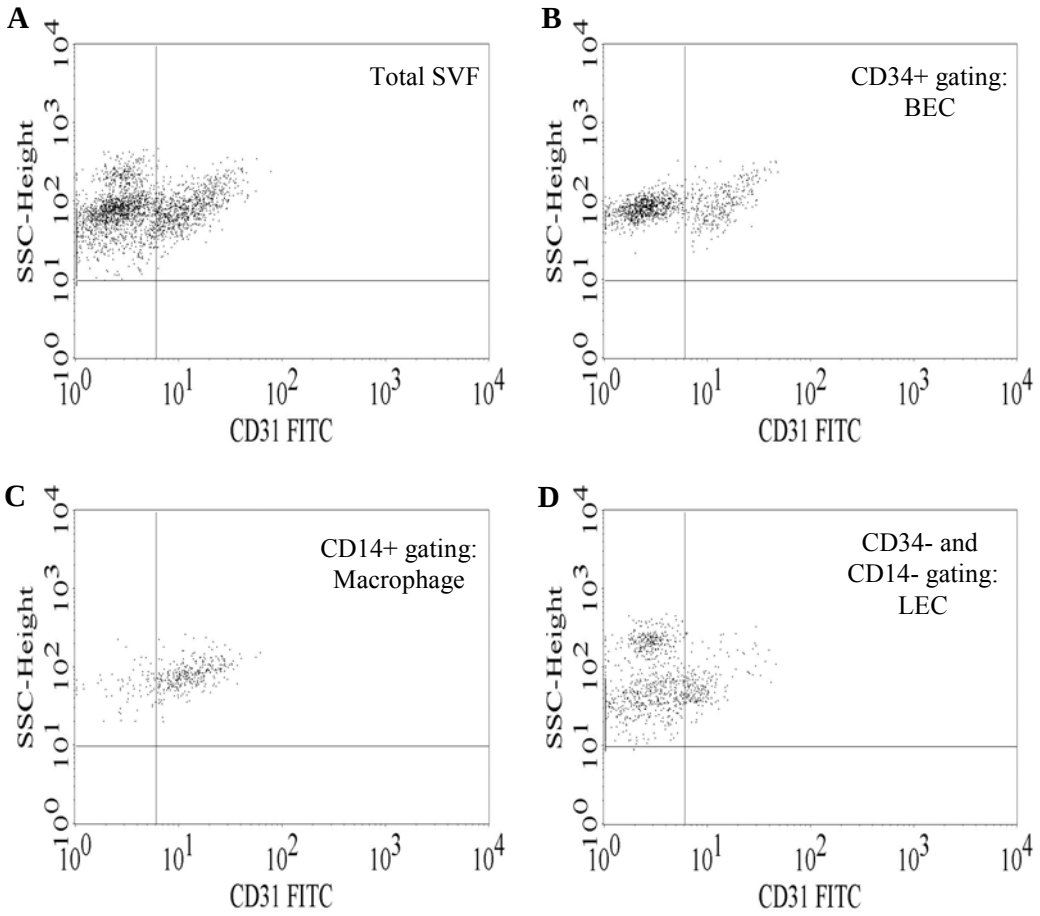

Figure 9: Characterization of the stroma-vascular fraction (SVF) of human adipose tissue *Freshly harvested cells from the SVF was triple-labeled with fluorescent antibodies directed against the endothelial cell marker CD31 (CD31-FITC), the stem/blood capillary endothelial cell marker CD34 (CD34-PerCP) as well as the macrophage marker CD14 (CD14-PE) and analyzed by flow cytometry. The dot plots represent the CD31-FITC fluorescence function of the side scatter (SSC) in the whole SVF (A), in the CD34-positive cell population (CD34+ gating) (B), in the CD14-positive cell population (CD14+ gating) (C) as well as in the CD34- and CD14-negative cell population (CD34- and CD14- gating) (D). Original dot plots from material obtained from one individual are shown.*

III.1.2 Characterization of the BEC and the LEC isolated from the human adipose tissue

To further characterize the BEC and the LEC from the human adipose tissue, we developed an approach based on immunoselection of the cells from the SVF of human adipose tissue using magnetic microbeads coupled to the antibody combination, CD34, CD31 and CD14. As depicted in **figure 10**, the distinct cellular populations were isolated by the alternative steps of cell depletion and selection.

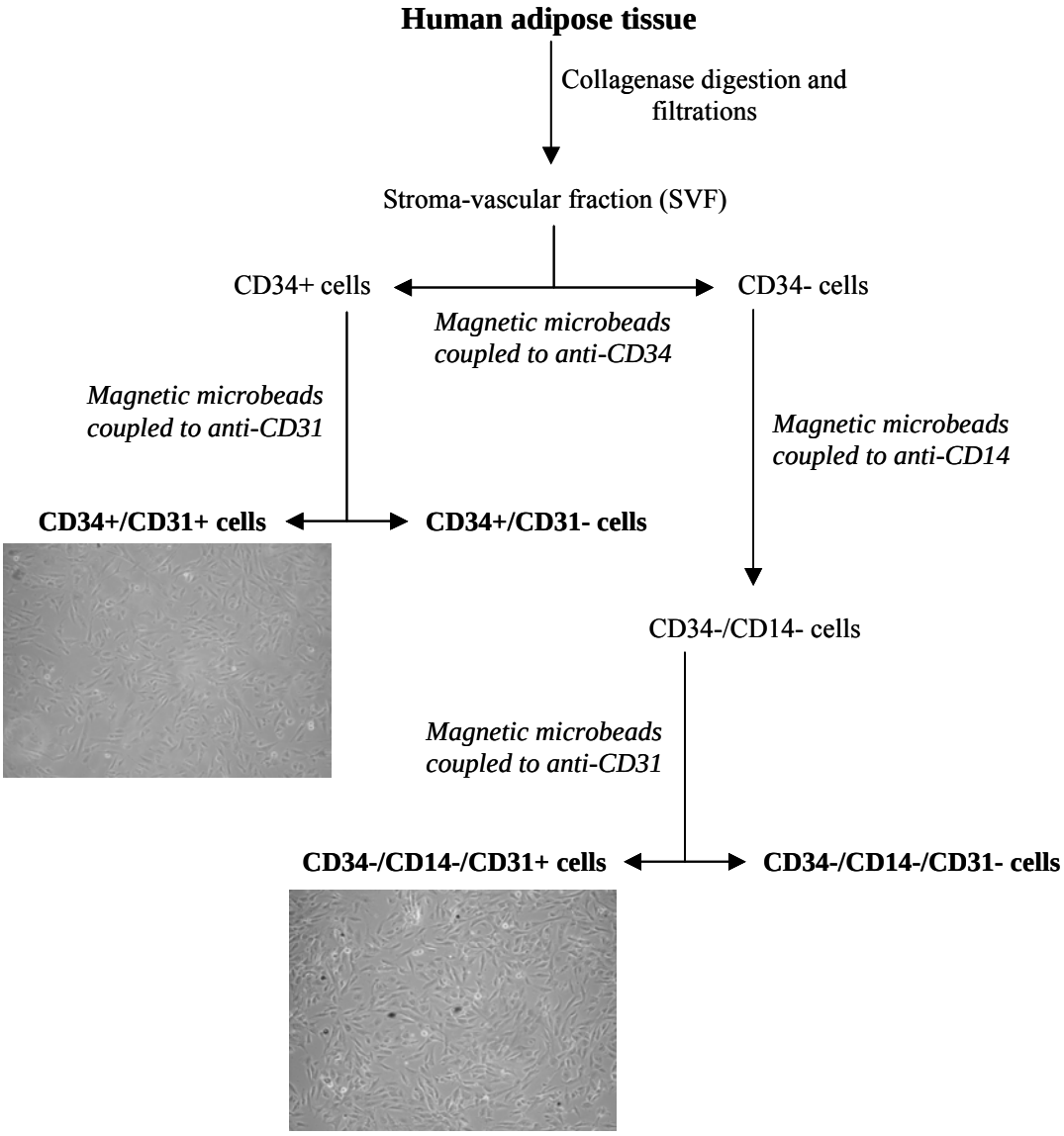

Figure 10: Protocol for the isolation of blood capillary (CD34+/CD31+ cells) and lymphatic capillary (CD34-/CD14-/CD31+ cells) endothelial cells from the SVF of human adipose tissue

Human adipose tissue was digested with collagenase, centrifuged and filtrated in order to obtain the cells from the SVF, as described in the method section. The CD34+/CD31+ cells were isolated by positive selection of the CD34+ cells followed by a positive selection of CD31+ cells. The CD34-/CD14-/CD31+ cells were isolated from the CD34-negative population by a positive selection of CD31+ cells after a depletion of CD14+ cells.

III.1.2.1 Common endothelial cell features

The expression of the endothelial genes VEGFR1, VEGFR2, vWf and Notch4 was assessed by real time-PCR in freshly isolated CD34+/CD31+ cells and CD34-/CD14-/CD31+ cells and compared with levels detected in human umbilical vein endothelial cells (HUVEC) and blood mononuclear cells (MNC). As depicted in **figure 11A**, VEGFR1, VEGFR2, vWf and Notch4 were detected in BEC and LEC at levels equivalent to those measured in HUVEC. The expression of VEGFR1, VEGFR2, vWf and Notch4 was not detected in MNC.

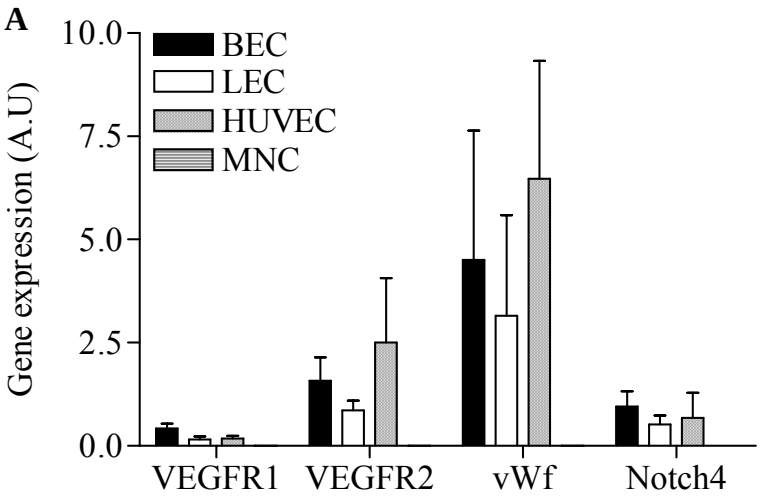

Figure 11A: Phenotypic characterization of blood capillary endothelial cells (BEC) and lymphatic capillary endothelial cells (LEC) from the SVF of human adipose tissue
Total RNA of freshly isolated BEC and LEC was reverse transcribed and used to perform real time-polymerase chain reaction using the primers for the common endothelial cell markers VEGFR1, VEGFR2, vWf and Notch4. The results obtained were normalized to the 18S expression. Results are the mean ± sem of at least 6 distinct adipose tissues.

Freshly isolated BEC and LEC were plated in low-serum endothelial cell growth medium and immunocytochemistry analyses were performed using the using the endothelial cell markers CD31 and vWf. In addition, the ability of the cells to accumulate fluorescent acetylated-low density lipoprotein (Ac-LDL), was addressed. Punctuate cytoplasmic expression of vWf was detected in both cell types (**figure 11B**, upper panels) as was a clear staining for CD31 at the cell membranes. Moreover, both BEC and LEC accumulated Ac-LDL and positive staining was detected at the periphery of the nuclei (**figure 11B,** lower panels).

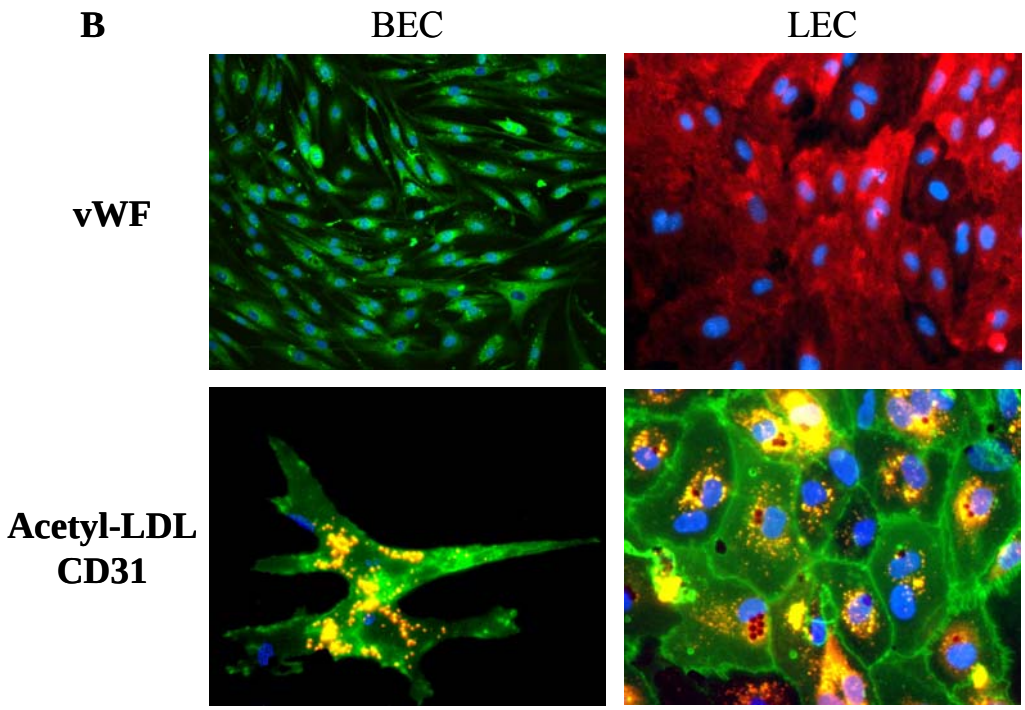

Figure 11B: Phenotypic characterization of blood capillary endothelial cells (BEC) and lymphatic capillary endothelial cells (LEC) from the SVF of human adipose tissue
(B) Immunohistochemistry using an antibody directed against vWf (green for BEC and red for LEC) and CD31 (green) as well as acetylated-LDL (red) uptake assay were performed on cultured BEC and LEC and analyzed by fluorescence microscopy. Photomicrographs representative from 4 experiments are shown.

III.1.2.2 Specific endothelial cell features

The expression of the specific lymphatic endothelial cell genes LYVE1, Prox1, desmoplakin, podoplanin, reelin and VEGFR3 was assessed in freshly isolated BEC and LEC by real time RT-PCR. As depicted in **figure 12A**, the lymphatic markers reelin and podoplanin were exclusively expressed in LEC whereas desmoplakin, LYVE-1 and VEGFR-3 transcripts were present in both cell types. Prox-1 expression could not be detected in either cell type. Freshly isolated BEC and LEC were plated in low-serum endothelial cell growth medium and immunocytochemistry analyses were performed using CD34, VEGFR3 and podoplanin. As expected, the expression of CD34 was detected exclusively in BEC. The staining obtained with the antibodies directed against VEGFR3 and podoplanin was markedly stronger in LEC as compared to BEC (**figure 12B**).

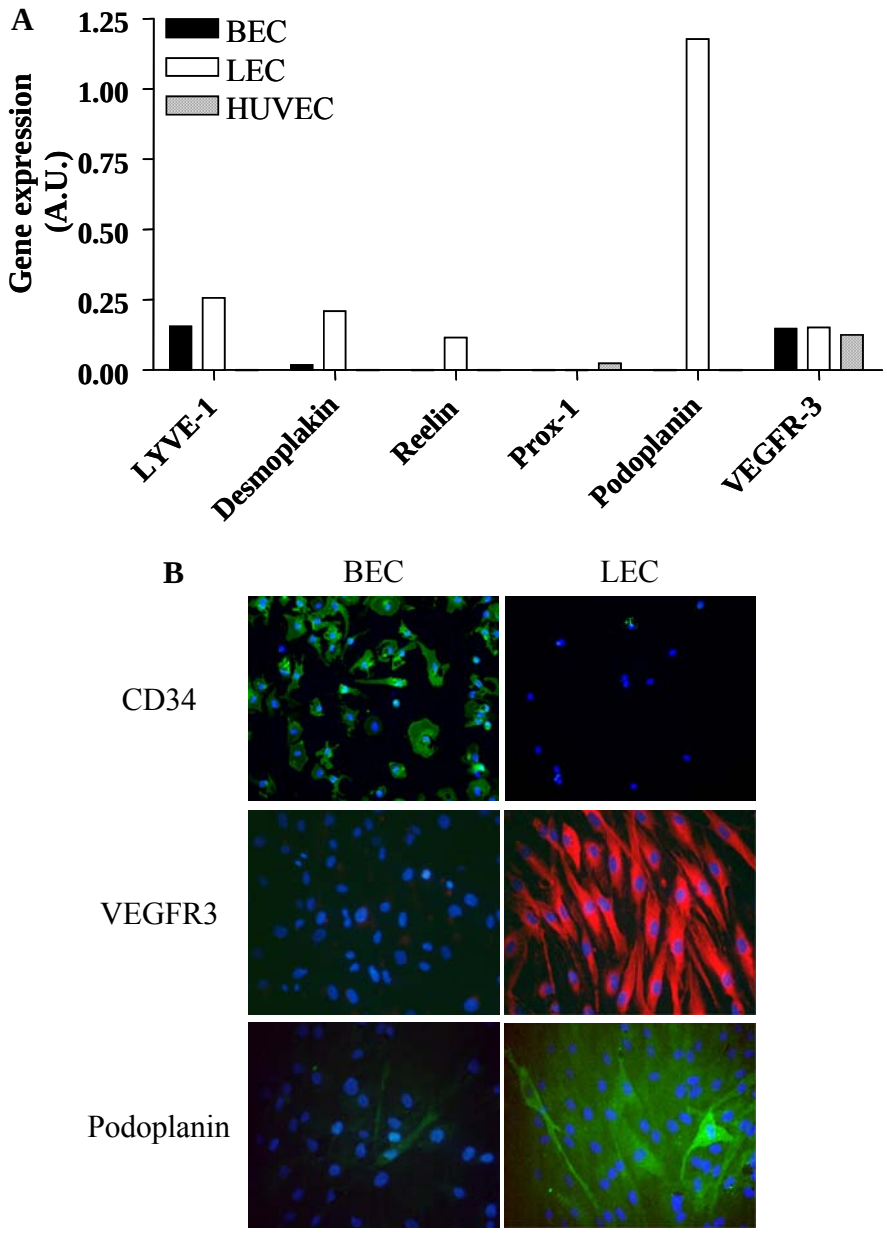

Figure 12: Phenotypic characterization of BEC and LEC from the SVF of human adipose tissue

(A) Total RNA of freshly isolated BEC and LEC was reverse transcribed and used to perform real time-polymerase chain reaction using the primers for the lymphatic endothelial cell markers LYVE-1, desmoplakin, reelin, Prox1, podoplanin and VEGFR3. The results obtained were normalized to the 18S expression. Results are the mean of at least 6 distinct adipose tissues.

(B) Immunohistochemistry using antibodies directed against CD34 (green), VEGFR3 (red) and podoplanin (green) was performed on cultured BEC and LEC and analyzed by fluorescence microscopy. Photomicrographs representative from 4 experiments are shown.

III.1.2.3 Influence of the state of adipose tissue growth on the endothelial cell number

The percentage of human adipose tissue derived-BEC and -LEC within the SVF was determined by flow cytometry analysis, in patients with different body mass indices (BMI), used as an estimate of adiposity. According to the world health organization's classification, two groups of patients were defined on the basis of the BMI, with patients with a BMI superior to 25 being defined as overweight/obese. The characteristics of both groups are indicated in **the Table 5**. The percentage of BEC and LEC was then normalized to the total number of cells in the SVF extracted after the collagenase digestion per gram of tissue. As depicted in **figure 13**, the total number of BEC remained constant between both groups, whereas the number of LEC was significantly lower in the SVF of patients with a higher BMI.

Lean	Overweight/Obese
N=61	N=42
Mean BMI: 22.15	Mean BMI: 28.68
Minimum BMI: 19	Minimum BMI: 25
Maximum BMI: 24.84	Maximum BMI: 43

Table 5: Characteristics of the lean vs overweight/obese groups

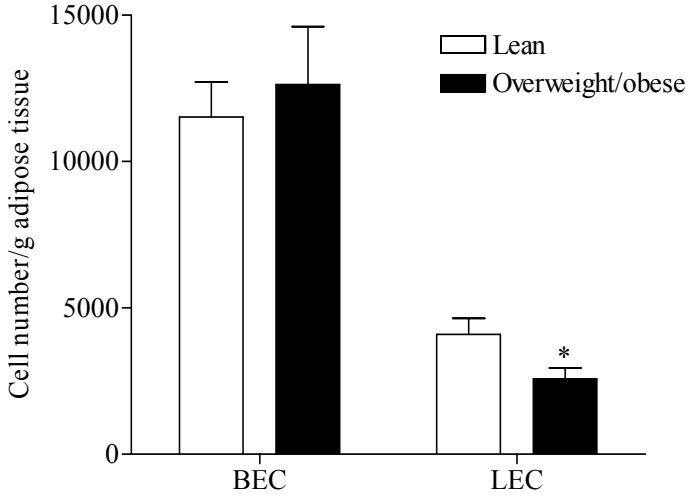

Figure 13: Influence of the body mass index (BMI) on the number of BEC and LEC present in the SVF of human adipose tissue.
*Number of BEC and LEC present in the SVF of human adipose tissue was calculated from results obtained by flow cytometry analysis and normalized to the total number of cells in the SVF extracted per gram of adipose tissue in individuals with a distinct BMI. Individuals were divided in 2 groups depending on their BMI (lean subjects with a BMI < 25, n=61 and overweight/obese subjects with a BMI ³ 25, n=42). Results are the mean ± sem, * p<0.05 obese/overweight versus lean.*

III-2. Evidence of the presence of endothelial cell progenitors in the human adipose tissue

The immunohistochemical analysis of human adipose tissue showed the presence of sparse CD34-positive cells within the stroma-vascular fraction. Flow cytometry analyses performed on the SVF of human adipose tissue also identified a cell population that expressed CD34 but did not express CD31 (see **figure 9B**, upper left panel). Since this cell population has never been described before and since CD34 is a marker for stem cells, we decided to further characterize the CD34+/CD31- cell population.

III.2.1. Characterization of the CD34+/CD31- cells within the stroma-vascular fraction of human adipose tissue

Cells expressing the primitive stem cell marker ABCG2 were detected in the SVF of human subcutaneous adipose tissue (9.7±3.1 % of the SVF, n=19) (**figure 14A**). In addition, the expression of the endothelial progenitor cell marker CD133 was detected (1.5±0.4 of the SVF, n=19) (**figure 14B**). Taken together with the presence of CD34 positive cells, these results strongly suggest that stem/progenitor cells are located within the SVF of human adipose tissue.

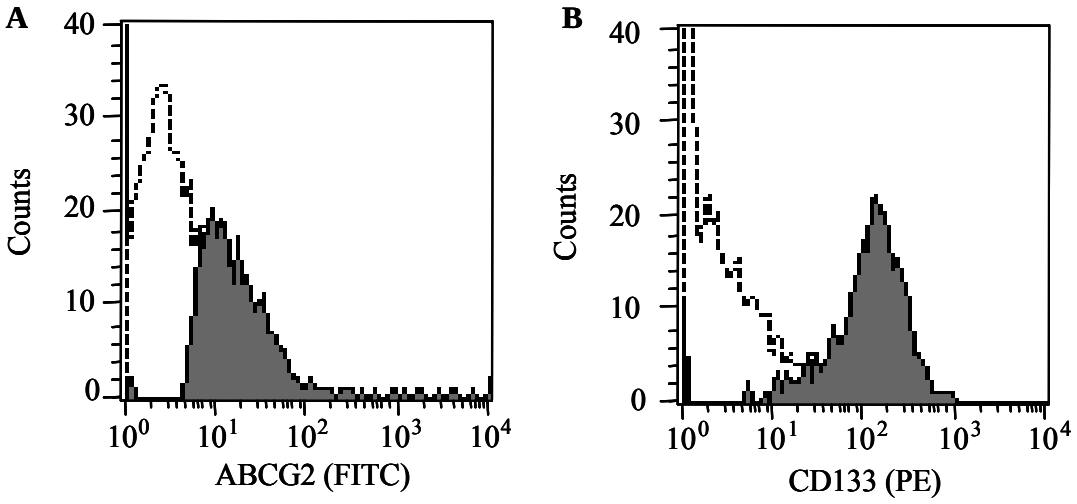

Figure 14: Expression of stem cell markers in the SVF of human adipose tissue*The cells of SVF freshly isolated from human adipose tissue were labeled with fluorescent antibodies directed against the stem cell markers ABCG2 (FITC) (A) and CD133 (PE) (B) and analyzed by flow cytometry. Histograms of the FITC and PE fluorescences (empty lanes correspond to the fluorescence signals obtained with the control isotype antibody and plain lanes correspond to the fluorescence signal obtained with ABCG2 and CD133 antibodies) representative from 19 experiments are shown.*

In order to further characterize the CD34+/CD31- cell population, a cell separation protocol was developed (**figure 15**). Using magnetic microbeads coupled to antibodies directed against CD34, we extracted the total CD34+ cells from the freshly digested SVF. Since the total CD34+ cell population contains the BEC that co-express CD34 and CD31, the total CD34+ cell population was depleted from the CD31+ cells using magnetic microbeads coupled to antibodies directed against CD31.

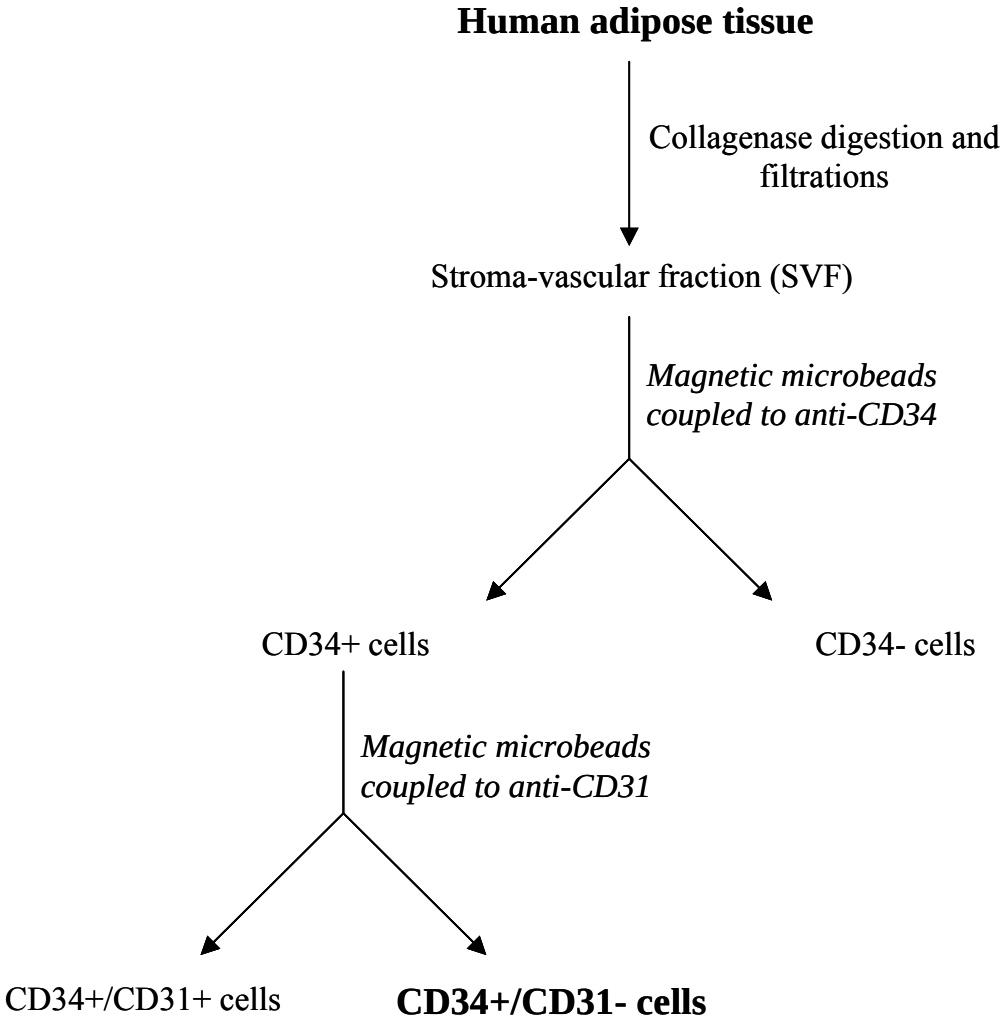

Figure 15: Protocol for the isolation of CD34+/CD31- cells from the SVF of human adipose tissue*Human adipose tissue was digested with collagenase, centrifuged and filtrated in order to obtain the cells from the SVF as described in the method section. The CD34+/CD31- cells were isolated by positive selection of the CD34+ cells followed by a negative selection of CD31+ cells.*

To verify the efficiency of the cell separation protocol, the expression of CD34 and CD31 on the freshly isolated cells was addressed by flow cytometry. No CD31 was detected whereas all cells expressed CD34, demonstrating the efficiency of separation of the CD34+/CD31- cell population (**figure 16A**). These cells were then further analyzed by double-color flow cytometry using antibodies directed against the leucocyte marker CD45 and the macrophage marker CD14. As seen in **figure 16B**, the CD34+/CD31- cells expressed neither CD45 nor CD14. While a few expressed the endothelial cell progenitor marker CD133 (1.4±0.2%,n=7, **Figure 16D**), 18±4 % expressed the stem cell marker ABCG2 (n=7, **Figure 16C**).

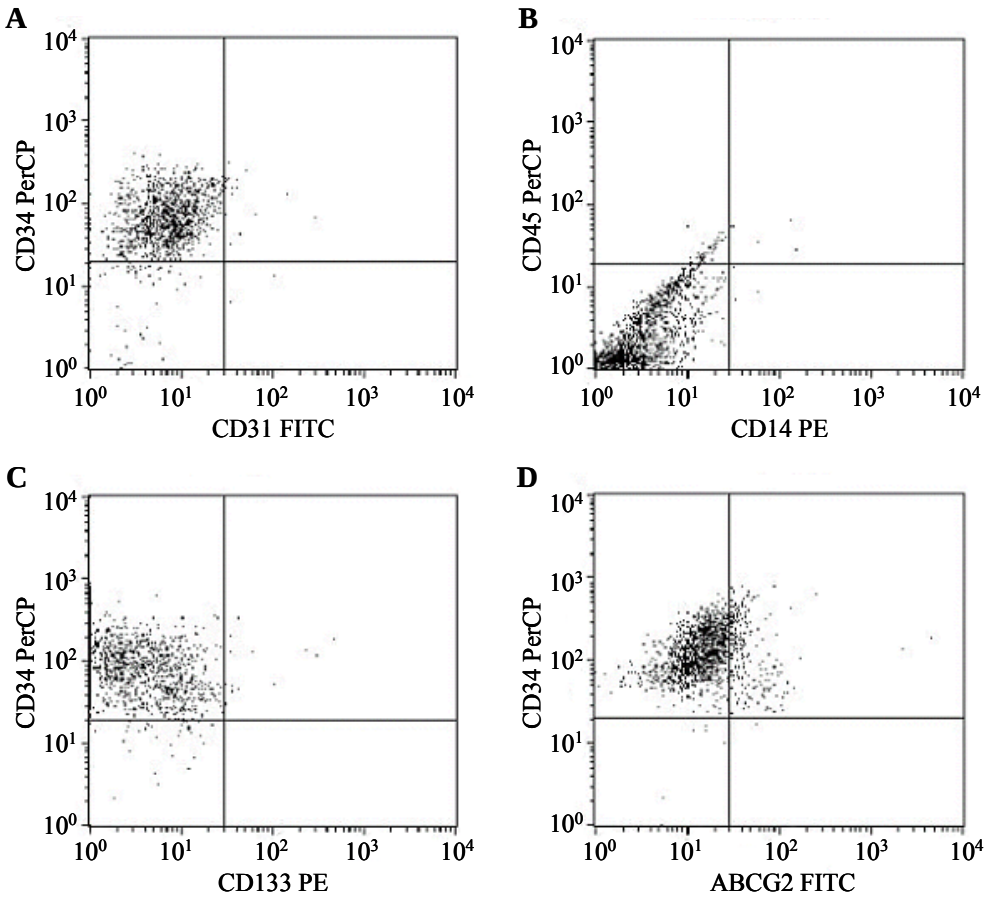

Figure 16: Characterization of the freshly isolated CD34+/CD31- cells from human adipose tissue

Double color FACS analysis was performed on the freshly isolated CD34+/CD31- subset from the SVF of human adipose tissue with antibodies directed against CD34 (PerCP) and CD31 (FITC) (A), the monocyte/macrophage marker CD14 (PE) and the hematopoietic marker CD45 (PerCP) (B), CD34 (PerCP) and CD133 (PE) (C) or CD34 (PerCP) and ABCG2 (FITC) (D). The respective dot-blot analysis from at least 7 independent experiments are shown.

III.2.2 Fate of the CD34+/CD31- cells in vitro

Freshly isolated CD34+/CD31- cells were plated on fibronectin in culture media, i.e. endothelial cell basal medium (EBM) containing 10% of fetal calf serum (FCS), endothelial cell growth medium (ECGM) or endothelial cell growth medium supplemented with vascular endothelial growth factor (VEGFA, 0.5ng/mL) and insulin-like growth factor (IGF, 20ng/mL). Cells were harvested and counted every 24 hours and the doubling time of the cell population was determined. The CD34+/CD31- cells exhibited a high proliferative capacity in culture (doubling time of 33 hours in BM/10%FCS), that was further enhanced in ECGM supplemented with VEGF and insulin-like growth factor (IGF) (doubling time of 31.5 hours and 30.5 hours, respectively, P<0.01, **Figure 17**).

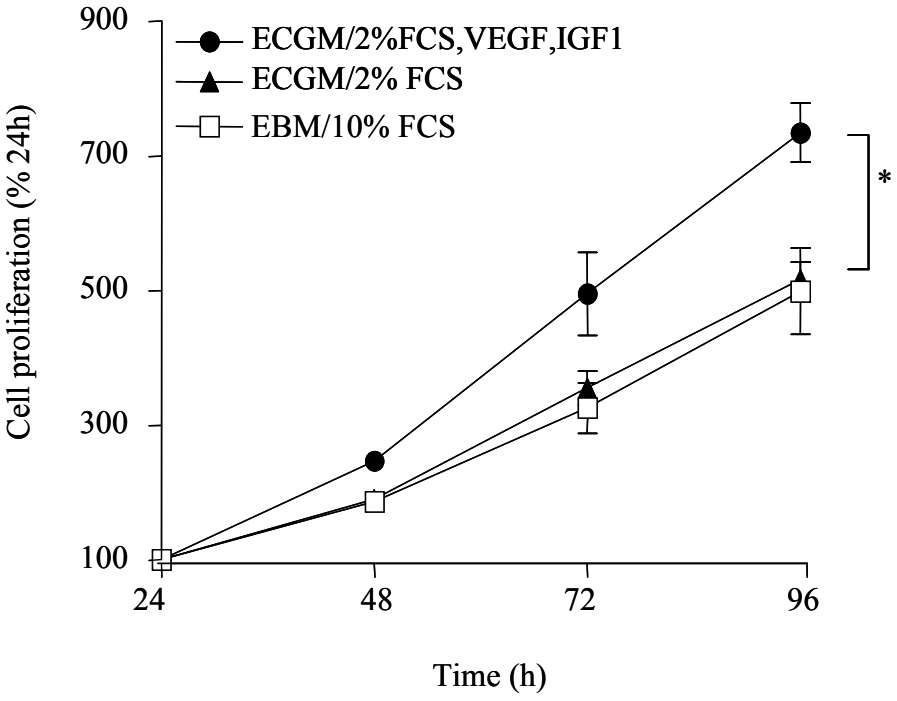

Figure 17: Phenotypic characterization of the CD34+/CD31- subset in culture
*CD34+/CD31- cells plated at low density (5000 cells/cm^2) were cultured in EBM/10%FCS or ECGM/2%SVF supplemented or not with VEGF A (0.5ng/mL) and IGF (20ng/mL). Cells were counted at the indicated times. The results are the mean ± sem of the percentage of cells counted after 24hours, n=5, * p<0.01.*

In order to determine their differentiation ability, CD34+/CD31- cells were plated at high density (20 000 cells/cm^2) on fibronectin in EBM/10%FCS, in ECGM or ECGM supplemented with VEGFA and IGF. After 10 days of culture, cells developed a spindle-shaped morphology, which is a feature of endothelial cells, in ECGM whereas the cells cultured in EBM/10%FCS formed cobblestone areas, which is characteristic of endothelial cells but also of adult stem cells (**Figure 18A** and **18B**). Immunocytochemical analyses demonstrated the expression of vWF (**Figure 18A**) and CD31 (**Figure 18B**) in cells cultured in ECGM, that was increased in the presence of VEGF and IGF (26%±11 and 14%±5 of cells positive for CD31 and vWF, respectively).

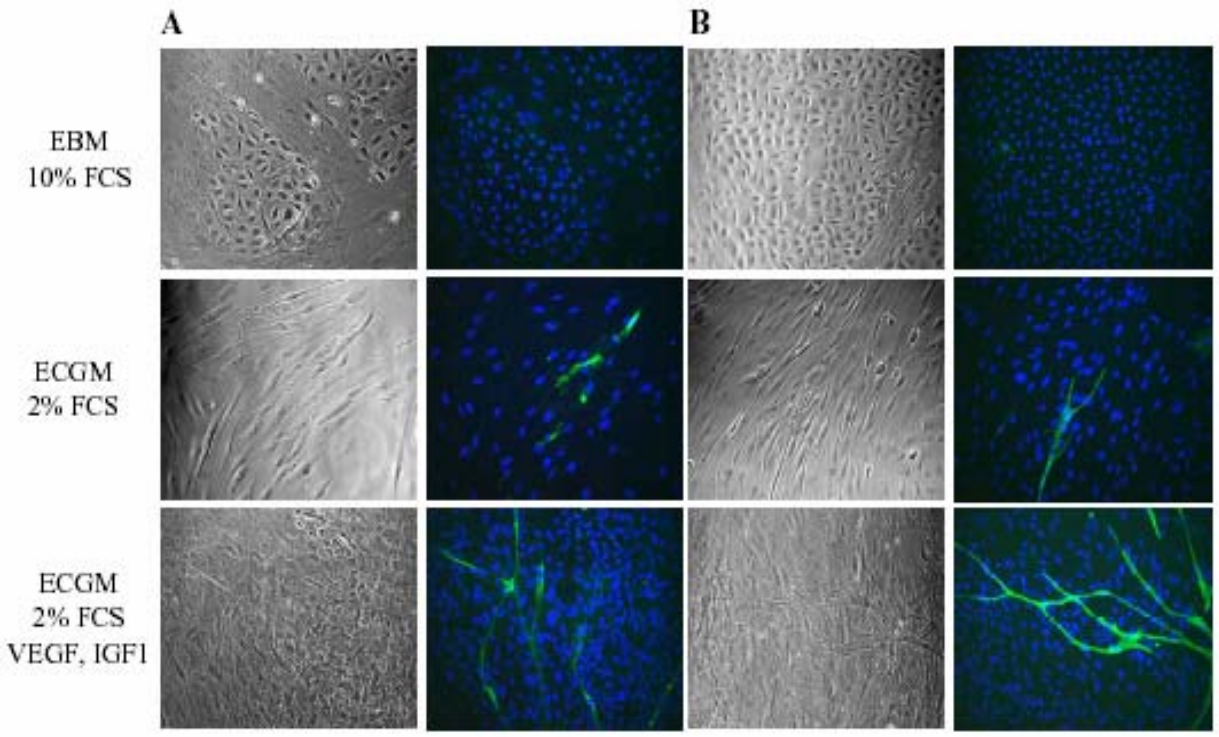

Figure 18: Phenotypic characterization of the CD34+/CD31- subset in culture
CD34+/CD31- cells plated at high density (20 000 cells/cm2) were cultured in EBM/10%FCS or ECGM/2%SVF supplemented or not with VEGF A (0.5ng/mL) and IGF (20ng/mL). Cells were analyzed after 10 days of culture by immunocytochemistry using antibodies directed against vWf (A) and CD31 (B). Representative photomicrographs are shown from n=10 independent experiments.

III.2.3. "In vivo" fate of the CD34+/CD31- cells

The fate of CD34+/CD31- cells was analyzed in vivo in the model of the mouse hindlimb ischemia. Athymic mice underwent deep and superficial femoral artery ligature of the right hindlimb. Twenty-four hours after surgery, CD34+/CD31- cells were freshly isolated from the SVF of human adipose tissue and injected into the tail vein. Saline, CD34-/CD14-/CD31- cells from human adipose tissue and BM-MNC were injected into animals that underwent the same procedure. Thereafter laser Doppler imaging was performed every week on both hindlimbs. The quantitative analysis of the Laser Doppler imaging revealed a significant, time-dependent increase in blood flow after the injection of CD34+/CD31- cells to the ischemic hindlimb (**figure 19A**) that was maximal after 2 weeks (2-fold increase, $P<0.05$) and equivalent to that observed following the injection of BM-MNC. The injection of the CD34-/CD14-/CD31-cells did not induce any significant improvement in the blood flow (**figure 19B**).

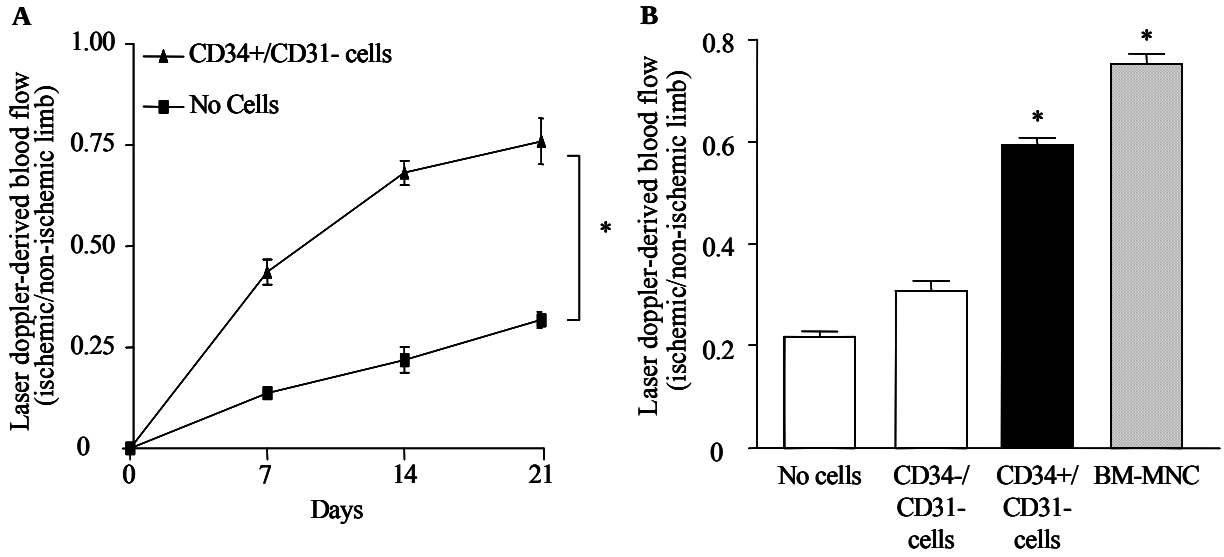

Figure 19: Blood flow improvement after injection of the CD34+/CD31- cells in mouse ischemic hindlimb

*Freshly harvested human adipose tissue-derived cells (CD34+/CD31- or CD34-/CD31-) or bone marrow-mononuclear cells (BM-MNC) were injected to mice 24 hours after inducing hindlimb ischemia. Relative blood flow in the ischemic limb was measured by Laser Doppler imaging analysis every 7 days (A) or after 14 days of ischemia (B) (n=12 for human adipose tissue-derived cells and n=7 for BM-MNC, * p<0.05).*

After 2 weeks, mice were sacrificed and adductor and the semi-membranous muscles were dissected to prepare tissue sections for immunohistochemical analysis. Antibodies directed against the endothelial cell marker CD146 were used to determine the capillary density of the muscle section that is represented by the ratio of capillaries/myocytes. Moreover, the incorporation of human cells into the capillary structures was analyzed by double staining using antibodies directed against CD146, that recognized endothelial cells, and human leucocyte antigen (HLA) that allows the identification of human cells. The capillary density significantly increased in the ischemic muscles when CD34+/CD31- cells from human adipose tissue or BM-MNC were injected as compared to treatment with buffer saline or CD34-/CD14-/CD31- cells from human adipose tissue (n>5, $P<0.001$ versus mice injected with CD34-/CD14-/CD31- cells, **figure 20A**). Moreover, HLA-positive cells were found in the CD146-positive vascular structures in muscle section of mice that had received the CD34+/CD31- cells (**figure 20B**), suggesting that the CD34+/CD31- cells from human adipose tissue incorporated into the murine vasculature.

Taken together, these data demonstrate that the CD34+/CD31- cells from human adipose tissue have the ability to promote the neovascularization that occurs in ischemic tissues. It is thus suggested that CD34+/CD31- cells exhibit progenitor cell properties.

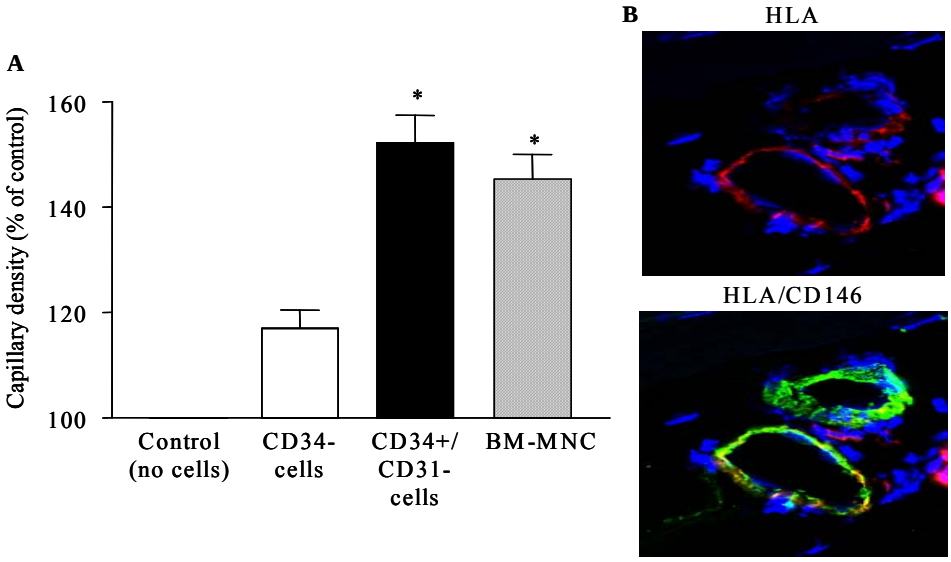

Figure 20: Neovascularization after injection of the CD34+/CD31- cells in mouse ischemic hindlimb

*Freshly harvested human adipose tissue-derived cells (CD34+/CD31- or CD34-/CD31-) or BM-MNC were injected to mice 24 hours after inducing hindlimb ischemia. A) Capillary density in the ischemic hind limb, 14 days after ischemia. Values are the mean ± sem of the percentage of the control (n>5, * p<0.01). B) Incorporation of CD34+/CD31- cells in the mouse vasculature 14 days after ischemia. Representative photomicrographs of immunohistochemistry analyses using anti-HLA-APC (red) and anti-CD146-FITC (green) antibodies are shown (n=7).*

III.3. Regulation of the human adipose tissue-derived BEC

III.3.1. Regulation of BEC proliferation

To determine whether local signals originating from adipocytes and progenitor cells (CD34+/CD31-) could regulate BEC proliferation, freshly harvested adipose tissue derived-BEC or cells that were passaged once, were plated at a low density and treated with conditioned medium (collected over 24 hours) from progenitor cells or from mature adipocytes. After 24 hours, BrdU incorporation, the amount of which is correlated with DNA replication, was determined. As a positive control, cells were treated with endothelial cell growth medium that contains 5% fetal calf serum, 10ng/mL epidermal growth factor and a mixture of endothelial cell growth factors. As shown in **figure 21**, the progenitor cells-conditioned medium did not induce BrdU incorporation in BEC while the medium conditioned by mature adipocytes induced a significant increase in BrdU incorporation. The extent of BrdU incorporation was similar in adipocyte-conditioned medium-treated cells and in cells cultured in the presence of low-serum endothelial cell growth medium (2-fold increase, n=27, $p<0.01$ and 3-fold increase, n=3, $p<0,05$, respectively).

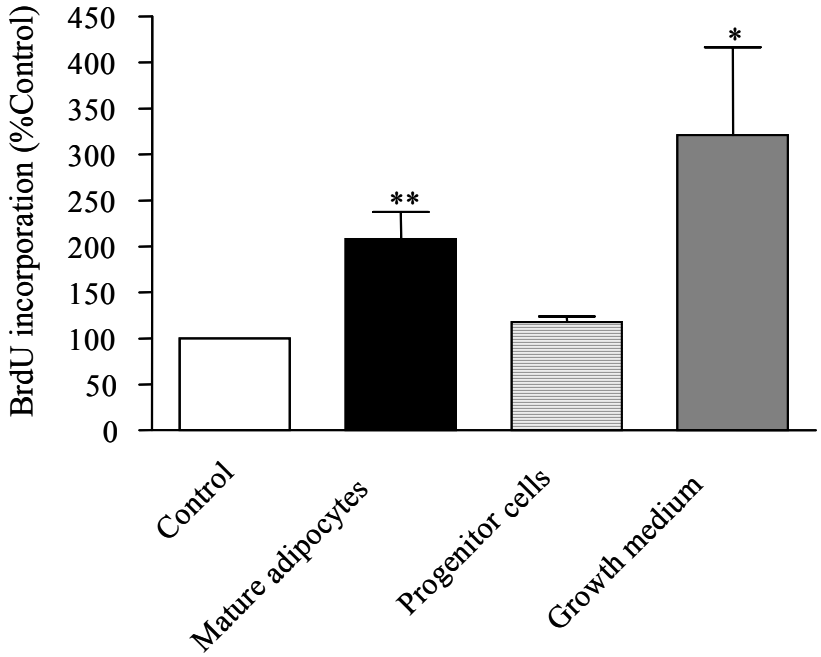

Figure 21: Proliferation capacity of BEC from human adipose tissue
Cultured BEC were treated with 24 h-conditioned medium from mature adipocytes or progenitor cells from human adipose tissue or endothelial cell growth medium for 29h and BrdU was added in the medium for 3 additional hours. The BrdU incorporation assay was thus performed as described in the method section. Results are the mean ± sem of the percentage of the control values, $p<0.05$ and ** $p<0.01$, n=11)*

The expression of the angiogenic factors VEGF A, VEGF C, VEGF D and the adipokines, leptin and adiponectin, was determined in freshly isolated mature adipocytes and progenitor cells by real time RT-PCR analysis. The angiogenic factor VEGF A was expressed at equivalent levels in both cell types. Significantly higher transcripts of VEGF C and VEGF D were detected in adipose tissue derived-progenitor cells as compared with mature adipocytes (0.35±0.17 vs 0.05±0.03, n=8, p<0,05 and 0.28±0.12 vs 0.007±0.007, n=8, p<0,05, respectively). In contrast, adiponectin and leptin were expressed exclusively in mature adipocytes (data not shown). The expression of the receptors for both leptin and adiponectin was also analyzed by real time RT-PCR experiments. The long form of the leptin receptor as well as both adiponectin receptors were detected in adipose tissue derived-BEC. Compared with the VEGF-receptor forms, VEGFR2 transcripts were expressed at the higher levels (**figure 22)**.

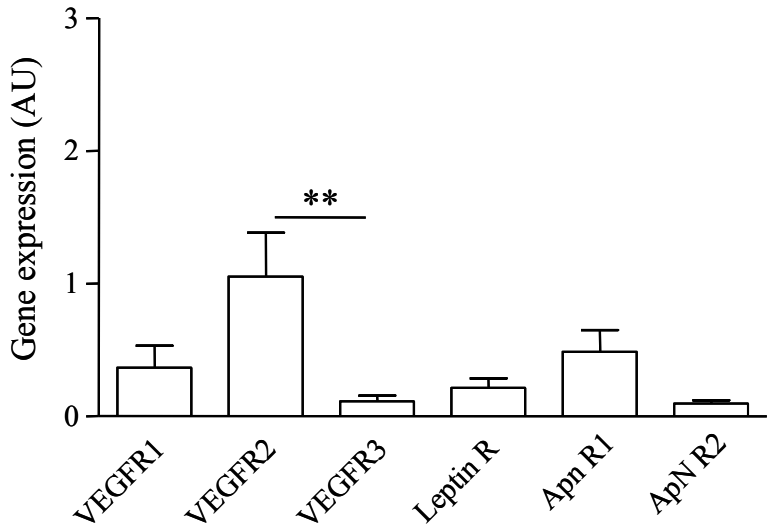

Figure 22: Expression of receptors to (lymph)angiogenic factors and adipokines in BEC from human adipose tissue

*The expression of the receptors of the VEGFs (VEGFR1, VEGFR2 and VEGFR3) and of the adipokines leptin (Leptin R) and adiponectin (ApN R1 and ApN R2) was measured by real time-PCR in freshly isolated BEC from human adipose tissue. Results were normalized to 18S expression and are the mean ± sem of at least 18 experiments. (** p<0.01)*

Finally, the response of BEC to angiogenic factors and adipokines was studied. Freshly harvested cells or cells that were passaged once, were plated at low density and treated with VEGF A, VEGF C, VEGF D, leptin and adiponectin at increasing concentrations (0.2, 2 or 20ng/mL for VEGF A, leptin and adiponectin and 20, 200, 1000ng/mL for VEGF C and VEGF D) or with a combination of leptin (2 ng/mL) and VEGF A (2 ng/mL). After approximately 30 hours, proliferation was assessed by BrdU incorporation. As depicted in **figure 23**, significant proliferative response was observed only in the presence of the combination of leptin with either VEGF A or bFGF (1,8-fold increase BrdU incorporation, n=6, p<0,05 and 2,4-fold increase BrdU incorporation, n=5, p<0,01, respectively), whereas treatment of the cells with the angiogenic factors alone did not affect the growth of the BEC (n=6).

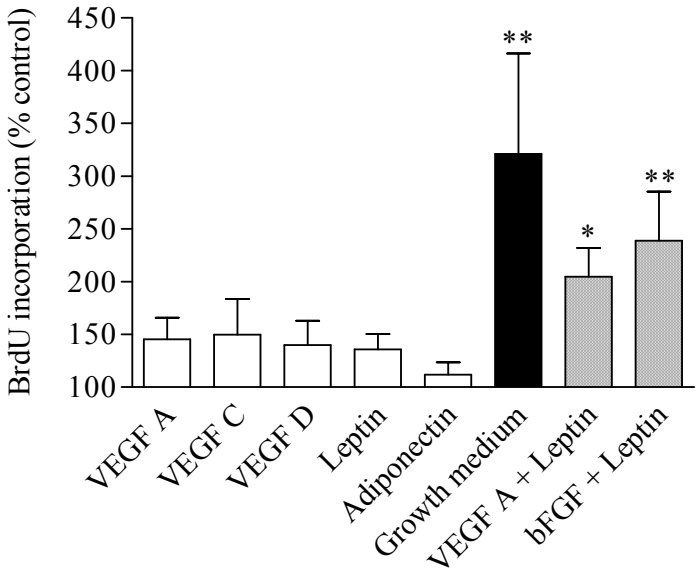

Figure 23: Proliferation capacity of BEC from human adipose tissue
*Cultured BEC were treated with recombinant VEGF A (2ng/mL), VEGF C (20ng/mL), VEGF D (20ng/mL), leptin and adiponectin (2ng/ml) alone or in combination (VEGF A + leptin, or bFGF + leptin) for 29h and BrdU was added in the medium for 3 additional hours. The BrdU incorporation assay was thus performed as described in the method section. Results are the mean ± sem of the percentage of the control values, * p<0.05 and ** p<0.01, n=4*

III.3.2. Regulation of BEC migration and tube formation

To assess the effects of angiogenic factors and adipokines on BEC migration and tube formation, adipose tissue derived-BEC were plated at high density on growth factor reduced matrigel. The cells were treated with VEGF A (2 ng/mL), VEGF C (200 ng/mL), VEGF D (200ng/mL), leptin and adiponectin (2 ng/mL). Cell migration and tube formation was followed by phase contrast microscopy after 3h, 6h and 24h. After 24 h, the number of tubes and branching points as well as the length of cytoplasmic extensions of the cells were measured. As shown in **figure 24**, leptin treatment led to a statistically significant increase in the length of tubes and number of branching points (2-fold increase, *p<0,05, n=3 and 3-fold increase, * p<0,05, n=3, respectively). VEGF A as well as adiponectin tended to enhance cell migration and tube formation but these effects did not reach statistical significance.

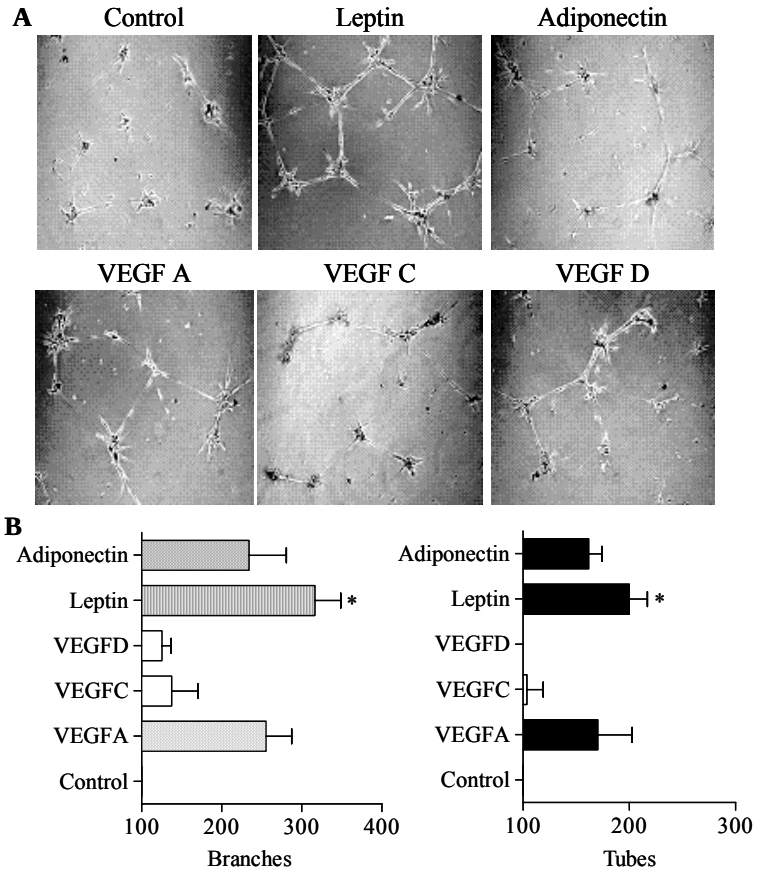

Figure 24: Tube formation capacity of BEC from human adipose tissue
BEC were plated on growth factor-reduced Matrigel and treated with recombinant VEGF A, VEGF C, VEGF D, leptin and adiponectin for 24h. Cells were observed under phase contrast microscopy. Representative photomicrographs from 4 independent experiments are shown. The length of cytoplasmic extensions (tubes) as well as the number of branching points (branches) were measured. (B) Results are the mean ± sem of the percentage of the control from 3 experiments are shown. (p<0.05)*

III.3.3. Signaling pathways stimulated by leptin in BEC

To analyze the potential signaling pathway involved in the leptin-mediated stimulation of BEC migration and tube formation, Western blot analysis was performed on crude protein extracts of cells treated with leptin (2, 5 and 15 minutes). The phosphorylation of the protein kinase Akt, reported to be involved in endothelial cell survival and migration as well as the phosphorylation of the extracellular regulated kinases 1 and 2 (Erk1/2), which are involved in endothelial cell proliferation were determined by the use of phospho-specific antibodies and normalized to the levels of the respective proteins. As depicted in **figure 25**, leptin did not affect the phosphorylation of Erk 1/2 at any time point. However, leptin increased the phosphorylation of Akt, that in a time-dependent manner was maximal after 5 min of treatment (1,5-fold increase, $p<0.05$, $n=3$). Similar experiments were performed using BEC treated with adiponectin (2 ng/mL). No effect of adiponectin on the phosphorylation of Erk1/2 or Akt was detected, whatever the time of treatment (n=3, data not shown)

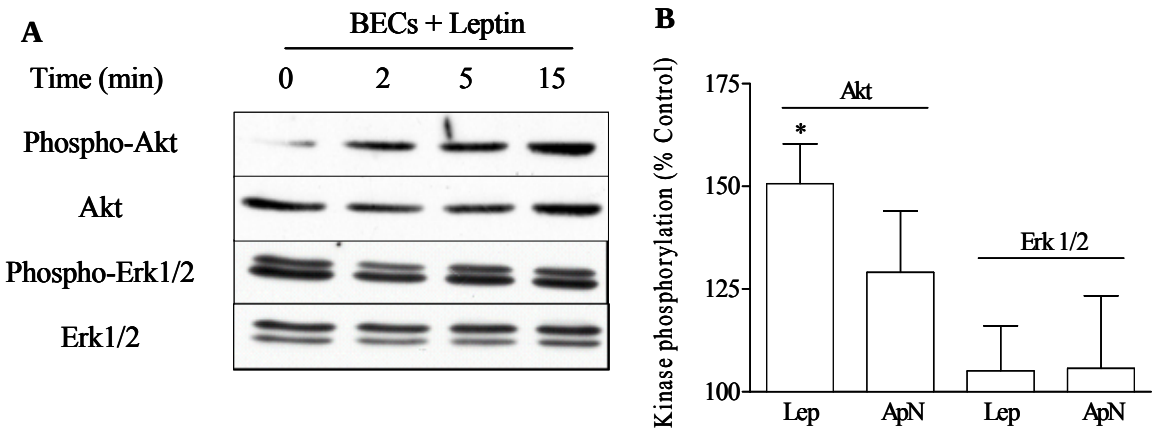

Figure 25: Effect of leptin on phosphorylation of Akt and Erk1/2 in BEC from human adipose tissue

Confluent BEC from human adipose tissue were treated with 2ng/mL of leptin for 2min, 5min or 15min. Total proteins were extracted and western blot analysis performed. The phosphorylation of Erk1/2 as well as Akt were analyzed using antibodies directed against the phosphorylated forms of the kinases. The blots were then stripped and re-probed with antibodies directed against the total Erk1/2 and Akt proteins. The kinase phosphorylation was calculated by densitometry analysis. Representative blots (A) as well as the mean ± sem of kinase phosphorylation versus control at 5 min are shown ($p<0.05$, $n=3$)(B).*

III.4. Regulation of the human adipose tissue-derived LEC

III.4.1. Regulation of LEC proliferation

To determine whether local signals originating from adipocytes and progenitor cells could regulate LEC proliferation, freshly harvested human adipose tissue derived-LEC or cells that were passaged once, were plated at low density and treated with conditioned medium from progenitor cells or from mature adipocyte cultures. After approximately 30h, BrdU was added to the cell culture medium and cells were incubated for 3 additional hours. After fixation, BrdU incorporation was determined. As positive control, cells were treated with endothelial cell growth medium that contains 5% FCS, 10ng/mL EGF and a mix of endothelial cell growth factors. As shown in **figure 26**, progenitor cells conditioned medium did not induce any changes in BrdU incorporation compared to control in LEC. However, the medium conditioned by mature adipocytes induced a significant increase in BrdU incorporation as did the low-serum endothelial cell growth medium (167%±37 of BrdU incorporation, n=8, p<0,01).

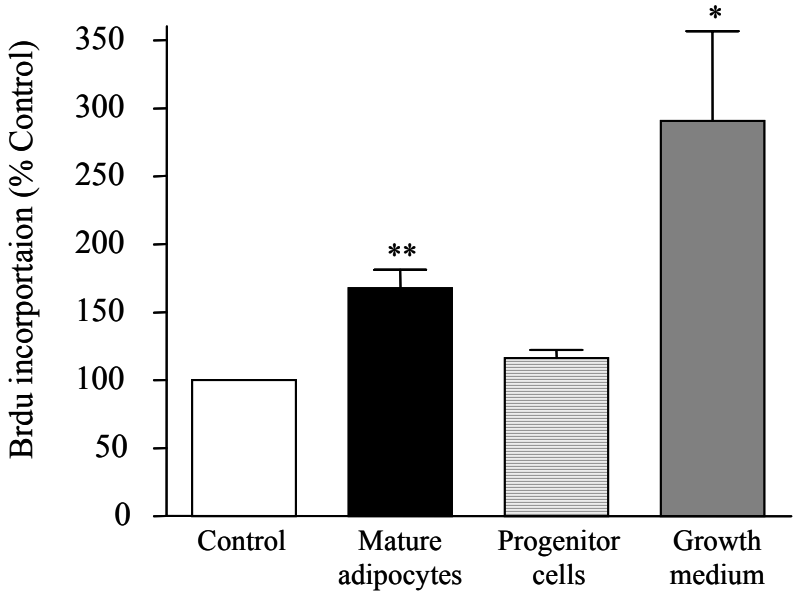

Figure 26: Proliferation capacity of LEC from human adipose tissue
Cultured LEC were treated with 24h-conditioned medium from mature adipocytes or progenitor cells from human adipose tissue or endothelial cell growth medium for 29h and BrdU was added in the medium for 3 additional hours. The BrdU incorporation assay was thus performed as described in the method section. Results are the mean ± sem of the percentage of the control values (p<0.05 and ** p<0.01, n=11)*

The expression of the leptin receptor and the 2 adiponectin receptors were then determined in adipose tissue derived-LEC by real-time RT-PCR. The expression of leptin receptor was very low and statistically significantly different from that detected in BEC ($p<0.001$, $n=16$), whereas the levels of transcripts for the adiponectin receptor 1, ApNR1, were high (**figure 27**).

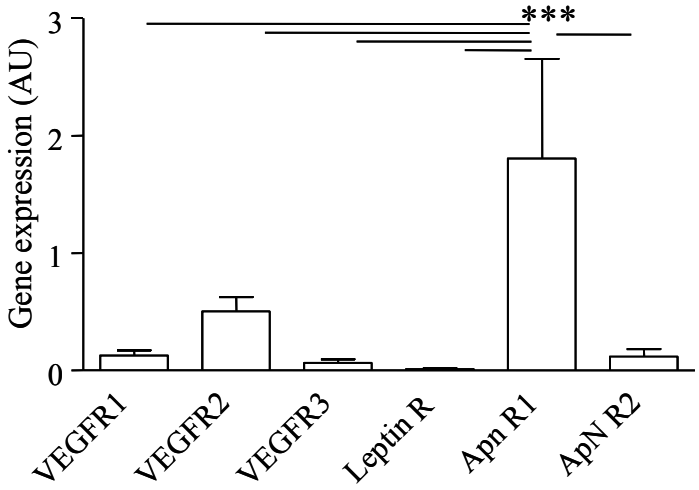

Figure 27: Expression of receptors to (lymph)angiogenic factors and adipokines in LEC from human adipose tissue

*The expression of the receptors of the VEGFs (VEGFR1, VEGFR2 and VEGFR3) and of the adipokines leptin (Leptin R) and adiponectin (ApN R1 and ApN R2) was measured by real time-PCR in freshly isolated LEC from human adipose tissue. Results were normalized to 18S expression and are the mean ± sem of at least 16 experiments. (*** p<0.001)*

Finally, angiogenesis was studied in adipose tissue derived-LEC. Freshly harvested cells or cells that were passaged once, were plated at low density and treated with VEGF A, VEGF C, VEGF D, leptin and adiponectin at increasing concentrations (0.2, 2 or 20ng/mL for VEGF A, leptin and adiponectin and 20, 200, 1000ng/mL for VEGF C and VEGF D) or with a combination of leptin (2 ng/mL) and VEGF A (2 ng/mL). Following incubation, proliferation was assessed by BrdU incorporation assay. As shown in **figure 28**, adiponectin, VEGF C and VEGF D induced a concentration-dependent increase in the BrdU incorporation that was maximal using 2ng/mL adiponectin (210±12%, $p<0.01$, $n=3$), 20ng/mL VEGF C (183±37%, $p<0.01$, $n=4$) and 20ng/mL VEGF D (152±27%, $p<0.05$, $n=4$). The other treatments did not exert any significant effect on the BrdU incorporation in LEC.

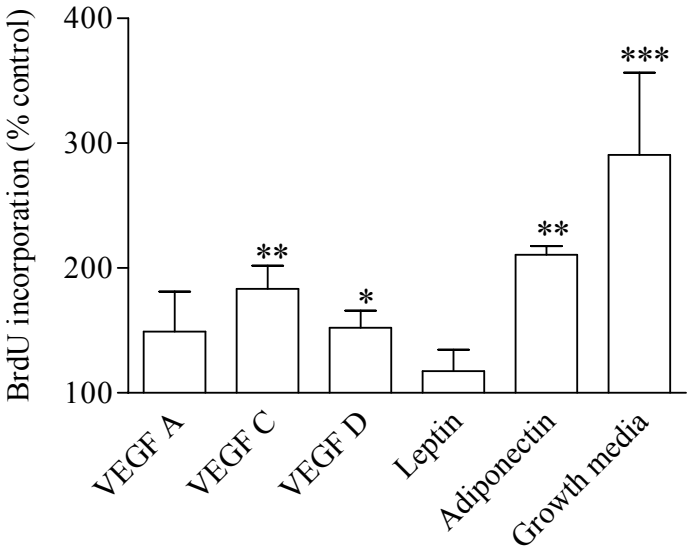

Figure 28: Proliferation capacity of LEC from human adipose tissue
*Cultured LEC were treated with recombinant VEGF A (2ng/mL), VEGF C (20ng/mL), VEGF D (20ng/mL), leptin and adiponectin (2ng/ml) alone or in combination (VEGF A + leptin or bFGF + leptin) for 29h and BrdU was added in the medium for 3 additional hours. The BrdU incorporation assay was thus performed as described in the method section. Results are the mean ± sem of the percentage of the control values, * p<0.05, ** p<0.01 and ***p<0.001, n=4*

III.4.2. Regulation of LEC migration and organization

To assess the effect of angiogenic factors and adipokines on LEC migration and organization, human adipose tissue derived-LEC were plated at high density on growth factor reduced matrigel. The cells were treated with VEGF A (2 ng/mL), VEGF C (200 ng/mL), VEGF D (200ng/mL), leptin and adiponectin (2 ng/mL). Endothelial cell tube formation was monitored by phase contrast microscopy after 3h, 6h and 24h. After 24 h, the number of tubes and of branching points as well as the length of cytoplasmic extensions of the cells were measured. As shown in **figure 29**, adiponectin and VEGF A treatment led to a statistically significant increase in tube length and the number of branching points (131%, *p<0.05, n=4 and 138%, *p<0,05, n=3, respectively). While VEGF C also tended to increase angiogenesis, these effects did not reach statistical significance.

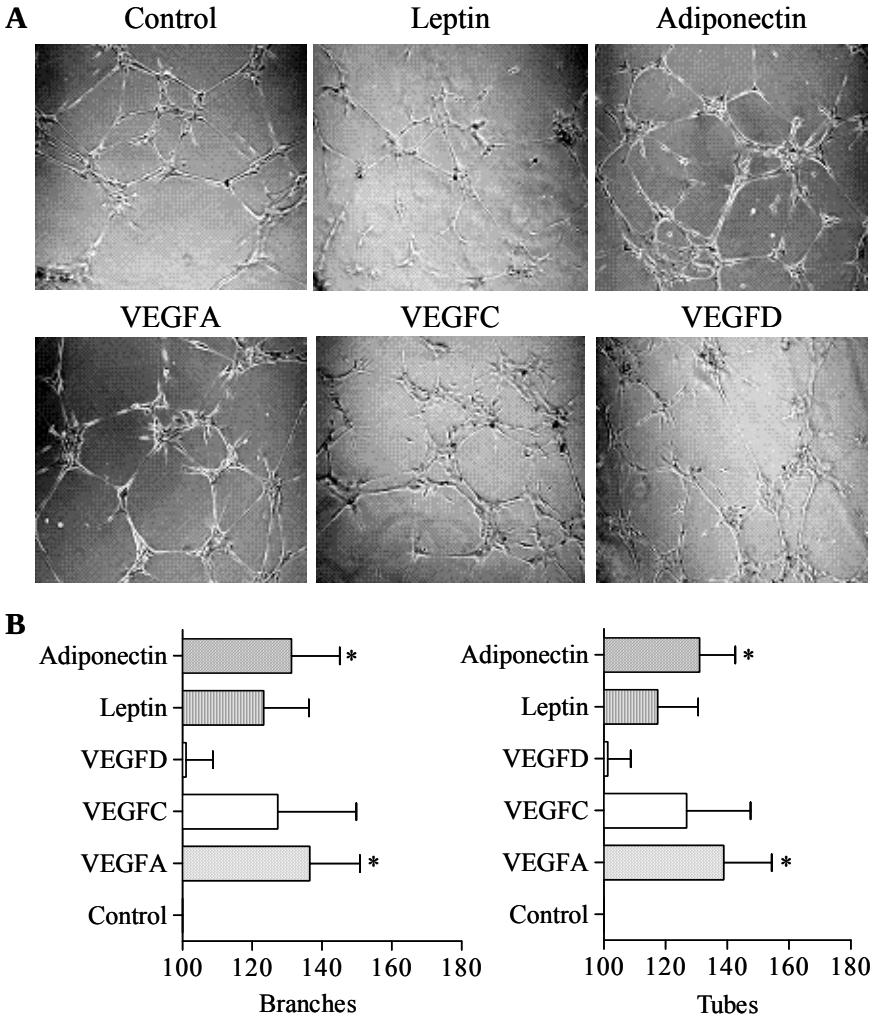

Figure 29: Tube formation by LEC from human adipose tissue
LEC were plated on growth factor-reduced Matrigel and treated with recombinant VEGF A, VEGF C, VEGF D, leptin and adiponectin for 24h. Cells were observed under phase contrast microscopy. Representative photomicrographs from 4 independent experiments are shown. The length of cytoplasmic extensions (tubes) as well as the number of branching points (branches) were measured. (B) Results are the mean ± sem of the percentage of the control from 3 experiments are shown. ($p<0.05$)*

III.4.3. Signaling pathways stimulated by adiponectin in LEC

To determine the signaling pathways that might be involved in the effect of adiponectin on adipose tissue derived-LEC, Western blot analyses were performed on protein extracted from LEC treated with 2ng/mL of adiponectin for 2, 5 or 15 minutes. The phosphorylation of Akt and Erk1/2 were then assessed by the use of specific antibodies and normalized to the total protein levels of Akt and Erk1/2. As shown in **figure 23**, adiponectin treatment induced a time-dependent phosphorylation of Akt with a maximal effect at 5 minutes (121±6%, $p<0.05$, $n=3$) whereas no statistical significant effects of adiponectin were observed on Erk1/2 phosphorylation.

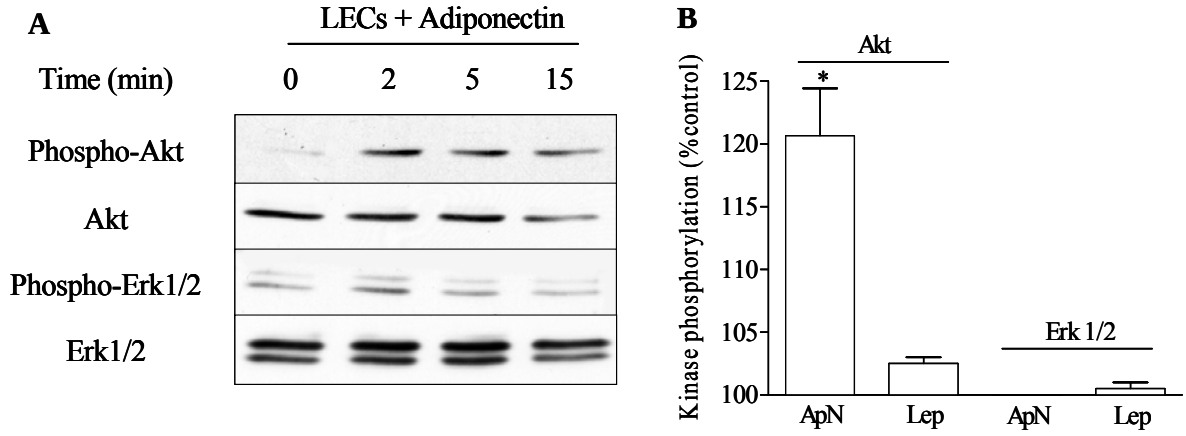

Figure 30: Effect of adiponectin on phosphorylation of Akt and Erk1/2 in LEC from human adipose tissue

Confluent LEC from human adipose tissue were treated with 2ng/mL of adiponectin for 2min, 5min or 15min. Total protein were extracted and western blot analysis performed. The phosphorylation of Erk1/2 as well as Akt were analyzed using antibodies directed against the phosphorylated forms of the kinases. The blots were then stripped and re-probed with antibodies directed against the total Erk1/2 and Akt proteins. The kinase phosphorylation was calculated by densitometry analysis. Representative blots (A) as well as the mean ± sem of kinase phosphorylation versus control at 5 min are shown ($p<0.05$, $n=3$)(B).*

IV. DISCUSSION

Obesity, the prevalence of which is dramatically increasing in industrialized as well as in developing countries, has become a very important challenge in the field of public health since it represents a risk factor for diseases such as type 2 diabetes, cardiovascular diseases and certain types of cancer. Obesity is the result of an excessive development of adipose tissue due to adipocyte hypertrophy and hyperplasia. Because of the central role of adipocytes in the development of the fat mass, most studies have focused on these cells in an effort to understand the mechanisms leading to increased fat mass deposition in human. However, several studies have highlighted the role of cells from the SVF, and in particular endothelial cells in the development and expansion of adipose tissue. Indeed, the development of obesity in ob/ob mice that normally occurs due to the absence of leptin is prevented by treatment with anti-angiogenic agents (156). Moreover, apoptotic agents that target the vasculature within adipose tissue in obese mice have been reported to reverse diet-induced as well as genetically-induced obesity (100). In both of the latter studies, the reduction in fat mass was associated with an improvement in metabolic functions such as increased lipid turnover leading to a normalized energy expenditure. These observations clearly demonstrate that the local vascular network plays a crucial role in the development and maintenance of the fat mass and strongly suggest that blood vessels may represent an attractive therapeutic target for regulating fat mass development.

In the present study, the characterization and remodeling of the endothelial cells of human adipose tissue was addressed.

IV.1. Characterization of the vascular network and endothelial cells type within human adipose tissue

It is well accepted that endothelial cells exhibit marked differences in their phenotype, surface markers and function depending on the organ, tissue and vessel type (for review see (4)). The examination of blood vessels by electron microscopy allowed to establish a classification of capillaries into three general categories. continuous, discontinuous and fenestrated capillaries (14). In fact, the morphology of the capillaries depends on the function of the organ in which they are. Indeed, endocrine and exocrine organs have fenestrated capillaries facilitating selective permeability required for efficient absorption, secretion and filtering whereas brain and retina contain continuous capillaries that represent an effective

barrier to fluid transfer (62). Endothelial cell heterogeneity also depends on the vessel type: vein, artery or capillary. Indeed, microvascular and macrovascular endothelial cells from the same organ differ in the expression of adhesion molecules, transporters and ability to form capillary-like structures (2). For example, in the lung, microvascular endothelial cells exhibit more calcium transporters and have better growth ability than their macrovascular counterparts (97) (60). Moreover, venous and arterial endothelial cells are also distinct. Increasing amount of data concern the lineage orientation of the venous and arterial endothelial cells. The separation of venous and arterial lineages appears in the early embryogenesis. It involves activation of two intracellular signaling pathways that are implicated in cell fate and developmental processes, i.e. the Notch- and the Ephrin-dependent pathways constituted by ligands (ephrin) and tyrosine kinase receptors (Ephrin A and B) (105). Endothelial cell expression of Notch 4 is restricted to arterial endothelium (185) whereas expression of Ephrin B is restricted to venous endothelial cells. It is also clear that endothelial cells from the lymphatic system exhibit also a high heterogeneity both in structure and protein expression pattern depending on their location and vessel type. Moreover, blood and lymphatic endothelial cells from a same organ show distinct expression of lineage-specific genes. For example, in human neonatal foreskin, the cell surface glycoprotein CD34 is exclusively expressed on vascular endothelial cells whereas lymphatic endothelial cells specifically express VEGFR3, podoplanin and LYVE-1 (144). Clearly, endothelial cells constitute an heterogeneous cell population.

There has not been a thorough analysis of the phenotype of the endothelial cells present in adipose tissue on their potential heterogeneity. There is some evidence indicating the presence of fenestrated capillaries within murine adipose tissues (31) although this observation has not been later further investigated. In order to characterize the vascular network in human adipose tissue and in particular the phenotype of the endothelial cells, fluorescence immunohistochemistry analysis were performed. A dense capillary network was observed in human adipose tissue as evidenced by the use of antibodies directed against the common endothelial cell markers CD31 and vWf. Blind-ended capillaries, a hallmark of the lymphatic capillaries, were also observed, indicating that both vascular and lymphatic systems were labeled by the CD31 and vWF antibodies. The use of the marker CD34, expressed on blood but not on lymphatic capillary endothelial cells (144,172), together with vWf allowed us to identify two distinct blood (CD34- and vWF-positive) and lymphatic (CD34-negative and vWF-positive) capillary networks. The lymphatic vasculature was also labeled by antibodies directed against two lymphatic endothelial cell-specific surface proteins; VEGFR3 (85) and

podoplanin (27,28). Taken together, these data demonstrate the presence of CD31-, vWF-, VEGFR3-, podoplanin-positive but CD34-negative endothelial cells within the human adipose tissue, which can be defined as lymphatic endothelial cells (LEC), and CD31-, vWF-, CD34-positive but VEGFR3- and podoplanin-negative cells which are vascular endothelial cells (BEC). It should be noted that staining with the antibody directed against the lymphatic endothelial cell marker, lymphatic vessel hyaluronan receptor (LYVE-1), was localized specifically at the periphery of larger vessels, suggesting that LYVE-1 was expressed by perivascular cells in the human adipose tissue. Several studies support our observation of a non-lymphatic expression of LYVE-1 since it is reported to be expressed by macrophages (161) and hepatic blood sinusoidal endothelial cells (127).

To further characterize both endothelial cell populations in human adipose tissue, we developed an immunoselection technique to isolate LEC and BEC from the SVF of adipose tissue based on immunoselection. LEC have been isolated from other tissues by positive selection using antibodies directed against podoplanin (103), LYVE-1 (144) or VEGFR3 (119). However, given the fact that LYVE-1 expression was not restricted to LEC in adipose tissue and that podoplanin and VEGFR3 antibodies also labeled adipocytes (data not shown), the use of such antibodies was not appropriate for the purification of LEC from adipose tissue. On the other hand, CD34 appeared to be a suitable marker to allow us to distinguish between BEC and LEC. Hirakawa et al separated human dermal BEC and LEC based on the difference of CD34 expression between the two cell types, isolating BEC using microbeads coupled to an anti-CD34 antibody (CD34+) and LEC by selecting CD31-positive cells present in the CD34-negative cell population (CD34-/CD31+)(73). We developed a similar approach with some additional depletion steps to avoid contamination with the non-vascular cells present in the stroma of adipose tissue: cells positive for CD34 but negative for CD31 and cells that were negative for CD34 but positive for CD31 and CD14, identified as macrophages (44). The BEC were isolated from the SVF of human adipose tissue by magnetic microbeads coupled to anti-CD34 antibody followed by a positive selection of the CD31 positive cells. The lymphatic endothelial cells were isolated from the CD34-negative population depleted from the CD14-positive cells before a positive selection step using the anti-CD31 antibody.

Both of the cell types thus isolated (BEC and LEC) expressed transcripts specific for the endothelial cells including the VEGF receptors, VEGFR1 and VEGFR2 as well as vWf and Notch-4. The specificity of these genes for endothelial cells was demonstrated by their absence in mononuclear cells isolated from human peripheral blood and their expression in the human umbilical vein endothelial cells. Moreover, both BEC and LEC were able to

accumulate acetylated low density lipoprotein (Ac-LDL), an ability that is restricted to endothelial cells (and macrophages) which express the Ac-LDL scavenger receptor (1,187). Altogether these results demonstrate that the BEC and LEC extracted from human adipose tissue exhibit some common features that are usually attributed to endothelial cells. The presence of podoplanin mRNA, a glomerular membrane mucoprotein described in podocytes as well as in LEC (27,28) and reelin, a glycoprotein that is secreted by Cajal-Retzius cells and also expressed in LEC (158) were detected exclusively in human adipose tissue derived-LEC. Desmoplakin, a protein that constitutes desmosomes present in lymphatic but not in vascular endothelial cells (162), was detected in both cell types, although at lower levels in BEC. Recent studies have described the presence of desmoplakin in BEC and more particularly in developing vessels and have attributed desmoplakin a role in blood vessel growth (33,200). Thus it appears that desmoplakin is not a marker that can be used to discriminate between lymphatic and vascular endothelial cells. VEGFR3 mRNA was expressed at similar levels in both BEC and LEC as well as in HUVEC, whereas VEGFR3 protein was detected exclusively in LEC. This observation is in agreement with the report by Podgrabinska et al. showing VEGFR3 protein expression exclusively in LEC (144). Finally, the transcripts encoding Prox-1 were detected neither in BEC nor in LEC. The lack of Prox-1 expression in human adipose tissue derived-LEC is intriguing since it is reported to be a key transcription factor involved in the commitment of endothelial cells to the lymphatic lineage (76). However, no study has assessed the expression of Prox-1 in mature adult LEC. One can thus speculate that the expression of such a transcription factor might be down-regulated once LEC are fully differentiated. Taken together, these results demonstrate that LEC from the human adipose tissue express a distinct pattern of cell markers, as compared to those expressed by classical human LEC. Other studies have also reported such an heterogeneity (119,139), suggesting that the phenotype of the LEC is strongly influenced by factors originating from the immediate microenvironment of the tissue in which the cells reside. However it is currently unclear whether these apparently distinct LEC populations, defined by their pattern of expression of several markers really do represent different cell types that have distinct functions and properties. Moreover it is highly likely that the isolation and maintenance of LEC in culture, a common approach used to study LEC, leads to the modulation of gene expression much in the same way that vascular endothelial cell develop an altered morphology and gene expression profile. A comparison of the transcript pattern of cultured LEC with freshly isolated LEC, the approach that we have performed in the present study, should provide additional information about the plasticity of the LEC within human adipose

tissue. Finally, to better characterize both the BEC and LEC populations extracted from human adipose tissue, large scale gene analysis might be an interesting approach to obtain new data regarding the specific patterns of gene expression that distinguish between vascular and lymphatic endothelial cells isolated from the same tissue.

In order to assess the impact of the stadium of adipose tissue development on vascular and lymphatic endothelial cells, the numbers of BEC and LEC, within the SVF of patients with distinct body mass index (BMI) was determined. The results showed that the number of BEC per gram of adipose tissue remains constant independent of the BMI whereas the numbers of LEC were reduced in overweight/obese subjects. These findings imply that in order to maintain an adequate density of vascular endothelial cells to support the expansion of the fat mass, endothelial cells proliferation and/or enhanced differentiation of endothelial progenitor cells might occur. On the other hand, the reduction in LEC number with the development of the adipose tissue suggests that the LEC are not submitted to remodeling with the development of obesity. Taken together, one can speculate that the vascular capillary network, but not the lymphatic system, develops in parallel with the fat mass and that obesity is associated with neovascularization within the adipose tissue without the concomitant expansion of the lymphatic capillary network.

IV.2. Processes involved in the remodeling of the endothelial cells in the human adipose tissue

The mechanisms involved in the control of the vascular remodeling and in the growing capacities of both LEC and BEC within the human adipose tissue were studied. In particular, the expansion of the vascular system either by vasculogenesis, which involves the differentiation of endothelial precursor cells, or by angiogenesis, which implicates mature BEC, as well as the lymphangiogenic capacities of LEC were addressed.

IV.2.1. Evidence for the presence of endothelial progenitor cells in human adipose tissue

Flow cytometry analysis demonstrated the presence of a cell type within the SVF of human adipose tissue that expressed CD34 but not CD31. This is noteworth as CD34 is expressed on capillary endothelial cells as well as being a hallmark of adult stem cells, as it is expressed on hematopoietic and progenitor cells (102). Moreover, we were able to demonstrate the existence of a cell population expressing other stem cell markers such as ABCG2, marker of the side population and CD133, expressed on hematopoietic and endothelial progenitor cells (193). The separation of CD34-positive cells from the CD31 positive cells allowed us to show that the leucocyte marker CD45 as well as the monocyte marker CD14 were absent from the CD34+/CD31- cells. Thus that these cells do not belong to the monocytic lineage. Some cells however co-expressed the stem cell markers ABCG2 and CD133, an observation that strengthens the possibility of a stem cell-like phenotype of such a population. It should be noted that not all the CD34+/CD31- cells expressed ABCG2 and CD133, suggesting that this cell population is probably constituted by distinct populations of stem/progenitor cells. The plasticity of the CD34+/CD31- cells was shown in vitro using distinct culture media. Indeed with high concentrations of serum, CD34+/CD31- cells spontaneously assumed a cobblestone morphology, a hallmark of mature endothelial cells but also of adult stem cells (143). Moreover, in low-serum culture medium, some CD34+/CD31- cells expressed the endothelial cell markers CD31 and vWf and formed tube-like structures. The latter effect was enhanced in presence of VEGF A and IGF, two growth factors known to trigger the differentiation of the progenitor cells isolated from human bone marrow into endothelial cells (87). In particular, VEGF A is able to associate with fibronectin which facilitates signal transduction of VEGF A, thus enhancing endothelial cell differentiation

(191). Thus, the CD34+/CD31- cells from human adipose tissue possess the capacity to differentiate into endothelial-like cells in vitro. The behavior of these cells in vivo was assessed using a model of ischemic hindlimb in nude mice. The surgical induction of ischemia within the hindlimb of nude mice has been shown to trigger homing signals for circulating endothelial progenitor cells leading to the induction of neovascularization (35,135). Our results showed that the injection of CD34+/CD31- cells from human adipose tissue efficiently elicited the recovery of the ischemic hindlimb in the same manner as bone marrow-derived mononuclear cells (BM-MNC). Moreover, since human leucocyte antigen (HLA)-positive cells were detected in the mouse vasculature, it appears that the CD34+/CD31- cells were able to participate in neovascularization by incorporating into the vasculature and potentially differentiating into endothelial cells within the newly formed vessels. Altogether, these results show that the CD34+/CD31- cells freshly isolated from the SVF of human adipose tissue exhibit progenitor cell properties and participate in the vasculogenesis of ischemic tissues. Other groups have made similar observations using the same model and non-separated cultured adipose-derived cells were also reported to rescue the ischemic hindlimb (32,129,142,147). The process responsible for such an effect was suggested to be mediated through direct incorporation of the human cells into the vessels (142), in agreement with our results, or in an indirect manner through the secretion of angiogenic factors such as VEGF A and HGF (147).

Our results demonstrate that endothelial progenitor cells are present in the CD34+/CD31- cell population. The presence of cells exhibiting stem cell-like properties in the human adipose tissue was initially suggested by the work of Zuk et al. who described the expression of markers from mesenchymal stem cells, such as Stro-1 and CD105, as well as the lack of expression of CD34 in the SVF of human adipose tissue in culture (203). Moreover, in the appropriate culture conditions, these cells were shown to express distinct phenotype features of various lineages: adipogenic, chondrogenic, myogenic as well as osteogenic (203) leading to the hypothesis that the SVF of human adipose tissue contains pluripotent adult stem cells that exhibit some similarity with the mesenchymal stem cells in the bone marrow (93,141,196). One potential confounding factor in these studies was that the cells had been expanded in vitro in the presence of high serum concentration, conditions usually associated with cell dedifferentiation and alterations in the expression of cell surface markers that define stem and stromal cells. Long term in vitro culture of cells from the SVF has also been associated with spontaneous transformation and immortalization leading to tumoriginicity in vivo. Our study was the first to demonstrate that non-expanded and selected cells from the

human adipose tissue also exhibit endothelial progenitor cell properties and to indicate that these cells would then represent an attractive cell population for therapeutic strategies to increase vascularization of damaged or ischemic tissues. Indeed, adipose tissue can be easily harvested and the proportion of the CD34+/CD31- that can be isolated is sufficient for re-injection to the same patient, thus avoiding inflammation reactions and additional expansion steps. However, additional studies performed by our group have demonstrated that the CD34+/CD31- cells are the only cells within the human adipose tissue SVF that are able to differentiate into adipocytes (164). Thus, the CD34+/CD31- cells appear to possess the ability to differentiate into two distinct lineages, endothelial cells and adipocytes. Such a population potentially also contributes to the growth of the adipose tissue by acting as a source of adipocytes, through adipogenesis as well as new capillaries through vasculogenesis. Nevertheless because of this bipotentiality, it is necessary to further characterize this cellular population in order to determine the factors involved in their commitment into the adipocyte or endothelial lineage and to better control the fate of the cells before potential therapeutic application to increase vascularization of damaged tissues can be considered further.

IV.2.2. Paracrine interactions involved in the control of BEC trophic capacities

To assess the local signals involved in the regulation of the growth of BEC within the adipose tissue, the effects of conditioned media originated from mature adipocytes or progenitor cells on BEC proliferation were analyzed. Indeed, adipocytes produce a wide range of pro-angiogenic factors (23) and progenitor cells present in the SVF of human adipose tissue can also stimulate angiogenesis through the production of angiogenic factors such as VEGF A and HGF (147). Our results clearly demonstrate that the proliferation of human adipose tissue derived-BEC increased in response to factors released from mature adipocytes but was not influenced by soluble factors derived from progenitor cells. Moreover, since the transcripts for the receptors of leptin and adiponectin, were identified in the human adipose tissue derived-BEC and since both adipokines have been described to exert proliferative effects in other models of human endothelial cells (22,133,167,168), we assessed the effects of leptin and adiponectin at increasing concentrations together with the different VEGF forms on BEC proliferation. While neither leptin, adiponectin nor VEGF forms alone affected the proliferation of BEC, leptin in combination with VEGFA or with bFGF led to a significant increase in BEC proliferation. This observation is in agreement with several studies performed on human capillary endothelial cells which showed that such cells do not exhibit

strong proliferative responsiveness to classical pro-angiogenic factors (202). Moreover, a synergistic effect of leptin with VEGF A and bFGF on BEC proliferation has been reported (31). Taken together, our results strongly suggest that leptin produced by the mature adipocytes is involved in the control of BEC proliferation in the presence of low concentration of VEGF A and/or bFGF, both of which are also produced by adipocytes. Growth-factor reduced Matrigel assays showed that leptin enhances BEC migration and the formation of tube-like structures whereas neither adiponectin nor any of the different VEGF forms exerted any effects. The classical intracellular signaling pathway activated by leptin involves the janus kinase/signal transducer and activator of transcription JAK/STAT pathway (for review see (57)). However, leptin has also been shown to activate other signaling pathways such as the mitogen-activated protein kinases (MAPK) cascade as well as the protein kinase B (PKB or Akt) (for review see (57)). In endothelial cells, the activation of the MAPK signaling pathway has been linked with survival and proliferation whereas the activation of Akt has been associated with cell migration (124). In BEC leptin alone failed to induce the phosphorylation of Erk1/2, a finding that is in agreement with our observations that leptin alone did not induce BEC proliferation. However, leptin induced a significant increase in Akt phosphorylation suggesting that the leptin-mediated increase in BEC migration and structural reorganization might be at least in part mediated through Akt-dependent signaling pathway, as in fact has already been suggested by studies on human umbilical vein endothelial cells (63).

The angiogenic properties of leptin have been described in various endothelial cell models. Indeed, leptin was shown to induce the proliferation (22) and migration (168) of human endothelial cells and to trigger neovascularization in vivo in various models such as the chick chorioallantoic membrane (22) and rabbit cornea (168). However, it remains to be demonstrated whether the local production of leptin within adipose tissue has any effect on the resident endothelial cells. Since the expression and production of leptin by the mature adipocytes is increased in obesity (40,132), it is tempting to speculate that leptin might be involved in the neovascularization process that occurs during adipose tissue growth and that mature adipocytes themselves can influence the remodeling and expansion of the vascular network. However, further experiments need to be performed to clearly demonstrate the involvement of leptin in angiogenesis within the adipose tissue. One important question that remains to be answered is whether the endothelial cells in the adipose tissue from obese patients are still responsive to leptin. Indeed, obesity in humans is characterized by an hyper-leptinemia and associated with the development of leptin resistance (50,181). Although

several studies suggest that the leptin resistant state might be restricted to some specific tissues (181), whether or not the cells within adipose tissue themselves develop resistance to the adipokine is not known.

IV.2.3. Paracrine interactions involved in the control of LEC trophic capacities

To characterize the local signals potentially involved in the control of the growth of LEC within the adipose tissue, the effects of conditioned media from mature adipocytes or progenitor cells on LEC proliferation were analyzed. As observed for BEC, adipocyte- but not progenitor cell-conditioned media markedly increased the proliferation of LEC. The lack of proliferative effect of the progenitor cell-conditioned medium was not expected since the transcripts for the main lymphangiogenic factors described, VEGF C and VEGF D, were actually expressed at higher levels in progenitor cells than in adipocytes. However, as the actual protein concentrations of VEGF C and VEGF D were not analyzed in our study, we can not exclude that growth factor production simply failed to attain the threshold required to initiate proliferation. Indeed, VEGF C is able to stimulate LEC proliferation but high concentrations are required, much higher than reported in the literature (119).

Interestingly, among the mRNA for the various receptors detected on LEC, levels of the adiponectin receptor-1 were highest. Treatment of LEC with increasing concentrations of adiponectin enhanced LEC proliferation whereas no effect of leptin was observed. The latter observation is probably related to the very low levels of leptin receptors that were detected in LEC. Moreover, the comparison of the transcript levels for the leptin receptor in BEC and LEC showed a statistically significant discrepancy suggesting that the expression of leptin receptor might be considered as a marker of vascular endothelial cells. The lack of effect of leptin on the LEC further supports the hypothesis that leptin is an angiogenic but not lymphangiogenic factor.

Adiponectin also significantly increased the number of tubes and branching structures in growth factor-reduced Matrigel assays. Until now, no studies had pointed out the potential role of adiponectin in the regulation of growth, migration and organization of human LEC. Several studies have described effects of adiponectin on vascular endothelial cells, albeit controversially and pro-angiogenic as well as pro-apoptotic effect of adiponectin have been reported (26,133). A recent study used loss- and gain-of-function genetic manipulations to show in an elegant manner in vivo that adiponectin is a pro-angiogenic factor (167). The discrepancies in the effects of adiponectin reported in vitro might arise from the different

origins and culture conditions of the endothelial cells used in the respective studies. Our results suggest that the effect of adiponectin is largely dependent on the type of endothelial cells studied since we did not observe any effect of adiponectin on BEC. It is tempting to speculate that the relative expression of the different adiponectin receptors might play a role as we observed a higher expression of the adiponectin receptor-1 in LEC than in BEC. Recently, T-cadherin has been suggested to constitute an active binding site for adiponectin (81). The determination of T-cadherin expression in BEC and LEC should provide information about the potential involvement of such a system in the lymphangiogenic effects of adiponectin. Another aspect that might play a role in the reported controversial effect of adiponectin is the nature of the recombinant protein used to treat the cells. Indeed, the first studies performed with adiponectin used human recombinant adiponectin produced in non-eucaryotic cells (16) that did not exhibit the post-translational features of the human adiponectin. This is important as adiponectin is a complex protein that oligomerizes to give different forms that can exert distinct effects at the cellular level (37,136,176,189). The concentration of adiponectin used might also play an important role as adiponectin is present in the plasma at high concentrations (micromolar range) and most of the studies used high adiponectin concentrations to treat the cells (26,133). However the plasma concentration of adiponectin reflects its time-dependent accumulation and not the rate of production and release of the adipokine by mature adipocytes (in the nanomolar range). Furthermore, plasma adiponectin has a long half-life, especially the high molecular weight forms. In the present study, we used nanomolar concentrations as we considered this reflected more accurately the local adiponectin concentration in adipose tissue.

Many of the effects of adiponectin have been shown to be mediated by the AMP-activated protein kinase (AMPK). Indeed, it has been shown that AMPK signaling mediates adiponectin-induced angiogenic and anti-apoptotic cellular responses in endothelial cells (98,133). There is a crosstalk between the AMPK and Akt and AMPK activation leads to the activation of Akt and the phosphorylation of both kinases is required for the induction of angiogenesis by adiponectin (133). Here we demonstrate that adiponectin induced Akt phosphorylation in adipose tissue derived-LEC. Although the effect of adiponectin on the AMPK in LEC needs to be studied, Akt activation via the AMPK signaling pathway may be involved in the adiponectin effect on LEC proliferation and migration

In the present study, we identified adiponectin as a novel lymphangiogenic factor in human adipose tissue. Other factors have been shown to exert lymphangiogenic effects and IL-7 as well as HGF can induce LEC proliferation, migration and tube formation in human dermal

cells (6,86). In addition, angiopoietin-1, a well characterized angiogenic factor, has recently been described to promote lymph vessel formation in addition to angiogenesis in the model of mouse cornea (125). Since adiponectin, an adipokine produced by adipocyte, is a lymphangiogenic factor and that the growth of the fat mass is not associated with an extension of the lymphatic vascular network, one can speculate that obesity is associated with an inhibition of the adiponectin-mediated stimulation of the trophic capacities of the LEC. Clearly different hypothesis might be suggested and adiponectin gene expression might be locally decreased or adiponectin-mediated cell signaling might be impaired with obesity. Adiponectin expression in adipocytes has been shown to be regulated by different hormones and cytokines. In particular, the pro-inflammatory cytokines TNFα and IL-6, the concentrations of which increase during the development of adipose tissue, decrease adiponectin expression (53,54). Thus the development of a chronic low-grade inflammatory state in the obese adipose tissue (138) might contribute to the down-regulation of adiponectin expression and lead to inhibition of lymphangiogenesis. Further studies are also needed to characterize the signaling pathways activated by adiponectin in order to determine whether the signal transduction or the expression/function of the adiponectin receptors are altered during the development of obesity. Interestingly, the levels of another lymphangiogenic factor angiopoietin-1 are reported to be depressed in the fat mass during obesity in mice (46). Such an effect might also contribute to a defect in the processes controlling lymphangiogenesis.

The lymphatic system plays an important role in tissue immune surveillance as well as in tissue fluid homeostasis since it absorbs the excess fluid and cells from tissues and restores them to the circulation (5,7). It has been demonstrated that the rate of adipose tissue lymphatic drainage is inversely correlated to its growth (157). In agreement with this, mice treated with inhibitors of VEGFR3 signaling (soluble VEGFR3 or anti-VEGFR3 antibody) which leads to the inhibition of lymphangiogenesis, diplayed a thicker subcutaneous adipose tissue layer (104,118). Moreover, a recent study has demonstrated that an insufficiency of the lymphatic system is associated with the development of obesity. Indeed, the Prox1 haploinsufficiency in mice, that led to lymphatic vascular defect, was associated with a late-onset development of obesity. (66). These observations have for the first time provided strong evidence that a lack or defect of lymphatic vascular system favors the development of the fat mass. However, the mechanisms underlying such an effect remain to be studied. Harvey et al. have shown that lymph itself stimulates adipogenesis, suggesting that delays in its removal will contribute to adipose tissue accumulation (66). These observations make the lymphatic vascular system of the adipose tissue another putative therapeutic target to modulate the accumulation of adipose

tissue. Indeed, one can speculate that the stimulation of lymphangiogenesis might restore a sufficient lymphatic drainage, thus preventing excessive accumulation of adipose tissue. A better understanding of the factors and mechanisms involved in the development of the lymphatic vasculature might then lead to the development of novel therapeutic tools to restrict the development of adipose tissue as well as the genesis of obesity-associated diseases.

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Die Ursache von Adipositas liegt im übermäßigen Wachstum von Fettgewebe, welches hauptsächlich aus Fettzellen, den Adipozyten, besteht. Die Zellen der stroma-vaskulären Fraktion, welche Vorläuferzellen, Makrophagen und Zellen des lokalen Gefäßnetzwerks enthält, sind außerdem an der Homöostase des Fettgewebes beteiligt. Insbesondere spielt das Gefäßsystem des Fettgewebes in Nagetieren eine wichtige Rolle im Fettgewebewachstum, da die Hemmung der Angiogenese in genetisch- und diät-induzierten fettleibigen Mäusen die Entstehung von Adipositas verhindert. Dennoch wurde das Gefäßsystem des menschlichen Fettgewebes bis heute nicht erforscht.

Durch immuno-histochemische Analysen am subkutanen menschlichen Fettgewebe konnten wir zwei verschiedene Gefäßsysteme identifizieren: das vaskuläre Netzwerk des Bluts und das lymphatische vaskuläre Netzwerk. Während die Endothelzellen von beiden Gefäßsystemen die gemeinsamen Endothelzellmarker von Willebrand factor (vWf) und CD31 (PECAM, Platelet Endothelial Cell Adhesion Molecule) exprimierten, konnten die Endothelzellen der Blutgefäße an der Expression des Markers CD34 (Stamm/Blutgefäß-Endothel-Zell-Marker) und die Endothelzellen der Lymphgefäße an der Expression der beiden lymphatischen Marker Podoplanin und VEGFR3 (Vascular Endothelial Growth Factor Receptor 3) spezifisch erkannt werden. Ausschließlich für den Marker CD34-positive Zellen und in Rosetten angeordnete CD31-positive Zellen, welche als residente Makrophagen wurden auch charakterisiert.

Um die beiden Gefäßsystemen des menschlichen Fettgewebes weiterhin zu erforschen, haben wir ein auf Immunoselektion basiertes Protokoll entwickelt. Es ermöglicht, Blut- (BEC) und lymphatische (LEC) Endothelzellen aber auch Makrophagen und CD34-positive Zellen spezifisch zu isolieren. Sowohl BEC als auch LEC exprimierten VEGFR1, VEGFR2, vWf und Notch4 und nehmen acetyliertes LDL auf. Darüber hinaus konnte in LEC die Expression von Genen, welche spezifisch für das Lymphgefäßsystem sind, wie Podoplanin, Reelin, VEGFR3, Desmoplakin, LYVE-1 nachgewiesen werden.

Durch fluss-cytometrischen Analysen des Anzahls von BEC und LEC im Fettgewebe von Patienten mit unterschiedlichen Body Mass Indices (BMI) wurde entdeckt, dass Fettleibigkeit von einer Erweiterung des vaskulären Netzwerks des Bluts im Fettgewebe begleitet wird, jedoch nicht von einer Erweiterung des lymphatischen vaskulären Systems.

Flusscytometrische Analysen belegen, dass es in der CD34-positive Stroma-Zellpopulation Zellen gibt, die den endothelialen Progenitor-Zellmarker CD133 und den primitiven Stammzellmarker ABCG2 exprimieren. Außerdem zeigten die CD34-positive Zellen eine signifikant stärkere Proliferation und Expression von Endothelzellmarkern wie CD31 und

vWf, wenn dem Kulturmedium zuvor die Faktoren Vascular Endothelial Growth Factor A (VEGF A) und Insulin-Like Growth Factor-1 zugefügt worden waren. Wurden Mäusen mit Hinterbeinischämie CD34-positive Zellen in vivo injiziert, beteiligten sich diese Zellen an der Neovaskularisation des ischämischen Hinterbeins. Eine signifikante Zunahme des Blutflusses im ischämischen Bein, gekoppelt an einer erhöhten Kapillardichte im ischämischen Muskel und einer Integration der menschlichen Zellen in die Vaskulatur der Maus waren erkennbar. Diese Ergebnisse weisen darauf hin, dass es unter den CD34-positive Zellen eine Population von endothelialen Progenitorzellen gibt, die -bei geeigneter Stimulation- zu Endothelzellen differenzieren.

Parallel dazu wurden die lokalen Faktoren untersucht, die potentiell an der Wachstumskontrolle, der Migration und der Organisation der ruhenden, aus dem Fettgewebe stammenden, BEC und LEC beteiligt waren. Sekrete der Adipozyten, jedoch nicht der CD34-positive Zellen, induzierten eine signifikante BEC- und LEC-Proliferation. Außerdem induzierte die Kombination von Leptin und VEGF A oder des basic Fibroblast Growth Factor eine signifikante Zunahme der BrdU-Inkorporation in BEC während Adiponectin, VEGF C und VEGF D bereits alleine konzentrationsabhängig die Proliferation von LEC induzierten. Leptin, und nicht Adiponectin, führte zu signifikant höherer BEC-Migration und Röhrenformung, während Adiponectin, und nicht Leptin, die LEC-Migration und -Organisation förderte. Dabei führte Leptin in BEC und Adiponectin in LEC zeitabhängig zu einer signifikanten Zunahme der Phosphorylierung der Kinase Akt. Diese Ergebnisse belegen, dass die beiden aus Adipozyten stammenden Adipokine Leptin und Adiponectin eine tragende Rolle in der Umverteilung von BEC bzw. LEC spielen.

Im Rahmen der Adipositas steigt die Plasmakonzentration von Leptin an während die Plasmakonzentration von Adiponectin sinkt. Unsere Ergebnisse deuten daraufhin, dass Leptin als lokaler pro-angiogenetischer Faktor identifizieren und Adiponectin als neuer lymphangiogenetischer Faktor im menschlichen Fettgewebe beschreiben konnte. Demnach könnten Veränderungen, in der Adipositas, der Adipokinfreisetzung durch Adipozyten am Umbau des vaskulären Netzwerks des Bluts und am ausbleibenden Wachstum des lymphatischen vaskulären Systems innerhalb des Fettgewebes beteiligt sein. Schließlich belegen die vorliegenden Ergebnisse das Vorhandensein einer Progenitor-Zell-Population in der Stroma-Fraktion des menschlichen Fettgewebes. Diese Progenitor-Zellen sind in der Lage sich an der Neovaskularisation ischämischen Gewebes zu beteiligen. Diese Population könnte im Hinblick auf zelltherapeutische Strategien eine interessante Alternative zu Stammzellen aus dem Knochenmark darstellen.

Alexandra MIRANVILLE

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Date of birth	29th April 1979 (Reunion Island, France)
Nationality	French
Marital Status	Single

I–ACADEMIC DEGREES

2002-2003 **Equivalence of Diplom Arbeit (preparatory year of PhD) in physiology**
"Characterization of the different cellular populations of the stroma-vascular fraction of human adipose tissue"
Project leader: Dr. Anne Bouloumié (Sofia Kovaleskaja award)
Institute of Cardiovascular Physiology, JW Goethe University, Frankfurt/Main, Germany
Chairman: Pr.Dr. Rudi Busse – Mentors: Dr. Anne Bouloumié, Pr. Dr. Jürgen Bereiter-Hahn

2000-2001 **Maîtrise in cellular biology and physiology specialized in animal physiology**
Université Paul Sabatier, Toulouse, France

1999-2000 **Licence in biology (BSC) with specialization in cellular biology**
Université Paul Sabatier, Toulouse, France

1997-1999 **Diplôme d'études universitaires générales (Diploma of general higher education) in life sciences**
Université de la Réunion, Saint-Denis de la Réunion, France

1997 **Baccalaureate in life sciences (French secondary school diploma)**
Lycée Roland Garros du Tampon, Reunion Island, France

II–PROFESSIONAL EXPERIENCE

Since 2003 **PhD student**
"Characterization and remodelling of the vasculature in human adipose tissue"
Project leader: Dr. Anne Bouloumié (Sofia Kovaleskaja award)
Institute of Cardiovascular Physiology, JW Goethe University, Frankfurt/Main, Germany
Chairman: Pr.Dr. Rudi Busse – Mentors: Dr. Anne Bouloumié, Pr. Jürgen Bereiter-Hahn

2001-2002 **Practical trainee (4 months)**
"Introduction of biotechnological data in the development of cosmetics"
Yves Rocher research laboratories

2001 **Practical trainee (2 months)**
"Influence of hypoxia on murine preadipocytes differentiation (3T3F442A)"
French National Institute for health and medical research (INSERM)- Obesity research Unit
Mentor: Dr. Anne Bouloumié

Technical know how

Cell culture	Immunoselection and primary culture of stroma-vascular cells (endothelial cells, macrophages, precursor cells) and adipocytes from human white adipose tissue and of murine preadipocytes (3T3F442A). Proliferation assay, migration assay (boyden chamber), tube formation assay (matrigel)
Protein analysis	*Protein extraction, immunoprecipitation and western blot* Gelatine zymography (matrix metalloproteinase activity) Immunocytochemistry Fluorescent activating cell sorter analysis (FACS)
Gene analysis	mRNA isolation, RT-PCR, real-time quantitative RT-PCR (Taqman®)
In vivo	Ligature of femoral artery in mouse hind limb and laser Doppler imaging Subcutaneous injection of matrigel plugs

Proficient with PC and Mac compatibles, including a number of software applications (Excel, Word, Prism, Adobe Photoshop, PowerPoint).

III–ARTICLES, COMMUNICATIONS, ABSTRACTS

Original publications

Curat CA, Wegner V, Sengenès C, **Miranville A**, Tonus C, Busse R, Bouloumié A
Macrophages in the human visceral adipose tissue: Increased accumulation with obesity and source of resistin and visfatin
Diabetologia 2006; 49(4): 744-7

Miranville, A.; Heeschen, C.; Sengenès, C.; Curat, C.A.; Busse, R.; Bouloumié, A.
Improvement of postnatal neovascularization by human adipose tissue-derived stem cells
Circulation 2004; 110 (3): 349-355

Curat, C.A.; **Miranville, A**.; Sengenès, C.; Diehl, M.; Tonus, C.; Busse, R.; Bouloumié, A.
From blood monocytes to adipose tissue-resident macrophages - Induction of diapedesis by human mature adipocytes
Diabetes 2004; 53(5): 1285-1292

Reviews

Bouloumié, A.; Curat, C.A.; Sengenès, C.; Lolmède, K.; **Miranville, A**.; Busse, R.
Role of macrophage tissue infiltration in metabolic diseases
Curr Opin Clin Nutr Metab Care 2005; 8(4): 347-354

Miranville A, Lafontan M, Bouloumié A
Leptin and angiogenesis (article in french)
Sang, thrombose, Vaisseaux 2004; 5(16): 243-247

Abstracts

Miranville A, Sengenès C, Curat CA, Heeschen C, Busse R, Bouloumié A
Is adipose tissue development due to its own local bank of stem cells?
Circulation Research 2004; 94:1523 (supplement data) (Poster presentation)
1st Annual Symposium of the American Heart Association council on basic cardiovascular sciences: Stress signals, molecular targets and the genome (July 2004, Stevenson, Washington, USA)
Travel award

Curat, C.A.; **Miranville, A**.; Sengenès, C.; Busse, R.; Bouloumié, A.
Fat tissue growth is an inflammatory process
International Journal of Obesity 2004; 28: S98 (Poster presentation)
13th European Congress on Obesity (May 2004, Prague, Czech republic)

Miranville, A.; Sengenès, C.; Curat, C.A.; Busse, R.; Bouloumié, A.
Human adipose tissue-derived stem cells improve postnatal neovascularization
International Journal of Obesity 2004; 28: S100 (Poster presentation)
13th European Congress on Obesity (May 2004, Prague, Czech republic)

Sengenès, C.; **Miranville, A**.; Curat, C.A.; Busse, R.; Bouloumié, A.
The adipo-angioblast: A common precursor cell in human adipose tissue
International Journal of Obesity 2004; 28: S103 (Poster presentation)
13th European Congress on Obesity (May 2004, Prague, Czech republic)

Curat, C.A.; **Miranville, A**.; Sengenès, C.; Busse, R.; Bouloumié, A.
Fat tissue growth is an inflammatory process
European Journal of Physiology 2004, 447:S29 (Oral presentation)
83rd Meeting of the German Physiological Society (March 2004, Leipzig, Germany)

Miranville A, Sengenès C, Curat CA, Busse R, Bouloumié A
Human adipose tissue stem cells improve blood flow in the mouse model of ischemic hind limb
European Journal of Physiology 2004, 447:S29 (Oral presentation)
83rd Meeting of the German Physiological Society (March 2004, Leipzig, Germany)

Sengenès, C.; **Miranville, A**.; Curat, C.A.; Busse, R.; Bouloumié, A.
The adipo-angioblast: A common precursor cell in human adipose tissue
European Journal of Physiology 2004, 447:S29 (Oral presentation)
83rd Meeting of the German Physiological Society (March 2004, Leipzig, Germany)

Curat, C.A.; **Miranville, A**.; Sengenès, C.; Bouloumié, A.
Obesity as an inflammatory state
Circulation 2003; 108(17): 107 (Poster presentation)
American Heart Association – Scientific Sessions (Nov 2003, Orlando, Florida, USA)

Miranville, A.; Sengenès, C.; Curat, C.A.; Busse, R.; Bouloumié, A.
Human adipose tissue-derived stem cells improve blood flow in the ischemic mouse hind limb
Circulation 2003; 108(17): 164 (Oral presentation)
American Heart Association – Scientific Sessions (Nov 2003, Orlando, Florida, USA)

VI–NON SCIENTIFIC ACTIVITIES

Oct 2000-Feb 2001:	Commercial at the OFUP (academic press office) Customer prospecting for subscription to scientific press After sales service
Jul-Aug 1999	Private teaching Revision of the one-year program of biology with one student

VII–LANGUAGES

French: native language
English: advanced
German: intermediate (Kleines Deutsches Sprachdiplom obtained in November 2005)

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Plus le temps passe et plus je me rapproche de mon but (Alexandra Miranville ;-))
Ti hach y coup gros bois (Proverbe de la Réunion)