Tampere University

OUTI HUTTALA

Human In Vitro Vascularization, Adipocytes and Vascularized Adipose Tissue

Three relevant models for studies of vascularization, adipocytes and their interaction

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ACADEMIC DISSERTATION
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To all those numerous people
who have inspired me in numerous ways during my research

There are only two ways to live your life. One is as though nothing is a miracle.
The other is as though everything is a miracle.
- Albert Einstein

ABSTRACT

Adipose tissue is a highly vascularized endocrine tissue. Due to the ever growing problem of obesity, the interest in determining mechanisms behind adipose tissue imbalances and identifying substances causing metabolic disturbances in adipose tissue is increasing. There are several drug treatments targeting vasculature and/or adipose tissue currently available and constantly new ones being developed including cancer, eye condition, insulin sensitizing and obesity treatments. Hence drug development needs reliable and relevant models for development and testing of these treatments. In addition, more attention has been focused on chemicals in our living environment that inadvertently affect adipose tissue. These obesogens need to be identified as they are especially harmful to fetuses and children. In order to identify these, relevant test systems are needed. Most adipose tissue related research is still conducted on animals or animal cells. Due to the species-to-species differences in adipose tissue regulation, cell models depicting human biology are greatly needed.

The aim of this study was to develop an *in vitro* vascularized adipose tissue model. A bottom-up approach was utilized, first optimizing the building blocks for the model and then moving on to combining these components. First, serum-free induction medium was developed for induction of *in vitro* vascularization (i.e. angiogenesis model) in human adipose stromal cells (hASC) and human umbilical vein endothelial cell (HUVEC) co-cultures. Second, a protocol for inducing insulin sensitive adipocytes from hASC was developed. In the third step, vasculature and adipocytes were combined using simplified induction protocols to provide a proof of concept model of vascularized adipose tissue and to determine the optimal culture time, timing of induction media, and timing of seeding of hASC and HUVEC. Finally, we combined the two optimal components, insulin sensitive adipocyte protocol and vasculature utilizing the culture set-ups that were found to be optimal for creating the vascularized adipose tissue model. We studied the predictivity of this model by exposing it to chemicals, including insulin, with known effects on adipose tissue, and compared responses to those found in literature.

Results showed that the angiogenesis model induced with this serum-free medium produced an extensive vascular network with morphology resembling vessels *in vivo* including the intact endothelial layer, basement membrane, 3D tubules

with lumen, extracellular matrix proteins and surrounding pericytes. The adipocytes differentiated from hASC with the new protocol accumulated lipids, showed mature adipocytes markers and responded to insulin by lipolysis inhibition and glucose uptake. The vascularized adipose tissue model contained an *in vivo*-like vascular network and mature adipocytes that were responsive to insulin. The interaction of adipocytes with vascularization was seen to result in faster maturation of adipocytes and better insulin sensitivity. Also the impact of chemicals on vascularized adipose tissue was different than on adipocytes alone.

The angiogenesis model with serum-free induction and the insulin sensitive adipocyte model are relevant *in vitro* models that can be used independently for studying angiogenesis and adipogenesis. The combined model, vascularized adipose tissue model, is a useful tool for adipose tissue research, including toxicological, drug discovery and cell biology research applications.

TIIVISTELMÄ

Rasvakudos on verisuonitettu endokriininen kudos. Kasvavan liikalihavuusepidemian takia rasvakudoksen toiminnan ja aineenvaihdunnan sekä näiden häiriötilojen tutkiminen ja rasvakudokseen vaikuttavien aineiden tunnistaminen on noussut entistä tärkeämmäksi tutkimusaiheeksi. Verisuonituksen ja rasvakudoksen toimintaan ja määrään pyritään tietoisesti vaikuttamaan lääkkeillä. Lääkkeiden toiminta täytyy osoittaa ennen kuin lääkkeet päätyvät markkinoille. Elinympäristössä on myös kemikaaleja, joiden vaikutuksia rasvakudokseen ei ole tiedostettu. On tärkeää tunnistaa nämä obesogeenit sillä ne ovat erityisen haitallisia sikiöille ja kehittyville lapsille. Jotta nämä voidaan tunnistaa, tarvitaan luotettavia testisysteemejä. Rasvakudokseen liittyvät tutkimukset tehdään kuitenkin edelleen useimmiten eläimillä tai eläinsoluilla. Lajien väliset erot rasvakudoksen säätelyssä ovat kuitenkin huomattavia ja siksi tarvitaan solumalleja, jotka kuvaavat ihmisen biologiaa

Tässä tutkimuksessa kehitettiin verisuonitettu rasvakudosmalli ihmisen soluista. Tutkimuksessa kehitettiin ensin erillään mahdollisimman luonnollisen kaltaiset *in vitro* verisuonet ja rasvasolut, jonka jälkeen nämä yhdistettiin verisuonitetuksi rasvakudokseksi. Verisuonten muodostumista varten kehitettiin seerumiton kasvatusliuos rasvan stroomasolujen ja napanuoran endoteelisolujen yhteisviljelmässä käytettäväksi (angiogeneesimalli). Seuraavaksi kehitettiin protokolla insuliiniherkkien rasvasolujen erilaistamiseksi rasvan stroomasoluista. Kolmannessa vaiheessa verisuonitus yhdistettiin rasvasoluihin yksinkertaisia menetelmiä käyttäen, jotta saatiin selville sopiva viljelyaika mallille, solujen siirrostusajankohta sekä erilaistuksen aikaansaavien kasvatusliuosten lisäysjärjestys verisuonitetun rasvamallin aikaansaamiseksi. Näitä parhaiksi havaittuja viljelyolosuhteita hyväksi käyttäen yhdistettiin luonnollisen kaltaiset verisuonet ja rasvasolut verisuonitetuksi rasvamalliksi. Tämän mallin ominaisuudet ja vaste kemikaaleille, myös insuliinille, tutkittiin ja varmistettiin, että malli ja sen toiminta ovat luonnollisen rasvakudoksen kaltaisia.

Tulokset osoittivat, että angiogeneesimalliin muodostui hyvä verisuoniverkosto, jossa suonissa oli endoteelikerros jota ympäröi tyvikalvo. Suonet olivat kolmiulotteisia putkia, joissa oli lumen ja soluväliaine ja perisyytit ympäröivät näitä

suonia. Rasvasolut keräsivät rasvaa, ilmensivät kypsän rasvasolun geenejä ja reagoivat insuliiniin lipolyysin estymisellä ja glukoosin sisäänotolla. Verisuonitetussa rasvakudosmallissa olevat suonet olivat luonnollisten suonien kaltaisia, rasvasolut olivat edelleen kypsiä rasvasoluja ja malli reagoi insuliinille oikein. Verisuonituksen vaikutus rasvasoluihin nähtiin rasvasolujen nopeampana kypsymisenä ja parantuneena insuliiniherkkyytenä. Verisuonten nähtiin myös vaikuttavan kemikaalivasteisiin mallissa.

Kaikki kolme mallia, verisuonet, rasvasolut ja verisuonitettu rasva ovat itsenäisesti käytettäväksi soveltuvia malleja angiogeneesin ja adipogeneesin tutkimiseen. Verisuonitettua rasvakudosmallia voidaan käyttää laajasti rasvakudoksen tutkimiseen mm. toksikologian, lääkekehityksen ja solubiologian aloilla.

TABLE OF CONTENTS

ABBREVIATIONS

3D	three dimensional
3R	reduction, refinement and replacement
AA	ascorbic acid
ANG +T	adipogenesis/angiogenesis medium
ANG -I	angiogenesis medium w/o insulin
ANG	angiogenesis medium
Angpt	angiopoietin
ATE	adipose tissue extract
ATEm	ATE medium
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
COLIV	collagen IV
ECM	extracellular matrix
EGM-2	endothelial cell growth medium-2
eNOS	endothelial nitric oxide synthase
FABP4	fatty acid binding protein 4
FBS	fetal bovine serum
FCS	fetal calf serum
FGF-2	fibroblast growth factor 2
Glut	glucose transporter
(h)ASC	(human) adipose stromal cells
hASCm	hASC medium
HE	heparin
HUVEC	human umbilical vein endothelial cells
HY	hydrocortisone
IGF-1	insulin-like growth factor I
IL	interleukin
IRS	insulin receptor substrate proteins
LPL	lipoprotein lipases
MMP	matrix metalloproteinase

MSC	mesenchymal stem cells
PDGFR(-β)	platelet derived growth factor (β)
PDK	phosphoinositide-dependent protein kinase
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PPAR	peroxisome proliferator–activated receptor
PRDM16	PR domain containing 16
RT	room temperature
SEM	scanning electron microscopy
SFM	serum-free medium
SMM	Smooth muscle myosin heavy chain,
t3	3,3',5-triiodo-l-thyronine sodium salt
TEM	transmission electron microscopy
TZD	thiazolidinedione
UCP-1	uncoupling protein 1
ve-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vWf	von Willebrand factor

ORIGINAL PUBLICATIONS

The thesis is based on the following articles, which are referred to in the text by their Roman numerals (**I-IV**):

Publication I **Huttala O**, Vuorenpää H, Toimela T, Uotila J, Kuokkanen H, Ylikomi T, Sarkanen JR, Heinonen T. Human Vascular Model with Defined Stimulation Medium – A Characterization Study. Altex 2015;32(2):125-36.

Publication II **Huttala O**, Mysore R, Sarkanen JR, Heinonen T, Olkkonen VM, Ylikomi T. 2016. Differentiation of human adipose stromal cells *in vitro* into insulin-sensitive adipocytes. Cell and Tissue Research 2016;366(1):63-74.

Publication III **Huttala O,** Palmroth M, Hemminki P, Toimela T, Heinonen T, Ylikomi T, Sarkanen JR. Development of versatile Human *In vitro* Vascularized Adipose Tissue Model with Serum-Free Angiogenesis and Natural Adipogenesis Induction. Basic & Clinical Pharmacology & Toxicology 2018;123 Suppl 5:62-71.

Publication IV **Huttala O,** Sarkanen JR, Heinonen T, Ylikomi T. Presence of vasculature results in faster insulin response in adipocytes in vascularized adipose tissue model. Submitted to ALTEX 2019 [Epub ahead of print]

1 INTRODUCTION

In recent years, the development of human research models that depict *in vivo* human tissues and biology has become increasingly important due to pressure from regulatory, scientific and ethical aspects to replace animal testing with non-animal approaches. Following the introduction of EU acts such as REACH and cosmetics regulations (European Union 2010, European Union 2009, European Union 2006), the need and interest for official *in vitro* tests has increased. The reduction, refinement and replacement (3R) principle, and ethical aspects they represent, have long been one motivation to reduce and replace animal tests with *in vitro* methods, with work in this field continuing (Hampshire et al. 2018, Mallia et al. 2018, Franco et al. 2018).

In addition to the regulatory and ethical aspects, more and more evidence shows the differences between human and rodent biological processes, with adipose tissue being one example tissue clearly presenting such differences (Heinonen 2015, Chandrasekera et al. 2014, Al-Awar et al. 2016). As adipose tissue and its metabolism differ so greatly between rodents and humans, it is of special importance to find replacements for animal tests in the field of adipose research.

Due to the continuously growing problem of obesity and related diseases (World health organization WHO 2018), biological adipose tissue research is more and more important and the need to develop new relevant therapeutics and understand the underlying mechanisms behind adipose related diseases is great. Among the diseases related to adipose tissue are diabetes mellitus, hypertension, stroke, cardiovascular disease, dyslipidemia, gallbladder disease, cancer, respiratory disease, arthritis and gout (Pi-Sunyer 2009). Health issues related to adipose tissue are linked to an increase in the amount of white adipose tissue rather than that of brown (Fruhbeck et al. 2009). Great efforts have been put into elucidating the pathophysiological mechanisms by which obesity induces or amplifies its major adverse consequences and for developing effective therapies towards obesity and its associated disorders (Cao 2014). Environmental factors, which might impact adipose tissue and be part of the increase in obesity, have also raised interest (Heindel et al. 2018, Muscogiuri et al. 2017, Janesick et al. 2016, Holtcamp 2012).

In addition to being energy storage, adipose tissue is an active endocrine organ, which is particularly closely associated with the vascular system. Enlargement of the

adipose tissue can be supported by new blood vessel formation, neovascularization, or by dilation and remodeling of the already existing capillaries (Christiaens et al. 2010). Studies have shown that induction of vessel expansion could counteract obesity and related metabolic complications (Robciuc et al. 2016, Elias et al. 2012). The attempt to introduce vascularization into *in vitro* adipocyte cultures has been challenging due to the commonly used chemical cocktails utilized for adipocyte differentiation. The compounds in this chemical cocktail have been shown to inhibit endothelial proliferation and tubule formation (Kang et al. 2009, Sheu et al. 2006).

While developing *in vitro* models, in addition to biological relevance, regulatory aspects have to be taken into consideration. The repeatability and reproducibility of an assay should be ensured already during development of the new methods. OECD has determined the criteria that *in vitro* tests should pass in order to be suitable for regulatory use (Environment Directorate 2005).

The ultimate aim of this study was to create a vascularized adipose tissue model to be used as a tool for adipose tissue research and chemical testing to better depict human biology. The individual optimized components; induced vascularization and differentiated adipocytes, should also be able to be utilized independently. These three models provide versatile tools for a variety of research applications.

2 LITERATURE REVIEW

2.1 Chracteristics of adipose tissue

Adipose tissue is typically divided into two main types, brown and white adipose tissue. Whereas white adipose tissue is the primary energy storer, brown is specialized in fat burning for heat generation and energy expenditure through non-shivering thermogenesis, where triglycerides are oxidized for heat production to protect against cold and simultaneously obesity (Hyvonen et al. 2014, Choi et al. 2010a, Lefterova et al. 2009).

The most well known characteristic of white adipose tissue is its energy storing ability. Adipose tissue is highly dynamic tissue, able to rapidly increase or decrease its mass in response to changes in the energy balance of the body (Hyvonen et al. 2014, Sun et al. 2011). This allows continuous expansion and regression of subcutaneous adipose tissue deposits throughout adult life (Harrington et al. 2004). In addition, adipose tissue insulates heat and serves as mechanical support for the body (Rosen et al. 2014). The loss of adipose tissue due to injury can impair normal functions, such as range of motion, but also affects the cosmetic and emotional well-being of the patient (Patrick 2001).

White adipose tissue is the predominant type of fat in adult humans (Lefterova et al. 2009) and brown is present mainly in infants (Hyvonen et al. 2014, Choi et al. 2010a). The amount of brown tissue decreases in humans as the body matures (Choi et al. 2010a). The morphological difference between white and brown adipocytes is that white stores lipids in one large lipid droplet, whereas brown contains several smaller lipid droplets (Hyvonen et al. 2014, Peirce et al. 2014). In brown adipocytes, mitochondrial uncoupling protein 1 (UCP-1) is responsible for energy expenditure (Fedorenko et al. 2012). In obesity, white adipose tissue suffers inflammation and oxidative stress associated with insulin resistance, leading to metabolic syndrome and other systemic alterations (Boyer et al. 2015, Wensveen et al. 2015). As most health issues related to adipose tissue are linked to white adipose tissue, a white adipose tissue *in vitro* model would be very beneficial to extrapolate mechanisms behind diseases such as those related to diabetes mellitus.

Although white adipose tissue is named "white", the color of the tissue is influenced by diet. Carotenoid and tocopherol, are known to accumulate in human adipose tissue in varying amounts depending on dietary intake; beta-carotene (provitamin A), lycopene and alpha-tocopherol (vitamin E) being the most predominant compounds (Chung et al. 2009, Parker 1988). These colorful and fat soluble compounds obtained through the diet, mainly from fruits and vegetables, change the color of adipose tissue more yellow than white (Kirton et al. 1975). More than just influencing the color of adipose tissue, carotenoids and their metabolic products have been shown to impact a variety of adipose tissue characteristics including lipid metabolism, metabolic diseases and insulin resistance (Bonet et al. 2015, Bonet et al. 2012, Landrier et al. 2012). Higher intake of carotenoids is associated with lower levels of low-grade inflammation in relation to overweightness, obesity and metabolic syndrome (Calder et al. 2011). Carotenoids and their metabolic products have also been shown to inhibit adipogenesis and the fat storage capacity of mature adipocytes through suppression of PPARγ and PPARγ target genes in adipocytes (Amengual et al. 2011, van Helden et al. 2011). Due to the inflammation and adiposity lowering effects of carotenoids, they have also been proposed as a treatment option for obesity related conditions. One example is Astaxanthin, a xanthophyll carotenoid, which was shown to prevent obesity and fatty liver disease (Ikeuchi et al. 2007).

Adipose tissue is one of the most vascularized tissues in the body (Cao 2013). In addition to transporting nutrients, oxygen and waste, vasculature produces local growth factors and cytokines, impacting adipose tissue functions (Cao 2014). In turn, adipose tissue secretes pro- and anti-angiogenic factors to modulate the vasculature (Christiaens et al. 2010, Cao 2014). In addition to vascularization, adipose tissue also interacts with interstitium. Interstitium has been proposed as a new organ in recent years, however there is still debate whether it can truly be counted as an organ (Benias et al. 2018, Neumann 2018). Regardless of the classification of the interstitium, the composition of interstitial fluid has great relevance on adipose tissue functions in health and disease development, due to their close proximity. Signaling molecules produced in other organs move through the interstitium prior to reaching adipocytes, for example insulin (Nielsen et al. 2005, Jansson et al. 1993), which modulates glucose and lipid metabolism in adipocytes. Adipocytes secrete compounds including those of glucose and lipid metabolism and these either accumulate in interstitial fluid or are transferred to other organs through blood or lymphatic vessels (Chakraborty et al. 2019). Dead adipocytes release free lipids to the interstitium, which are scavenged by macrophages (Cinti et al. 2005). Accumulation

of macromolecules into the interstitial fluid are suspected to be a link between obesity and local inflammation and further, leading to the development of metabolic disturbances related to obesity (Angrim et al. 2013, Ouchi et al. 2011).

Adipose tissue influences both lipid and glucose metabolism. Adipose tissue secretes factors, which mediate physiological processes such as appetite, inflammatory responses, glucose metabolism, systemic insulin sensitivity, angiogenesis, blood pressure and reproductive function (Gu et al. 2013, Lau et al. 2005). In healthy adipose tissue, the release of fatty acids from adipose tissues into circulation and the uptake and oxidation in peripheral tissues are in homeostasis (Unger 2003). In obesity, energy intake exceeds the storage capacity of adipose tissue, which triggers an inflammatory response in expanding tissue as well as increased lipolysis, leading to alterations in circulatory fatty acid concentrations and glucose (Danforth 2000).

The size of fat storage in the body is highly variable, ranging from 5 to 60% of total body weight (Lee et al. 2013). Depots of adipocytes can be found in stereotypical locations throughout the body. The depots include subcutaneous (e.g. arm and gluteal adipose tissue), visceral (intra-abdominal) and ectopic (e.g. intramuscular) adipose tissue. Subcutaneous adipose tissue depots are the main adipose tissue deposits consisting of >80% of total body fat in humans (Lee et al. 2013). The different depots differ significantly from each other. Fat depots have differences in their abilities to store and release fatty acids, synthesize and secrete adipokines and in their plasticity (Lee et al. 2013, McLaughlin et al. 2011, Fain et al. 2004, Rosen et al. 2006). These characteristics also define the metabolic outcomes of homeostatic changes in the depots. For example, an increase in subcutaneous adipose tissue is not associated with increased risk of insulin resistance, whereas visceral adipose tissue is (McLaughlin et al. 2011). Subcutaneous adipose tissue is known to be less metabolically active than visceral adipose tissue (Fain et al. 2004). Visceral adipose tissue adipocytes are sensitive to lipolytic stimuli, whereas in structural depots such as those around the eyes and in heel pads, do not release stored lipids easily (Rosen et al. 2006).

2.1.1 Cells and components in adipose tissue and their functions

Adipose tissue does not contain only adipocytes. In adipose tissue, 20-60% of the cells are mature adipocytes, 10% resident macrophages and the rest are other stromal vascular cells (Hyvonen et al. 2014, Lanthier et al. 2014, Karastergiou et al. 2010).

Stromal vascular cells include fibroblasts, immune cells, leukocytes, endothelial cells, pericytes, adipose stem cells and extracellular matrix (ECM) components (Hyvonen et al. 2014, Choi et al. 2010a, Karastergiou et al. 2010, Astori et al. 2007, Poulos et al. 2010). In addition, adipose tissue contains blood vessels, lymph nodes and nerves (Corvera et al. 2014, Kim et al. 2008, Bartness et al. 2014). Adipocytes are supported by an ECM composed of collagen types I, II, III, IV, V, and VI and other ECM proteins (Nakajima et al. 1998, Flynn et al. 2006). The ECM forms fat lobules in adipose tissue (Bucky et al. 2008).

In white adipocytes the unilocular lipid droplet can occupy 95% of the cell volume and thus determine the size of the cell, ranging from 20 to 200 micrometers (Lee et al. 2013, Sugihara et al. 1987a). Only vertebrates have specialized cells that are recognized as adipocytes (Ottaviani et al. 2011). Triglycerides are the major storage molecules for fatty acids and used in addition to energy needs as synthesis material for membrane lipids (Yen et al. 2008). Characteristics of mature adipocytes are the ability for lipogenesis and release of fatty acids in lipolysis, insulin sensitivity and secretion of adipocyte-specific proteins. The lipolysis process can be induced by epinephrine, norepinephrine, ghrelin, growth hormone, testosterone, and cortisol binding activating adenylate cyclase, or by a drop in circulating insulin levels (Holm et al. 2000).

In circulatory blood, triglycerides are transported in multi-molecular lipoprotein particles (Mead et al. 2002). Lipoprotein lipases (LPL) released by adipocytes are needed for the utilization of circulating triglycerides by adipocytes (Frayn et al. 2003, Kersten 2014). LPL is transported to capillary endothelium (Kersten 2014), where it catalyzes the hydrolysis reaction that produces non-esterified fatty acids and 2-monoacylglycerols (Mead et al. 2002, Rutkowski et al. 2015). Several binding and transport proteins, such as CD36 and fatty acid transport protein family members participate in transporting non-esterified fatty acids into adipocytes (Rutkowski et al. 2015). These non-esterified fatty acids are esterified and form triglycerides for storage within the lipid droplets in adipocytes (Rutkowski et al. 2015).

In lipolysis, triglycerides are broken into glycerol and free fatty acids (FFA) that can then be oxidized locally or by other organs (Rosen et al. 2014, Rutkowski et al. 2015). Lipolysis is driven by β-adrenergic signaling in adipocytes. The binding of catecholamines to β-adrenergic receptors increases the concentration of cellular cyclic adenosine monophosphate, which in turn activates lipolytic enzymes through the stimulation of protein kinase A (PKA) (Zechner et al. 2005). Adipose triglyceride lipase, hormone-sensitive lipase and monoglyceride lipase are three examples of major lipolytic enzymes (Rosen et al. 2014). Adipose triglyceride lipase initiates

hydrolysis of triglyceride that produces diacylglycerols and free fatty acids (Zechner et al. 2005), while hormone-sensitive lipase and others complete the process of lipolysis and generate glycerol and free fatty acids (Rosen et al. 2014).

Human adipose stromal cells (hASC) are cells from the stromal vascular fraction, adipose mesenchymal stem cells, that have multi-potent differentiation potential and can differentiate into bone, cartilage, muscle, and adipose tissue (Kokai et al. 2014, Tsuji et al. 2014). Expanded ASC include also progenitor cells that can differentiate into mature endothelial cells and participate in blood vessel formation (Lindroos et al. 2011). The name adipose stromal cell was recommended by the Mesenchymal and tissue stem cell Committee of the international society, and designated for the stromal vascular fraction cells (Dominici et al. 2006). hASC can be obtained in high numbers from adipose tissue, cultured and expanded *in vitro* (Girandon et al. 2011). As adipose stromal cells are obtained from adult tissue, their use does not raise the ethical issues such as the use of embryonic stem cells. This makes them an interesting source of cells for regenerative medicine (Tsuji et al. 2014). In addition, ASC are genetically more stable in a long-term culture than e.g. mesenchymal stem cells (MSC) from bone marrow (Lindroos et al. 2011). ASC can be obtained from different fat depots and from various species, human and animal adipose tissue, which enables the study of differences between adipose tissue depots and species (Gregoire et al. 1998, Armani et al. 2010).

ASC also have immunomodulatory effects, which is a positive feature in tissue engineering. ASC have been shown to participate in host defense (Saillan-Barreau et al. 2003). They are capable of changing the inflammatory profile of macrophages *in vitro* into an anti-inflammatory phenotype, hence suppressing inflammatory response (Lindroos et al. 2011, Hanson et al. 2011, Gonzalez-Rey et al. 2010). ASC are known to secrete several soluble factors, such as angiogenic factors (e.g. vascular endothelial growth factor, VEGF), anti-apoptosis factors (e.g. insulin-like growth factor I, IGF-1), hematopoietic factors (e.g. colony stimulating factors and interleukins), and hepatocyte growth factor (Ong et al. 2013).

The definition of ASC requires them to be (1) plastic-adherent in standard cell-culture conditions; (2) multipotent *in vitro*, i.e., able to differentiate into adipocytes, chondrocytes and osteoblasts; and (3) positive for CD73, CD90, and CD105, and negative for CD11b or CD14, CD19 or CD79α, CD34, CD45, and HLA-DR on their surface immunophenotype (Dominici et al. 2006). Time and/or passage of ASC *in vitro* causes changes in the surface antigen profile (Mitchell et al. 2006). ASC and MSC surface immunophenotypes resemble each other with a similarity greater than 90% after two or more passages *in vitro* (Gimble et al. 2007). ASC display stromal

markers, such as CD29, CD44, CD73, CD90 and CD166 (Gimble et al. 2007), pericyte markers, such as CD140a and CD140b, and cytoskeletal markers, such as vimentin and α–smooth muscle actin (Traktuev et al. 2008). The presence of CD34 on human ASC is not found in MSC (Pittenger et al. 1999). To separate ASC from bone marrow MSC, they should be positive for CD36 and negative for CD106 (Bourin et al. 2013).

A specific subpopulation of stromal cells has been identified that efficiently differentiates into adipocytes and forms functional adipose depots *in vivo*. These cells show Lin^-, $Sca1^+$, $CD34^+$, $CD24^+$, $a7^-$, and a platelet derived growth factor receptor (PDGFR) α^+ expression pattern (Tang et al. 2008, Joe et al. 2010, Rodeheffer et al. 2008). Similarly to this more efficient subpopulation, differences between ASC derived from different fat depots have been found, which might explain differences in the expansion of these depots in response to nutrient excess (Joe et al. 2009).

2.1.2 Adipokines

Adipose tissue secretes numerous growth factors, hormones, pro- and anti-inflammatory cytokines, enzymes and matrix proteins as well as coagulation and complement factors (Poulos et al. 2010, Traktuev et al. 2008, Kilroy et al. 2007, Trayhurn 2005). The factors secreted by adipose tissue are called adipokines. Adipose tissue secretes proteins including leptin, adiponectin, resistin, tumor necrosis factor α, interleukin (IL) 6 and 1β, plasminogen activator inhibitor-1, fatty acid binding protein 4 (FABP4), VEGF, hepatocyte growth factor, transforming growth factor β, fibroblast growth factor (FGF) and matrix metalloproteinases (MMP), and tissue inhibitors of metalloproteinases (Fasshauer et al. 2015, Karastergiou et al. 2010,). These factors function in an endocrine, paracrine and autocrine manner and have a variety of effects including angiogenic, adipogenic and immune reactions. They are secreted by adipocytes, macrophages, stroma vascular fraction cells and preadipocytes (Fasshauer et al. 2015).

Leptin and adiponectin are the most abundant adipokines produced and released by adipocytes (Karastergiou et al. 2010, Galic et al. 2010). Although they have opposing functions, they both regulate whole body energy homeostasis via the hypothalamus (Galic et al. 2010, Coelho et al. 2013). They both also have metabolic effects on multiple different tissues (Galic et al. 2010).

Leptin is a peptide hormone mainly produced and secreted by mature adipocytes (Gregoire 2001). Specifically, the subcutaneous fat depot is a major source of leptin

(Wajchenberg 2000). The main effect of leptin is regulation of energy balance, which is mostly mediated by receptors located in the central nervous system (Coelho et al. 2013, Gregoire 2001, Kershaw et al. 2004). As the size of adipocyte lipid storage increases, the expression and levels of leptin increase (Tritos et al. 1997, Mantzoros et al. 1997). Thus, leptin concentrations are higher in obese individuals and increase along with increased energy intake. In addition to the role in energy homeostasis regulation, leptin has versatile effects on other tissues as well, as it regulates endocrine systems, the immune system, hematopoiesis, angiogenesis and bone development (Kershaw et al. 2004).

Adiponectin stimulates appetite and reduces energy expenditure and thus has the opposite functions to leptin (Coelho et al. 2013). Adiponectin is secreted exclusively by mature adipocytes (Wang et al. 2008, Hu et al. 1996). Adiponectin is expressed more in VAT than SCAT (Freedland 2004, Motoshima et al. 2002). There is a significant negative correlation between circulating adiponectin levels and body weight, insulin resistance and blood pressure (Fasshauer et al. 2014). Two adiponectin receptors have been reported, adipoR1 and adipoR2 (Yamauchi et al. 2003). The biological effects of these receptors depend on the blood concentration of adiponectin and also tissue specificity (Itoh et al. 2011, Kadowaki et al. 2005). Adiponectin has gained a lot of interest due to its insulin sensitizing, anti-inflammatory, and antiapoptotic properties (Fasshauer et al. 2014, Ye et al. 2013). Peroxisome proliferator–activated receptor (PPAR) γ ligands, which can function as insulin sensitizers have also been shown to increase adiponectin expression and plasma concentration *in vivo* and *in vitro* (Maeda et al. 2001).

FABP4 is a cytoplasmic fatty acid chaperone expressed primarily in adipocytes and also associated with the development of insulin resistance and atherosclerosis (Wu et al. 2014, Cao et al. 2013, Garin-Shkolnik et al. 2014). FABP4 levels are higher in visceral fat than subcutaneous, and also higher in obese adipose tissue with diabetes than healthy. (Garin-Shkolnik et al. 2014). FABP4 attenuated adipocyte differentiation by negatively regulating PPARγ levels occurs in macrophages and adipocytes by triggering ubiquitination and subsequent proteosomal degradation of PPARγ (Garin-Shkolnik et al. 2014).

As adipose tissue also produces and secretes many proangiogenic factors, the regulation of angiogenesis in adipose tissue is possibly based on the local balance between proangiogenic and antiangiogenic factors produced by the tissue itself (Christiaens et al. 2010). Angiogenic factors produced by adipose tissue include different members of the VEGF family, hepatocyte growth factor, placental growth

factor, FGF-2, angiopoietin-1 and -2 (Angpt-1 and 2), leptin, platelet derived growth factor-β (PDGF-β), and transforming growth factor-α (Christiaens et al. 2010).

2.1.3 Adipogenesis

New adipocytes develop through adipogenesis. Adipogenesis begins with a determination phase in which stem cells transform into committed preadipocytes (Rosen et al. 2006). Commitment is followed by a growth arrest, which is followed by the signalling of a specific mixture of adipogenic and mitogenic factors needed for the terminal differentiation step (Gregoire 2001). The second phase of adipogenesis is terminal differentiation, in which preadipocytes transform into insulin-sensitive, lipid synthesizing and releasing mature adipocytes that secrete adipocyte-specific secretory products (Rosen et al. 2006, Stephens 2012).

The differentiation process includes chronological changes in the expression of early, intermediate and late mRNA and protein markers (Gregoire et al. 1998). Activation of over 2000 genes is required to reach the status of a fully mature adipocyte (Guo et al. 2000). Commitment is established by intracellular factors including Zfp423 and S6K1, early B cell factor 1 and transcription repressor TCF7L1 (Chen et al. 2013). Commitment of stem cells to preadipocytes also involves bone morphogenetic protein signaling (Huang et al. 2009), whereas WNT signalling (Christodoulides et al. 2009, Prestwich et al. 2007) and the hedgehog signalling pathway inhibit the commitment phase (Cousin et al. 2007, Pospisilik et al. 2010). The formed preadipocytes express CD29, CD34, SCA1, PDGFR-α and CD24 (Chen et al. 2013, Berry et al. 2014).

In the differentiation of preadipocytes into mature adipocytes, the nuclear receptor PPARγ is a master regulator, playing a critical role in systemic lipid and glucose metabolism (Tontonoz et al. 1994). The expression of PPARɣ is induced by C/EBPβ and C/EBPδ (Rosen et al. 2014, Rosen et al. 2006, Bucky et al. 2008). PPARγ and C/EBP-β activate, then C/EBP-α, which directly induces many adipogenic genes and activates PPARγ (Rosen et al. 2006). The differentiated adipogenic state is maintained by the activation of C/EBP-α induced by PPARγ, which creates a positive feedback signal (Daquinag et al. 2011). C/EBPα is considered to have an important function in the activation and maintenance of terminally differentiated adipose tissue (Wu et al. 1996).

Activated PPARγ and C/EBPα induce genes expressed in mature adipocytes involved in insulin sensitivity, lipogenesis and lipolysis. These include FABP4, CD36,

fatty acid synthetase, insulin regulated glucose transporter (Glut) 4, leptin, lipoprotein lipase (LPL), and adiponectin, all of which contain peroxisome proliferator response elements (Daquinag et al. 2011, Tontonoz et al. 2008, Lowe et al. 2011, Lefterova et al. 2008).

PPARγ has two different isoforms that are expressed: PPARγ1 and PPARγ2. PPARγ2 is the more adipocyte-specific isoform of PPARγ (Ren et al. 2002, Escher et al. 2001), and it is transcriptionally regulated by nutrition (Ren et al. 2002, Werman et al. 1997). PPARγ2 is shown to be critical for the storage of lipids in adipocytes instead of other organs, and hence, its proper expression is highly important in obesity (Medina-Gomez et al. 2007).

Also transcriptional factors including Krox20, KLF4, TLE3 and CREB promote adipogenesis (Chen et al. 2013). TLE3, a target gene of and a cofactor for PPARγ enhances adipogenesis by activating PPARγ and suppressing WNT activity/expression, but TLE3 also competes with PR domain containing 16 (PRDM16) protein for PPARγ binding, which promotes white adipogenesis and inhibits brown adipocyte formation (Villanueva et al. 2013). The regulation of adipogenesis includes also other relevant signaling molecules besides transcriptional factors. The activation of the mitogen-activated protein kinase kinase/extracellular signal regulated kinase signaling pathway and p38 mitogen-activated protein kinase signaling pathway are mecessary for the adipogenesis process (Bost et al. 2005, Farmer 2005). In addition to these pathways, insulin, IGF-1, glucocorticoids and factors increasing intracellular cyclic adenosine monophosphate (cAMP) concentration act as promoters of adipogenesis (Gregoire et al. 1998). Insulin and lipids activate sterol-regulatory element binding protein 1c and this in turn also induces PPARɣ and subsequently adipogenesis (Daquinag et al. 2011). Sterol-regulatory element binding protein 1c further activates a variety of genes together with PPARɣ and C/EBPPα.

The morphological transformation of fibroblast-like shaped preadipocytes into more spherical preadipocytes is also a regulator of adipogenesis (Gregoire 2001). This shape changing leads to changes in ECM that promotes expression of C/EBPα and/or PPARγ (Gregoire 2001, Selvarajan et al. 2001). During the adipogenesis process, the number of mitochondria significantly increases (Wilson-Fritch et al. 2003) to facilitate increased metabolic demands (Wilson-Fritch et al. 2004).

At the terminal phase of differentiation, enzymes of triacylglycerol synthesis and degradation are activated and unilocular lipid storage is formed (Rosen et al. 2014, Gregoire 2001, Rosen et al. 2000). Expression of adiponectin (Flynn et al. 2008) and glucose transporter Glut4 are connected to the terminal stage of differentiation

(Gregoire et al. 1998). Adiponetin is a hormone that decreases the amount of FFA glucose and triacylglycerol in serum (Fruebis et al. 2001). Adiponectin is only expressed and secreted from adipose tissue (Kershaw et al. 2004). Another adipokine, leptin is produced and secreted mainly by mature adipocytes (Gregoire 2001). FABP4 in adipocytes has been found to be a critical link between hormone action, lipid metabolism and cellular functions (Maeda et al. 2001, Furuhashi et al. 2008). This is seen in the terminal stage of adipocyte differentiation where lipid accumulation elevates FABP4, which reduces PPARγ (Garin-Shkolnik et al. 2014) by triggering ubiquitination and subsequent proteosomal degradation of PPARγ (Rodriguez et al. 2007, van Beek et al. 2007, Yang et al. 2007).

2.2 Characteristics of blood vessels

Vascularization is essential for the growth and repair of organs and tissues. In adipose tissue, the vascular network needs to remodel itself in response to the shrinking or enlargement of the tissue to ensure sufficient blood flow to the tissue. Enlargement of the adipose tissue can be supported by new blood vessel formation (neovascularization) or by remodeling and dilating of the already existing capillaries (Christiaens et al. 2010).

The majority of substance exchange in tissues occurs through capillaries. In blood vessel walls, endothelial cells act as a selective barrier between the blood stream and tissues (Goddard et al. 2013). Vesicular trafficking, complex junction rearrangements and refined cytoskeletal dynamics control the dynamic and sensitive regulation of endothelial permeability (Goddard et al. 2013). This allows substance exchange to be adjusted with regards to environmental changes, facilitates immune surveillance and enables the deposition of matrix proteins outside the vascular wall when repair is needed (Goddard et al. 2013). In addition to this barrier function, endothelial cells have also role in the control of haemostasis and vasomotor tone (Lehle et al. 2010).

2.2.1 Cells and structure of blood vessels

All blood vessels are lined with endothelial cells regardless of size. Mature vasculature also has recruited mural cells, deposited extracellular matrix and has organ-specific specialization of cells, such as surface receptors and inter-endothelial junctions.

Endothelial cells are stable cells, however during neovascularization the turnover time of endothelial cells is accelerated and can be as fast as 5 days (Kalluri 2003). Endothelial cells vary greatly in their gene expression profile and function depending on their location (Lehle et al. 2010). Markers of endothelial cells from different tissues have been identified and can be used to selectively isolate specific subpopulations. Endothelial cell markers include von Willebrand factor (vWf) antigen, CD31, CD144, vascular endothelial growth factor receptor (VEGFR) 2, and endothelial nitric oxide synthase (eNOS) (Lehle et al. 2010, Szoke et al. 2012). VEGFR2 is widely expressed in endothelial cells regardless of their source, but VEGFR3 expression is shown to be excluded from arterial cells (Lehle et al. 2010).

Human umbilical vein endothelial cells (HUVEC) are widely used in vascular studies because they are relatively easy to obtain and culture. As they are derived from the umbilical vein, they are macrovascular cells. The morphology of endothelial cells is cobblestone-like (Szoke et al. 2012). The health condition of the umbilical cord donor from which HUVEC are obtained has been shown to influence function of the cells. HUVEC isolated from healthy newborns with type 1 diabetic mothers showed a 20–40% reduced resistance to shear stress, changes in plasma membrane, decreased rates of glucose uptake and an increase in the size of mitochondria, Weibel Palade bodies and rough reticulum (Lehle et al. 2010). HUVEC obtained from pregnancies resulting in healthy newborns with a family history of diabetes mellitus type 2 showed deficient nitric oxide synthesis and altered expression of eNOS mRNA (Lehle et al. 2010). For these reasons donors of primary cells for tissue engineering need to be carefully selected.

The basement membrane surrounding endothelial cells in blood vessels is an approximately 50 - 100 nm thick specialized extracellular matrix that separates endothelial cells from surrounding tissue (Morris et al. 2014). The main components of basement membrane are collagen IV, perlecan, laminins and nidogens (Timpl et al. 1996, Halfter et al. 2013). Basement membrane supports and stabilizes vessels but also helps control the permeability of capillaries (Senger et al. 2011).

Pericytes are support cells that surround the endothelial layer and participate in regulation of blood flow at a capillary level (Sarkar et al. 2012, Stapor et al. 2014). Capillaries lack the smooth muscle layer present in larger blood vessels. However, pericytes express actin and desmin, which are two proteins important in smooth muscle function, and are able to contract (Sarkar et al. 2012, Stapor et al. 2014). Pericytes also influence vascular permeability by altering endothelial gene expression (Daneman et al. 2010).

Maturate networks are branched and capable of expanding to meet local, tissue-specific demands (Jain 2003). Capillaries are the smallest blood vessels of the body consisting of a single layer of endothelial cells surrounded by basement membrane and pericytes (Jain 2003). The diameter of capillaries is about the same as that of one red blood cell (Ross et al. 2010), about 4-10 μm (Ko et al. 2007). Capillaries can only deliver oxygen and nutrients to a distance of up to 200 μm and thus angiogenesis is required to reach cells further away during new tissue formation (Carmeliet et al. 2000). Two types of capillaries exist, fenestrated and continuous. Fenestrated capillaries are more permeable than continuous capillaries (Risau 1998).

2.2.2 Angiogenesis

The formation of blood vessels is a vital process in growth and organ development. Formation of new blood vessels can occur by two different processes; vasculogenesis and angiogenesis. In the embryo, a primitive vascular network is formed by vasculogenesis in which endothelial precursor cells form new vessels. The process where these vessels sprout is called angiogenesis and is the main mechanism of new blood vessel development in adult individuals. It creates a network of arteries, veins and capillaries which enable the exchange of gases and metabolites. To reach the organization of a fully functional vessel system, the immature vascular network must mature at the level of the vessel wall. This maturation involves recruitment of mural cells, deposition of extracellular matrix and organ-specific changes in cell features, such as surface receptors and inter-endothelial junctions (Carmeliet et al. 2011, Ucuzian et al. 2007).

The basic method of angiogenesis consists of an increase in the number of endothelial cells, their migration through the extracellular matrix, intercellular junction formation, lumen formation, organization of perivascular cells, anastomosis with existing vessels, and finally the circulation flowing through the new blood vessel (Corvera et al. 2014, Carmeliet et al. 2011, Ucuzian et al. 2007). In order for the cascade of angiogenesis to occur, a wide range of pro- and anti-angiogenic factors (growth factors, chemokines, angiogenic enzymes, endothelial specific receptors, and adhesion molecules) are needed (Liekens et al. 2001).

Angiogenesis is initiated, when endothelial cells detect angiogenic signals, which are released in response to hypoxic, metabolic or pathophysiological conditions by inflammatory, tumour or tissue specific cells (Carmeliet et al. 2011, Mammadov et al. 2011, Auerbach et al. 2003, Auerbach 2008). There are numerous angiogenic

factors involved in angiogenesis including fibroblast growth factors (e.g. FGF-2), vascular endothelial growth factor (VEGF), Angpt-1 (Ucuzian et al. 2007). The activated endothelial cells start releasing proteases such as MMP family members and the plasminogen activator system, which leads to basement membrane degradation (Ucuzian et al. 2007, Goding 2009). Due to the activity of MMPs, pericytes detach from basal membrane and ECM is degraded (Jain 2003, Carmeliet et al. 2011). Nitric oxide dilates vessels followed by VEGF increasing the permeability of the endothelial cell layer in existing vessel (Jain 2003, Carmeliet et al. 2011). This leads to the release of plasma proteins out of the vessel and this leaked plasma serves as a matrix for endothelial cells as they move with the help of integrin interactions (Jain 2003, Carmeliet et al. 2011). One endothelial cell acts as a tip cell leading the endothelial cells according to signals such as VEGF receptors, neuropilins and Notch ligands DLL4 and JAGGED1 (Carmeliet et al. 2011). VEGF and other mitogenic signals also lead to the proliferation of endothelial cells thus providing enough cells to form the endothelial layer for new vessel branches (Corvera et al. 2014, Jain 2003). The action of VEGFs is controlled by the Notch-signaling pathway, which is responsible for the responsiveness of endothelial cells to VEGFs. Thus, the development of angiogenic sprouts is mostly mediated by the dynamic interactions of VEGFs, Notch and their ligands (Corvera et al. 2014). The initiation of lumen development in newly developed endothelial cords is triggered by the apical-basal polarity of the endothelial cells (Charpentier et al. 2014).

Immature vessels are stabilized in the final steps of angiogenesis by recruiting mural cells, either pericytes or smooth muscle cells, and by forming ECM (Ucuzian et al. 2007, Gerhardt et al. 2003). Four pathways have been recognized to be involved in the stabilization process. Transforming growth factor β induces genes, whose products are necessary for basement membrane formation (Holderfield et al. 2008). PDGF, PDGFR-β, sphingosine-1-phosphate-1 (S1P1) and endothelial differentiation sphingolipid G-protein coupled receptor 1 (EDG1) enable the recruitment of mural cells (Jain 2003, Carmeliet et al. 2011). The angiopoietin 1-Tie-2 pathway signal further stabilization of endothelial cell–mural cell interactions (Jain 2003).

When sufficient neovascularization has occurred, the endothelial cells become quiescent, and the vessels remain, or regress, if no longer needed (Liekens et al. 2001). This is induced by the down-regulation of angiogenic factors or increased local concentrations of angiogenesis inhibitors (Liekens et al. 2001).

Due to the complex nature of the formation and maintenance of the vascular network, it is sensitive to disturbances caused by regulatory failures but also due to

external effects such as exposures to environmental chemicals. The results are seen in ischemia in myocardial infarction, neurodegenerative disorders and obesity-associated disorders that are caused by inadequate vessel maintenance or growth causes (Potente et al. 2011). Excessive vascular growth or abnormal remodeling are linked to conditions such as cancer, inflammatory disorders and eye diseases (Potente et al. 2011). Moreover, failures in embryonic vascular development can cause various problems from benign vascular malformation to embryo lethality and congenital defects (Knudsen et al. 2011). Exposure to various chemicals can also lead to inhibition of the angiogenesis process (Toimela et al. 2017). The effect of angiogenesis influencing chemicals can be seen on multiple levels of angiogenesis (Knudsen et al. 2011). To identify an adverse outcome pathway for each type of vascular adverse outcome, the effects of known chemicals have to be studied on gene and protein expression level (remodeling, ECM interactions), cell level (motility, proliferation), organ level and whole organism level (Knudsen et al. 2011). Accurate and validated *in vitro* angiogenesis models are already available and shown to recognize angiogenesis and vasculogenesis inhibiting chemicals (Toimela et al. 2017, Sarkanen et al. 2011).

2.3 Obesity and adipose tissue

Obesity and overweightness are associated with type 2 diabetes, multiple cancer types, sleep apnea, asthma, degenerative joint disease, hypertension, renal failure, stroke, and cardiovascular disease, gallbladder disease, respiratory disease, arthritis and gout (Pi-Sunyer 2009, Switzer et al. 2013, van Baak 2013). Understanding the mechanisms behind changes detected in adipose tissue biology during obesity is important as fat tissue acts as an endocrine organ that regulates systemic metabolism and hence influences the whole body (Kershaw et al. 2004).

Obesity is characterized by an increase in the amount of adipose tissue and changes in adipose tissue metabolism caused by the increase. In rat studies obesity has been seen to develop first by enlargement of the existing adipocytes, hypertrophy and then by an increase in adipocyte numbers, hyperplasia (Cleary et al. 1979). When adipocytes enlarge they become insulin-resistant, hyperlipolytic and resistant to the anti-lipolytic effect of insulin (Ibrahim 2010). Visceral tissue contains more large adipocytes in contrast to subcutaneous, which contains more small adipocytes. Small adipocytes are more insulin-sensitive and have a high avidity for free fatty acid and triglyceride uptake, preventing their deposition in non-adipose tissue (Misra et al.

2003, Marin et al. 1992). Adipocytes of visceral adipose tissue are lipolytically more active than in subcutaneous in both lean and obese subjects (Van Harmelen et al. 1997). As visceral adipose tissue drains directly into the portal vein system it contributes more to plasma free fatty acid levels, thus providing an important link between visceral adipose tissue and the developmental of insulin resistance and type 2 diabetes (Van Harmelen et al. 1997). Due to these biological connections, the increase in visceral adipose tissue is more dangerous than an increase of subcutaneous adipose tissue.

The growth of adipose tissue is supported by either new blood vessel formation, or by dilating and remodeling of existing blood vessels (Christiaens et al. 2010). In adipose tissue, angiogenesis can be a response to hypoxia caused by proliferation and/or adipocyte enlargement, or the result of developmental and/or metabolic signals in adipose tissue (Corvera et al. 2014). There are several studies supporting the role of hypoxia as a trigger for angiogenesis (Corvera et al. 2014, Trayhurn 2013, Pasarica et al. 2009). However, angiogenesis/vasculogenesis precedes adipogenesis and blood vessel ECM develops before adipose tissue ECM development in fetal development (Hausman et al. 2004).

Hypoxia in expanding adipose tissue (Yin et al. 2009) also leads to accelerated apoptosis and necrosis in adipose tissue in obesity (Strissel et al. 2007). The enlargement of adipocyte cell size and increase in cell death are associated with chronic inflammation and insulin resistance in obesity. These in turn, inhibit adipogenesis of pre-adipocytes (Yin et al. 2009, Yun et al. 2002). The lack of sufficient vascularization further contributes to hypoxia and inflammatory responses in the adipose tissue of obese individuals (Ye 2011).

Obesity leads to changes in the levels of adipokine secretion, and recruitment of macrophages that release pro-inflammatory cytokines such as tumor necrosis factor α, transforming growth factor b and IL-6 (Bastard et al. 2002). These increasing levels of cytokines contribute to obesity-associated complications including insulin resistance (Yue et al. 2005). In insulin-resistant obese humans and animals, leptin levels are increased (Tritos et al. 1997, Mantzoros et al. 1997) and adiponectin levels are decreased (Fasshauer et al. 2014). The reported anti-diabetogenic activity of leptin and adiponectin is based on their common capacity to enhance insulin action in both skeletal muscle and liver. In accordance with this anti-diabetogenic categorization, leptin administration to humans with severe lipodystrophy partially reverses their insulin resistance and hyperlipidaemia (Oral et al. 2002).

In obesity, there is also an increase in the number of immune cells present in adipose tissue. Macrophage infiltration into adipose tissue has been shown to

temporally precede systemic insulin resistance in mice (Xu et al. 2003). However, T-cell accumulation, impaired glucose tolerance and insulin sensitivity preceded macrophage accumulation in another mouse study (Kintscher et al. 2008). Macrophage infiltration has been shown to positively correlate to the size of adipocytes, with a higher presence of hypertrophic adipocytes as opposed to hyperplastic adipocytes (Wang et al. 2001).

Lymphangiogenesis (formation of lymphatic vessels), a process similar to angiogenesis, is also linked to obesity and its modulation has also been proposed as a way of controlling obesity and related complications. The relationship between lymphatic vessels and obesity is bidirectional, e.g., defects in lymphatic function contribute to the development of obesity and vice versa. Impaired lymphatic function in mice has been shown to lead to activation of genes related to adipocyte differentiation and adipose tissue hypertrophy and proliferation (Aschen et al., 2012, Harvey et al. 2005). On the other hand, lymphatic function is impaired in obese patients (Scallan et al. 2015, Greene at el. 2012). Impairment of lymphatic vessels can lead to the development of lymphedema. Lymphedema results in a reduced ability to clear macromolecules within the interstitial space by the lymphatic system, which might have a role in the development of obesity related inflammation in hypertrophic adipose tissue (Arngrim et al. 2013). Due to the versatile interactions of adipose tissue and lymphatic vessels, VEGF-D, a growth factor inducing lymphangiogenesis, has been proposed as a treatment option for adipose tissue related complications. Overexpression of VEGF-D in adipose tissue in mice leads to enhanced glucose clearance, lower insulin levels, reduced liver triglycerides and a reduced number of macrophages in adipose tissue (Chakraborty et al. 2019). They also exhibited a more rapid and increased glycerol flux from adipose tissue, suggesting that lymphatic vessels take part in the clearance of glycerol from interstitial fluids (Chakraborty et al. 2019).

2.4 Functions of insulin

Insulin is a hormone secreted by the β cells of islets of Langerhans in the pancreas. Insulin maintains normal blood glucose levels by inducing cellular glucose uptake, modulating carbohydrate, lipid and protein metabolism, and increasing cell division and growth through its mitogenic effects (Wilcox 2005). Insulin is secreted from the pancreas in response to a rise in blood glucose levels in a bi-phasic manner; the first response is a rapid phase of insulin secretion (fed state), which is followed by a more

sustained and less intense release (basal state) of the hormone (Kahn et al. 1997). For insulin-dependent tissues such as muscle and adipose tissue, insulin is essential for the intra-cellular transport of glucose (Wilcox 2005).

2.4.1 Effects of insulin on adipose tissue

The regulation of adipose tissue metabolism is one of the central functions of insulin (Cohen 2006). Insulin signals the abundance of exogenous energy, which leads to suppression of fat breakdown and an increase in its synthesis in adipose tissue. When circulating glucose concentration is high, insulin binds to its receptor and causes the translocation of Glut4 from cytosol to the cell surface, which allows glucose transportation into adipocytes (Rutkowski et al. 2015, Watson et al. 2007). Insulin-stimulated Glut4 is the predominant glucose transporter type in adipocytes although basal levels of the insulin-independent glucose transporter Glut1 is also present at the cell membrane of adipocytes (Rutkowski et al. 2015). Adipocyte-specific Glut4 deletion in adipocytes has been shown to cause skeletal and hepatic insulin resistance, indicating that Glut4 mediated glucose uptake into adipocytes is critical in glucose homeostasis (Abel et al. 2001). Glucose is not only needed for ATP production but also for effective adipocyte lipid packaging (Bederman et al. 2009, Guan et al. 2002).

Insulin stimulates the uptake of amino acids and glucose into adipocytes initiating signaling via effectors like phosphatidylinositol-3 kinase (PI3K) and AKT1/2 (Kim et al. 2004, Garofalo et al. 2003). Binding of insulin to the insulin receptor elicits autophosphorylation of the receptor, leading to binding of various scaffold proteins, including insulin receptor substrate proteins (IRS) (Taniguchi et al. 2006, Cohen 2006). Phosphorylation of IRS1 leads to its association with the p85 regulatory subunit of PI3K. This association leads to the recruitment of the p110 catalytic subunit of PI3K to plasma membrane, resulting in conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate. This in turn, facilitates additional signaling events by binding to phosphoinositide-dependent protein kinase (PDK) 1, PDK2 and AKT. Colocalization of PDKs and AKT leads to the activation of AKT by phosphorylation at Thr308 of PDK1 and Ser473 of PDK2, and phosphorylation of downstream targets such as glycogen synthase kinase-3 and the AS160 Rab GTPase-activating protein, which in turn interact with the small GTPase RAB10 to facilitate translocation of Glut4 to the cell surface (Sano et al. 2007). (Muoio et al. 2008) Hyperstimulation of adipocytes with

insulin has been shown to induce a negative feedback response, downregulating Glut4 by a protein kinase CK2-dependent mechanism (Ma et al. 2014). Insulin has also been shown to inhibit FABP4 release from adipocytes *in vitro*, in mice and in humans, and that FABP4 and insulin form an endocrine loop coordinating the β-cell response to obesity (Wu et al. 2014).

In lipid metabolism, insulin promotes the synthesis and storage of triglycerides and inhibits their catabolism, lipolysis (Rutkowski et al. 2015, Watson et al. 2007, Choi et al. 2010b). The inhibition of lipolysis is achieved by activating phosphodiesterase 3B, which inactivates the function of cAMP (Choi et al. 2010b). Thus downstream activation of PKA, adipose triglyceride lipase and hormone-sensitive lipase is inhibited (Choi et al. 2010b). Insulin inhibits lipolysis also indirectly through increased uptake of glucose (Rutkowski et al. 2015). Puryvate, a metabolite of glucose enters the citric acid cycle but a portion is also metabolized into lactate. As lactate exits adipocytes, it initiates an autocrine and paracrine lactate loop that mediates insulin-dependent inhibition (Ahmed et al. 2010). Formation of cAMP and down-stream activation of PKA is inhibited by lactate binding to the G protein-coupled receptor GPR81, thus leading to the inhibition of lipolysis (Ahmed et al. 2010).

2.4.2 Effects of insulin on vasculature

Insulin also has effects on vasculature. In conduit arteries insulin increases arterial plasticity/compliance and blood pressure; in resistance arterioles blood pressure and total blood flow to tissues; pre-capillary arterioles tissue perfusion, and capillaries exchanges of nutrients, oxygen and hormones between the plasma and tissue interstitium (Zheng et al. 2015). These effects are significant especially in resistive vessels and precapillary arterioles in fat tissue (Safar et al. 2013). Peripheral arteries are surrounded by more muscle cells and hence are more sensitive to the actions of insulin than central elastic arteries and arterioles, which have less muscle cells surrounding them.

Vascular endothelial cells express insulin receptors, IGF-1 receptors and hybrid insulin/IGF-1 receptors. Binding of insulin to insulin receptors modifies the balance of vasodilation and vasoconstriction activities. Insulin signals vasodilation through the PI3K/AKT/eNOS signal pathway, which leads to an increase in the production and bioavailability of nitric oxide. The vasoconstriction by insulin is induced through endothelin-1 via the mitogen-activated protein kinase (MAPK) pathway (Muniyappa

et al. 2013). Nitric oxide production causes a decrease in vascular tone and vascular smooth muscle cell proliferation, adhesion of inflammatory cells and platelet aggregation to endothelium (Katusic et al. 2014).

2.5 *In vitro* models as relevant research tools

In vitro (in glass) cell studies are performed with cells outside a living organism, generally in flasks, Petri dishes, and cell culture well plates. *In vitro* cell models are developed for multiple reasons. Firstly, studies performed in animals do not exactly depict human biology and hence a relevant human model should be developed. Secondly, ethical reasons encourage the use and development of better *in vitro* models to reduce and replace the need for test animals (Hampshire et al. 2018, Mallia et al. 2018, Franco et al. 2018). The use of *in vitro* models has also increased by the introduction of EU legislations such as REACH and cosmetics regulations (European Union 2010, European Union 2009, European Union 2006). However, the validity of *in vitro* models needs to be confirmed through characterization of cell identity, physiological architecture and functionality, after which formal validation should be performed to show repeatability, reproducibility and relevance for the intended purpose (Bale et al., 2014; Hartung, 2011, Environment Directorate 2005).

When developing *in vitro* models, all aspects of the cell culture have to be carefully thought through. For developing relevant human cell models, the use of human cells is important. Especially so, when effects of tested chemicals might be caused by metabolites of the chemical (Ginis et al. 2004, Mestas et al. 2004, Rangarajan et al. 2003). Moreover, an important advantage of *in vitro* models is the possibility to create patient-specific systems using patient derived cells. These personalized models could be used to explore treatment options and study disease mechanisms and metabolic parameters that vary between patients.

Defined medium composition and the developmental stage of the model system are critical, especially when the test model will be used for chemical safety studies. Serum is not a necessary component as human MSC have been shown to maintain their immunophenotype and multilineage potential in serum-free medium (Patrikoski et al. 2013, Mark et al. 2013). Serum-free medium with xeno-free and defined supplements is critical for *in vitro* models to decrease variation between experiments due to unknown components in medium, and further, unknown binding properties of these components (Shen et al. 2013, Brunner et al. 2010, Gottipamula et al. 2013, van der Valk et al. 2010).

Biomaterials are also often used in *in vitro* cultures. Many biomaterials have been developed and used in tissue engineering (Choi et al. 2010a). These biomaterials are relevant especially in implantable tissue engineered applications. However, in toxicological studies the additional components such as biomaterial scaffolds may interfere with cell–cell interactions or cause unwanted and unknown interactions between materials and the studied chemicals or the cells in the system (Norotte et al. 2009, Higgins et al. 2003, Williams 2008).

Several *in vitro* models have been developed to mimic angiogenesis and vascular structures as well as adipogenesis and adipose tissue. Current models include 2D and 3D culture systems and range from simple monocultures to models containing multiple cell types. Models of *in vitro* angiogenesis and vascular structures are presented in Table 1. For adipose tissue research, some vascularized adipose tissue models have been developed. Current human cell *in vitro* vascularized adipose tissue models are listed in Table 2.

Table 1. Examples of different *in vitro* angiogenesis and vasculature mimicking cultures

Assay	Cells	Biomaterial	Serum	Reference
Collagen Assay	Primary human endothelial cells (e.g., human umbilical vein endothelial cells or human coronary artery endothelial cells	type I Collagen	FBS	(Evans 2015)
Matrigel Assay	Primary human endothelial cells (e.g., human umbilical vein endothelial cells or human coronary artery endothelial cells	Matrigel	FBS	(Evans 2015)
Gelatin assay	Microvascular Endothelial Cells or human umbilical vein endothelial cells	gelatine	FBS	(Bahramsoltani et al. 2016)
Endothelial+ Fibroblast Coculture Assay	Primary human endothelial cells (e.g., human umbilical vein endothelial cells or human coronary artery endothelial cells + Primary human fibroblast cells or The BJ human foreskin fibroblasts/human embryonic lung fibroblasts (HLF-1)	none	FBS	(Sarkanen et al. 2011, Evans 2015, Bishop et al. 1999, Guo et al. 2014)
Endothelial+ hASC or smooth muscle cell Coculture Assay	primary endothelial cells + hASC/coronary artery SMCs and human aortic SMCs	none	FBS/ no serum	(Merfeld-Clauss et al. 2010, Sarkanen et al. 2012a), Original publication I
3D spheroid	Clustered HUVEC	collagen matrix	not available	Promocell, Heidelberg, Germany
Microbead angiogenesis assay	HUVEC	cytodex-3-microcarrier beads, collagen matrix	FBS	(Mombeinipour et al. 2013)
Cryopreserved blood vessels	Vessel cells	-	Fetal calf serum (FCS)	(Muller-Schweinitzer 1994)
Chip microchannels	HUVEC + primary fibroblasta	fibrin	FBS	(Ryu et al. 2015)
***In vitro* vessels by bioprinted biomaterials**	Endothelial cells			(Song et al. 2014, Kim et al. 2017, Haase et al. 2017)
3D printing *in vitro* vessels with microfluidics	HUVEC+murine fibroblast 10T1/2 cells	gelatin methacrylate	FBS	(Song et al. 2014, Kim et al. 2017, Haase et al. 2017, Yang et al. 2016)

Table 2. Existing ways of producing vascularized adipose tissue *in vitro*

Cells	Biomaterial	Adipogenesis induction	Serum	Reference
hASC +HUVEC	silk scaffold	7 days: DMEM/F-12, 3% FBS, 1% penicillin–streptomycin, 33 µM biotin, 17 µM pantothenate, 1 µM insulin, 1 µM dexamethasone, 500 µM IBMX, and 5 µM TZD	FBS	(Kang et al. 2009)
liquefied adipose tissue	silk scaffold	DMEM/F-12, 10% FBS, 1% abtibiotic-antimycotic, 1µM insulin, 500 µM IBMX, 1 µM dexamethasone, 50µM indomethacin	FBS	(Abbott et al. 2016)
human pre-adipocytes+human endothelial cells	fibrin	Endothelial medium, 10% FCS, (±VEGF, FGF-2)	FCS	(Borges et al. 2007)
dermal fibroblast+ bone marrow MSC+ hASC+ HUVEC	no	DMEM-high glucose, 100 mm indomethacin, 500 mm IBMX, 0.5 mg/ml insulin, 3 mm dexamethasone, 270 µm ascorbate 2-phosphate, and 10% FBS	FBS	(Sorrell et al. 2011)
hASC+microvascular endothelial	no	DMEM/F12, 3% FCS, 250 µM AA, 100 nM insulin, 0.2 nM T3, 1 µM dexamethasone, 0.25 mM IBMX and 1 µM rosiglitazone	FCS	(Aubin et al. 2015)
hASC+HUVEC	Collagen I/ alginate microspheres	DMEM, 1 µM dexamethazone, 0.5 mM IBMX, 10 µM insulin	no	(Yao et al. 2013)
hASC+dermasl microvascular endothelial cells	Collagen I	Adipocyte Basal Medium with high glucose, 1 µmol dexamethasone, 1 µg/mL human insulin, 500 µmol IBMX and 100 µmol indomethacin, suppl. A	no	(Volz et al. 2018a)

2.5.1 Differences between human and animal adipose tissue

A wide variety of animal models have been developed for studies of adipose tissue and obesity (Al-Awar et al. 2016, Lai et al. 2014). Although they have increased understanding of the basic biology of tissues and mechanisms behind diseases, animal models have ethical problems (Heinonen 2015, Chandrasekera et al. 2014, Al-Awar et al. 2016, Sade 2011).

There is more and more evidence that the complex pathways of lipid metabolism are mostly species specific and results obtained translate poorly to humans (Chandrasekera et al. 2014, Al-Awar et al. 2016, Lai et al. 2014, Bergen et al. 2005). As adipose tissue and its metabolism differ so greatly between rodents and humans

(Chandrasekera et al. 2014), it is of special importance to find replacements for animal tests in adipose research. Differences are also seen in the mouse derived cell lines 3T3-L1 and 3T3-F442A, which undergo adipogenesis during *in vitro* culturing but lack the single large lipid droplet characteristic of mature *in vivo* adipocytes (Serlachius et al. 2004) and instead have a fibroblast-like phenotype (Sugihara et al. 1987b, Sugihara et al. 1986). Additionally murine differentiated cells secrete only 1-2% of the leptin secreted by primary mature adipocytes (MacDougald et al. 1995) and do not work in a similar manner in human and mice (Bowles et al. 2001). The correct endocrine functions are important in research models of adipose tissue as there is emerging interest in studying environmental chemicals and their impact on adipose tissue. Endocrine disruptor chemicals are one class of chemicals known to cause various health impacts by affecting endocrine organs including adipose tissue (Kabir et al. 2015).

3 AIMS OF THE STUDY

The goal of this study was to develop an insulin sensitive vascularized adipose tissue model that could be used for biomedical research and chemical testing. The specific aims were the following:

1. To develop a serum-free medium for inducing *in vitro* vasculature (angiogenesis model) based on the co-culture of hASC and HUVEC (I)
2. To develop a differentiation method for producing insulin sensitive adipocytes (II)
3. To develop a proof of concept model of vascularized adipose tissue; finding the optimal culture time, timing of adipogenesis induction and angiogenesis media, and timing of cell seeding. (III)
4. To create a protocol for producing an insulin sensitive vascularized adipose tissue model by combining the optimal culture conditions, insulin sensitive adipocyte protocol and vasculature induced with serum-free medium (IV)
5. To confirm that the developed test model is suitable for testing chemicals by studying the effects of known chemicals on the model (IV)

4 MATERIALS AND METHODS

4.1 Ethical considerations

This study conforms to the principles outlined in the Declaration of Helsinki. Human adipose tissue samples were obtained from surgical operations and human umbilical cords were received from caesarean sections with individual written informed consent from Tampere University Hospital, Tampere, Finland. The use of hASC and HUVEC were approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland with the permit numbers R03058 and R08028, followed by permits R15161 and R15033, respectively.

4.2 Materials

Cell culture reagents: EGM™-2 Endothelial Cell Growth Medium-2 (EGM-2, Lonza Group Ltd), collagenase I (Invitrogen, Paisley, Scotland, UK), DMEM/F12 (Gibco, Invitrogen, Carlsbad, CA, USA), human serum (Lonza Group Ltd, Basel, Switzerland), Human serum (PAA), L-Glutamine (Gibco), 3,3',5-Triiodo-L-thyronine sodium salt (T3, Sigma), Bovine serum albumin (BSA, PAA), BSA (Biosera (Boussens, France), BSA (Roche Diagnostics Corporation, Indianapolis, USA). Sodium puryvate (Gibco), ITS Premix (BD), Penicillin/streptomycin (Gibco), MycoAlert® Mycoplasma Detection Kit (Lonza Group Ltd); ascorbic acid (AA), heparin (HE) sodium salt from bovine intestinal mucosa and hydrocortisone (HY), Transferrin, Seleniuous acid purchased from Sigma; Vascular endothelial growth factor (VEGF, R&D systems), FGF-2 (R&D systems), Troglitazone (Trogl; Sigma-Aldrich, St. Louis, MO, USA), Tryple™ express (Gibco), Ficol-paque plus (GE Health care, Buckinghamshire, UK), Triton-X100 (MP Biochemicals, Ohio, USA), PBS (Gibco), DPBS (Gibco), Chemically defined lipid concentrate (Life technologies, Carlsbad, CA), UpCell plates (ThermoFisher), insulin (Sigma).

Chemicals: Rosiglitazone (Cayman Chemical), Chlorpyrifos (Sigma), Prochloraz (Sigma), Mancozeb (Sigma), Butylparaben (Sigma), 15-Deoxy-Δ12,14-Prostaglandin

J2 (Sigma), Bisphenol A (Sigma), Rt2 First Strand Kit (Qiagen), Bis-(2-Ethylhexyl) Phthalate (Sigma), Tributyltin Chloride (Sigma).

Commercial analysis kits: Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), Cell Proliferation Reagent WST-1 (Roche Life Science, Indianapolis, IN, USA), AdipoRed Assay reagent (Lonza), Quantikine ELISA for adiponectin, leptin and aP2 (R&D Systems, Abingdon, UK), Human Total Adiponectin/Acrp30 Quantikine ELISA Kit (R&D Systems), Human Leptin Quantikine ELISA Kit (R&D Systems) and Human FABP4 Quantikine ELISA Kit (R&D Systems), EnzyChrom Adipolysis Assay Kit (BioAssay Systems, Hayward, CA, USA).

FACS reagents: CD73, CD90 and CD105, eNOS- phycoerythrin IgG1 (PE, #560103), CD144-FITC IgG1 (#560411), CD73-Phycoerythrin-Cyanine IgG1 (PE-CY7, #561258), CD309-PE IgG1 (#560872), CD68-FITC IgG2b (#562117), CD90-FITC IgG1 (#561969), CD105-V450 IgG1 (#561447), CD34-APC IgG1 (#561209), CD140b-PE IgG2a (#558821), CD31-V450 IgG1 (#561653), CD45-PE IgG1 (#560975), CD14-FITC IgG1 (#561712). Isotype controls, Mouse IgG1-PE-CY7 (#557872), mouse IgG1-PE (#559320), mouse IgG1-FITC (#555748), mouse IgG2b-FITC (#556655), mouse IgG1-V450-(#642268), mouse IgG1-APC (#550854), mouse IgG2a-PE (#551438) all from BD Biosciences (Erembodegem,Belgium); NG2-PE IgG1 (#FAB2585P), vWf-A2-allophycocyanin IgG2b (APC, #IC27641A) and mouse IgG2b-APC (#IC0041A) from R&D Systems. , BD™ CompBeads (BD).

Commercial PCR related reagents: RNase-free DNase set (Qiagen), RNAeasy minikit (Qiagen), Human Angiogenesis RT2 Profiler™ PCR Array (Qiagen, Valencia, California, USA), PureLink RNA Mini kit (Ambion/Life Technologies), VILO kit (Invitrogen), PureLink DNase treatment (Life Technologies), RNA QC kit v2.0 (Qiagen, Venlo, the Netherlands), iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), iQ™ SYBR Green Supermix (Bio-Rad), high sensitivity DNA analysis kit (Agilent Technologies, Santa Clara, CA, USA), ITaq universal SYBR green one-step kit (Biorad), 96.96 Dynamic Array Chip for Gene Expression (Fluidigm).

Other reagents: [3H]-2-deoxy-D-glucose (Perkin Elmer, Waltham, MA, USA), OptiPhase HiSafe 2 oscillation liquid (Perkin Elmer), formaldehyde (Thermo Fisher Scientific), Fluoroshield™ with DAPI (Sigma), NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher Scientific).

Equipment: Varioskan™ Flash Multimode Reader (Thermo Scientific), flow cytometer FACSCAnto II (BD), BioRad CFX96 Real Time System (BioRad

Laboratories, USA), Cell-IQ (Chipman tech., Tampere, Finland), µPlate (Thermo Fischer), QIAxcel Advanced (Qiagen), Agilent 2100 Bioanalyzer (Agilent Technologies), Fluidigm Biomark HD (Fluidigm), liquid scintillation counter Wallac1410 (Perkin Elmer), Nikon Eclipse inverted fluorescence microscope with Digital Sight DS-U2 camera (Nikon, Tokyo, Japan), LSM710 with Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany),

Programs: BD FACSDiva™ Software (BD), CFX96 Real-Time System software (Bio-Rad), Microsoft Excel 2010 and 2013 (Microsoft Corporation, Redmond, WA, USA), Fluidigm's BioMark Data Collection Analysis program (version 4.1.3), Cell-IQ Analyzer (CM Technologies Oy), ImageJ software (National institutes of health, NIH, Maryland, USA), GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA), PCR Array Data Analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php), ZEN 2012 software (Carl Zeiss), Adobe Photoshop CS3 software (Adobe Systems Incorporated, San Jose, CA, United States), NIS Elements (Nikon, Tokyo, Japan)

4.3 Cells and extracts from primary tissues

4.3.1 Isolation and culture of human adipose stromal cells (I,II, III and IV)

hASC used in the study were heterogenous cell populations obtained by isolating stromal vascular fraction cells of human adipose tissue. hASC were isolated from human adipose tissue by using a mechanical and enzymatic procedure described previously (Sarkanen et al. 2012a). Briefly, human adipose tissue specimens were mechanically cut into small pieces and enzymatically digested with 0.15% collagenase I in DMEM/F12. hASC were cultured in hASC medium (Table 3). Cells were tested for mycoplasma contamination before experimental use. Seeding density of hASC was 20000 cells/cm^2 for experiments, unless otherwise stated. hASC were used at passage 2 for experiments.

4.3.2 Isolation and culture of human umbilical vein endothelial cells (I,III and IV)

HUVEC used in the study were isolated from donated umbilical cords. HUVEC were isolated by cannulating the umbilical cord vein and infusing the vein with 0.05%

collagenase I as described previously (Sarkanen et al. 2011). HUVEC were cultured in EGM-2 Medium. Before use cells were tested for mycoplasma contamination. Seeding density of HUVEC was 4000 cells/cm2 and HUVEC were at passage 4 in co-cultures.

4.3.3 Adipose tissue extract (II, III and IV)

Adipose tissue extract (ATE) was produced as described previously (Sarkanen et al. 2012b) but with a modification to the ratio of medium to fat. Briefly, the human adipose tissue sample was manually cut into small pieces. The sample was then incubated in DMEM/F12 containing 2 parts manually dissected fat and 3 parts DMEM/F12, for 24 h at 37 °C in 5 % CO humified atmosphere. This was then centrifuged at 200g for 3 min and liquid filtered through a 0.2-μm filter. The protein content of the extract was analyzed with a BCA kit using BSA as a standard. Results were measured after 30 min incubation at 37 °C at 562 nm. The ATE was stored at −20 °C until used. Batches were not pooled.

4.3.4 Quality control of isolated cells (I)

hASC were cultured in hASC medium for 6-7 days and HUVEC were cultured in EGM-2 medium for 3 days prior to surface marker expression analysis by flow cytometry. Flow cytometric analyzed 250,000 cells per sample.

The cells were washed once with 1% BSA in PBS, centrifuged at 131 x g for 5 min, after which they were stained either for intracellular markers (eNOS and vWf) or surface markers (all other studied markers available with FACS). Fixation and permeabilization were only performed when staining intracellular markers. The fixation was conducted in 2 % paraformaldehyde in PBS at room temperature (RT) followed by centrifugation of 500 x g for 5 min. Permeabilization was performed by 10 min incubation in 0.1% Triton-X100 in PBS at RT followed by centrifugation of 500 x g for 5 min. Cells were washed once or twice with 1% BSA before addition of antibodies. Labelled antibodies were added into cell suspension in cold 1% BSA and incubated on ice for 30 min in the dark. Cells were washed once with 1% BSA and twice with PBS. Surface marker stained cells were centrifuged at 200 x g for 5 min and intracellular marker stained cells were centrifuged for 5 min at 500 x g. Flow cytometry analysis was performed with cells suspended in ice cold PBS and 5000

events were analyzed per sample. Compensation was done with compensation particles.

Positive expression was obtained by gating 98% of event isotype control results and then inverting the gate to obtain a percentage of positively stained cells in samples. The results were calculated as percentages with SD.

For continuous quality control, hASC batches were analyzed for their expression of markers CD73, CD90 and CD105 before experimental use (Original publications II-IV) to ensure the isolated population fulfilled the minimal criteria set by The International Society for Cellular Therapy (Dominici et al. 2006).

4.4 Differentiation methods

4.4.1 Angiogenesis model i.e. vasculature component (I)

Co-culture of hASC and HUVEC was established as described previously (Sarkanen et al. 2012a) but with the addition of serum-free angiogenesis medium. Different angiogenesis induction media were developed and tested to find the best medium composition for angiogenesis stimulation. The most optimal medium chosen and currently utilized in the model (Toimela et al. 2017) is depicted here. The time sequence of the culture protocol of the angiogenesis model and its control is depicted in Table 4.

hASC were seeded in EGM-2 followed by seeding of HUVEC in EGM-2 medium after 1-3 hours. The day after plating, angiogenesis medium was applied to the co-culture (see Table 3). Results were compared to previously used EGM-2 induced vasculature and to the basal serum-free medium of the amgiogenesis medium (undifferentiated cells control).

During the development of the angiogenesis medium, the concentrations of AA, HE and HY were optimized. The tested concentrations of AA were 0, 50, 100, 200, 500, 1000 and 2000 μg/ml; of HY 0, 20, 200, 1000, and 2000 ng/ml and of HE 0, 50, 500, 10000 and 50000 ng/ml. The co-cultures were grown for 6 days prior to analysis. Stimulation medium was changed once during the 6 day culture.

Table 3. Culture media used in the study, their abbreviations and content.

Medium	Abbreaviation	Content
hASC medium	hASCm	DMEM/F12 10 % Human serum 2 mM L-glutamine
Serum-free medium	SFM	DMEM/F12 2.56 mM L-glutamine 0.1 nM T3 ITS Premix: 1.15 µM: 6.65µg/ml insulin 6.65 µg/ml Transferrin 6.65 ng/ml seleniuous acid 1 % BSA 2.8 mM Sodium puryvate
ATE medium	ATEm	1800 µg/ml ATE DMEM/F12 10 % Human serum 2 mM L-glutamine 100 IU/ml Penicillin (50 IU/µl in III and IV) 0.1 mg/ml streptomycin (50µg/µl in III and IV)
Angiogenesis medium	ANG	Serum-free medium supplemented with 200 µg/ml AA 0.5 µg/ml HE 5.5 µM: 2 µg/ml HY 10 ng/ml VEGF 1 ng/ml FGF-2
Angiogenesis medium w/o insulin	ANG -I	DMEM/F12 2.56 mM L-glutamine 0.1 nM T3 6.65 µg/ml Transferrin 6.65 ng/ml Seleniuous acid 1% BSA 2.8 mM Sodium puryvate 200 µg/ml AA 0.5 µg/ml HE 2 µg/ml HY 10 ng/ml VEGF 1 ng/ml FGF-2
Adipogenesis/angiogenesis medium	ANG +T	Angiogenesis medium supplemented with 9 µM Troglitazone

Table 4. Time sequence of the culture of the angiogenesis model, insulin sensitive adipocytes and vascularized adipose tissue and their control protocols. Ficol=Ficol Paque Plus

Protocol name	Day 0	Day 1	Day 3/ Day4	Day 6/ Day7	Day8	Day 10	Day 14
Undifferentiated cells control for angiogenesis model	seeding hASC & HUVEC in EGM-2	SFM	SFM	analysis			
Previous angiogenesis model	seeding hASC & HUVEC in EGM-2	EGM-2	EGM-2	analysis			
Angiogenesis model	seeding hASC & HUVEC in EGM-2	ANG	ANG	analysis			
Undifferentiated hASC	seeding hASC in hASCm	hASCm	SFM	SFM	-	SFM	analysis
Previous adipocyte protocol	seeding hASC in hASCm	ATEm	ATEm	ATEm	-	ATEm	analysis
Insulin sensitive adipocyte protocol	seeding hASC in hASCm	ATEm	ANG +T	-	-	ANG -I	analysis
Undifferentiated cells control for vascularized adipose tissue	seeding hASC in hASCm	hASCm + 25µl Ficol	SFM +25µl Ficol	HUVEC in EGM-2	SFM +25µl Ficol	SFM+25µl Ficol	analysis
Adipocyte control for vascularized adipose tissue model	seeding hASC in hASCm	ATEm +25µl Ficol	ANG+T +25µl Ficol		ANG+T +25µl Ficol (chemical exposure)	ANG –I + 25µl Ficol (chemical exposure)	analysis
Vascularized adipose tissue model	seeding hASC in hASCm	ATEm +25µl Ficol	ANG+T +25µl Ficol	seeding HUVEC in EGM-2	ANG+T +25µl Ficol (chemical exposure)	ANG –I +25µl Ficol (chemical exposure)	analysis

4.4.2 Insulin sensitive adipocytes (II)

To develop a protocol for insulin sensitive adipocytes differentiated from hASC, several media were tested with different combinations. Here the protocol producing the most promising adipocytes is depicted.

hASC were plated in hASC medium (Table 3). The following day, differentiation began according to Table 4 utilizing differentiation media seen in Table 3. A combination of hASC medium and serum-free medium was used as the

Undifferentiated cell control and the Previous adipocyte protocol was used as a reference for the differentiation (Table 4). The total culture time was 14 days, except for the long-term culture, which was cultured for 12 weeks. For the long-term culture there were no medium changes after day 10. Due to the medium loss caused by evaporation, DMEM/F12 was added when needed and once a week 0.04 v/v % of a chemically defined lipid concentrate was added to supply energy.

4.4.3 Combining vascularization and adipocytes (III)

Several protocols were tested and compared for their ability to create vascularized adipose tissue. Protocols varied in cell number, cell plating time-point and the differentiation process (adipogenesis induced prior to angiogenesis or vice versa). Adipogenesis was induced with the ATE medium and angiogenesis with angiogenesis medium (Table 3). The most prominent from these two protocols were continued with and are presented in Table 5. Three types of controls were used: Undifferentiated hASC+HUVEC (Table 5), vasculature control i.e. Angiogenesis model (Table 4) and adipocyte control i.e. Previous adipocyte protocol (Table 4). All cells were seeded in EGM-2. HUVEC were seeded on top of hASC in a 50 µl aliquot, either on day 0 or day 7 depending on the protocol (according to Table 5). Differentiation media were changed on days 4, 8 and 11.

Table 5. Plating and differentiation details of different protocols for combining vascularization and adipocytes

Protocol name	hASC plating	Seeding density (cells/cm²)	HUVEC plating	Seeding density (cells/cm²)	Medium on days 1-7	Medium on days 8-14
Undifferent-iated hASC+ HUVEC	Day 0	40000	Day 0	4000	SFM	SFM
P1	Day 0 Day 7	20000 20000	Day 7	4000	ATE m	ANG
P6	Day 0	20000	Day 0	4000	ANG	ATEm

4.4.4 Vascularized adipose tissue model (IV)

Serum-free ANG medium and the differentiation media sequence of the Insulin sensitive adipocyte protocol were combined with the culture conditions of P1 to create optimized vascularized adipose tissue. P1 was optimized by lowering the number of seeded hASC to 20000 cells/cm². For vascularized adipose tissue hASC were plated on day 0. On days 1, 4, 8 and 11 media were changed according to Table 4. On day 7 HUVEC were plated after first removing 100µl of culture media from wells and then adding HUVEC in 100µl of EGM-2. Undifferentiated control contained hASC and HUVEC cells cultured in basic media corresponding to media used in the differentiation (Undifferentiated cells control for vascularized adipose tissue, Table 4). Adipocytes were differentiated with the Insulin sensitive adipocyte control protocol with the addition of one replenishment of medium and macromolecule ficol (Adipocyte control for vascularized adipose tissue, Table 4). Total medium volume used per well was 500µl and after each media change, Ficol-paque plus was added to each well at 25µl per 500µl of culture media. The different media compositions can be seen in Table 3. Endpoint analyses were performed at day 14.

4.5 Chemical exposure (IV)

The effect of the nine chemicals Rosiglitazone, Chlorpyrifos prochloraz, Mancozeb (MANC), Butylparaben, 15-Deoxy-Δ12,14-prostaglandin J2, Bisphenol A, bis-(2-ethylhexyl) phthalate and tributyltin chloride were studied for their impact on lipid accumulation, mitochondrial activity and amount of vasculature in the vascularized

adipose tissue. The effects of selected chemicals were compared between adipocytes and vascularized adipose tissue also at the gene expression level, in addition to the above mentioned analysis. Details of chemicals are found in Table 6.

Chemical exposures were performed on day 8. After one week of exposure, analyses were performed. The purity of chemicals used was acknowledged when making dilutions. Vehicle controls for all chemicals were exposed to the same diluent concentration in which the chemicals were diluted. EC50 values were calculated using GraphPad Prism 6.05 software using the "log(inhibitor) vs. response - Variable slope" equation.

Table 6. Information and concentrations of chemicals used in this study.

Chemical	CAS number	Concentrations tested	Diluent
15-Deoxy-Δ12,14-prostaglandin J2	87893-55-8	3,15;1;0,315;0,1;0,0315;0,01;0,003;0,001 µg/ml	0.5% DMSO
BIS-(2-ETHYLHEXYL) PHTHALATE	117-81-7	100, 31.6, 10, 31.7, 1, 0.32, 0.1, 0.0032 µg/ml	0.5% DMSO
Bisphenol A	80-05-7	99.4, 31.45, 9.95, 3.15, 0.5, 0.3, 0.0995, 0.03 µg/ml	0.5% DMSO
Butylparaben	94-26-8	500, 158, 50, 16, 5, 1.5, 0.5, 0.15 µg/ml	medium
Chlorpyrifos	2921-88-2	100, 31, 10, 3.1, 1, 0.3, 0.1, 0.03 µM	0.5% Ethanol
Mancozeb	8018-01-7	0.5, 0.16, 0.05, 0.016, 0.005, 0.0015, 0.0005, 0.00015 µg/ml	medium
Prochloraz	67747-09-5	5000, 1000, 500, 250, 50, 5, 0.5, 0.05µM	0.5% Ethanol
Rosiglitazone	122320-73-4	139.9, 44, 14, 4.4, 1.4, 0.44, 0.14, 0.044 µM	0.5% DMSO
TRIBUTYLTIN CHLORIDE	1461-22-9	325.5, 103, 32.5, 10.3, 3.25, 1.04, 0.33, 0.11 ng/ml	0.5% DMSO

4.6 Analysis methods for both vasculature and adipocytes

4.6.1 WST-1 (II, III, IV)

WST-1 reagent was added to culture medium at a ratio of 10µl/100µl of medium on day 14. After 1.5 h (adipocytes and controls) or 1h (vascularized adipose tissue and controls) incubation at 37 °C in 5 % CO, the absorbance was measured at 450 nm as a multipoint measurement.

4.6.2 PCR (I, II, III, IV)

For gene analysis by quantitative real-time PCR, cell cultures were washed with PBS and total RNA was extracted from cultures using an extraction kit following the manufacturer's protocol. Genomic DNA contaminations were eliminated during RNA isolation with reagents suitable for use with RNA isolation kits. Reverse transcription of total RNA to cDNA was performed following manufacturer's instructions for cDNA transcription kit use.

Gene expression assessment of the angiogenesis model utilized the Human Angiogenesis RT2 Profiler™ PCR Array for 84 key angiogenesis related genes. The previous angiogenesis model was used as a control for expression change calculations in the web portal designed for the kit. A two-fold change compared to the housekeeping gene GAPDH was considered relevant.

The analysis of adipocytes and controls was performed with the gene-specific primer pairs seen in Table 7. Relative gene expression results were calculated by normalizing Ct values with the geometric mean of two housekeeping genes SDHA and 36B4.

For combining of adipocytes and vasculature, RNA was first analyzed for its purity by the 260/280nm absorbance ratio and quality of RNA samples was checked using QIAxcel. Primer concentration was 300 nM, and the amount of template was 30 ng. Thermal cycling conditions were as follows: 95°C 3 min., 95°C 10 sec., 51–65°C (gradient) 15 sec., 72°C 30 sec., repeated 40 times. Melt curve analysis was performed at 55–95°C (0.5°C increments/10 sec.). The quality of amplified DNA was studied with micro-capillary electrophoresis using a DNA analysis kit according to manufacturer's instructions. The relative quantification of mRNA expression data was calculated using the ΔΔCt method with the following equation:

$2^{(Ct(\text{mean of housekeeping genes SDHA and 36B4, studied protocol})-Ct(\text{Gene of interest, studied protocol}))} /$

$2^{(Ct(\text{mean of housekeeping genes SDHA and 36B4, control})-Ct(\text{Gene of interest, control}))}$

For expression analyses, the previous adipocyte protocol was used as a control.

In the vascularized adipose tissue model, gene expression studies of insulin exposed cultures were performed by adding 500µM insulin for 24h prior to RNA isolation. A one-step PCR kit was utilized directly after RNA isolation using 30 ng of template and primers at a concentration of 300nM. Melt curve analysis was performed for each run. Results were calculated with the ΔΔCt method and 36B4 as a housekeeping gene. In calculations, unexposed and insulin exposed samples were analyzed using undifferentiated cells as the control and chemical exposed cultures were compared to corresponding vehicle controls. To inspect stage of vascular maturation in the model, a panel of 30 genes was analyzed with the fluidigm system. The details of the fluidigm analysis methods and primers used can be found in Original publication IV.

Table 7. Primer sequences used in this study.

Gene	Primer sequence	Publication used in
36B4	Forward- ATGCTCAACATCTCCCCCTTCTCC Reverse- GGGAAGGTGTAATCCGTCTCCACAG	II, III,IV
ADCY5	Forward- TCTCCTGCACCAACATCGTG Reverse- CATGGCAACATGACGGGGA	II
Adiponectin	Forward- GGCCGTGATGGCAGAGAT Reverse- CCTTCAGCCCGGGTACT	II, III, IV
FABP4	Forward- GCTTTTGTAGGTACCTGGAAACTT Reverse- ACACTGATGATCATGTTAGGTTTGG	II, III, IV
CIDEA	Forward- GATGCCCTCGTCATCGCTAC Reverse- GCGTGTTGTCTCCCAAGGTC	II
Glut4	Forward- TGGGCGGCATGATTTCCTC Reverse- GCCAGGACATTGTTGACCAC	II, III
Leptin	Forward- GCCCTATCTTTTCTATGTCC Reverse- TCTGTGGAGTAGCCTGAAG	II, III
PGC1-α	Forward- GCTTTCTGGGTGGACTCAAGT Reverse- GAGGGCAATCCGTCTTCATCC	II
PPARγ	Forward- GATCCAGTGGTTGCAGATTACAA Reverse- GAGGGAGTTGGAAGGCTCTTC	II, III, IV
PPARγ2	Forward-CAGTGTGAATTACAGCAAACC Reverse- ACAGTGTATCAGTGAAGGAAT	III, IV
Prdm16	Forward- CGAGGCCCCTGTCTACATTC Reverse- GCTCCCATCCGAAGTCTGTC	II
RIP140	Forward- GGATCAGGTACTGCCGTTGAC Reverse- CTGGACCATTACTTTGACAGGTG	II
SDHA	Forward- CATGCTGCCGTGTTCCGTGTGGG Reverse- GGACAGGGTGTGCTTCCTCCAGTGCTCC	II, III
UCP1	Forward- CAATCACCGCTGTGGTAAAAAC Reverse- GTAGAGGCCGATCCTGAGAGA	II
Glut4	Forward- CGT CGG GCT TCC AAC AGA TA Reverse- CAC CGC AGA GAA CAC AGC AA	IV
Leptin	Forward- AGG GAG ACC GAG CGC TTT C Reverse- TGC ATC TCC ACA CAC CAA ACC	IV
PPARα	Forward- GGC GAA CGA TTC GAC TCA AG Reverse- TCC AAA ACG AAT CGC GTT GT	IV
Glut1	Forward- GCT GTG CTT ATG GGC TTC TC Reverse- CAC ATA CAT GGG CAC AAA GC	IV

4.6.3 Immunocytochemistry (I, II, III, IV)

To visualize structures in the angiogenesis model, insulin sensitive adipocytes and vascularized adipose tissue model, immunocytochemical staining was performed. Angiogenesis models were fixed with 70% ethanol with a 20 min incubation at RT or with 4% formaldehyde for 20 min at RT. After fixation, cells were permeabilized with 0.5% Triton-X100 at RT for 15 min and non-specific binding sites were blocked with 10% BSA at RT for 30 min. Primary antibodies were diluted with 1% BSA, applied to cells and incubated for 1h at RT or overnight at +4°C. Cells were then incubated with secondary antibodies diluted in 1% BSA in DPBS for 40 min at RT. Washes between treatments were performed with PBS or DPBS. Nuclei were stained with DAPI in the bedding reagent (II, IV) and with NucBlue in the long-term culture of insulin sensitive adipocytes (unpublished). The antibodies used can be found in Table 8.

Table 8. Antibodies used in immunostainings for this study

Antibody	Produced in	Target	Used dilution	Product number, Manufacturer	Publication
anti- human von Willebrand factor IgG	rabbit	endothelial cell	1:100	F3520, Sigma	I, III, IV
anti- human smooth muscle actin (clone 1A4)	mouse	common pericytic marker	1:200	M0851, DAKO	I
anti- human smooth muscle myosin heavy chain (clone hSM-V)	mouse	vascular smooth muscle cell marker	1:800	M7786, Sigma	I
anti- human collagen IV (clone COL-94)	mouse	basement membrane marker	1:500	C1926, Sigma	I, III, IV
anti- human occludin (clone 1G7)	mouse	tight junctions	1:300	WH0004950M1, Sigma	I
anti- human vascular endothelial cadherin (CD144, Clone 55-7H1)	mouse	Vascular adherens junctions	1:50	555661, BD Pharmingen	I
anti- human platelet derived growth factor receptor-β (clone PDGFR-B2)	mouse	pericytic and smooth muscle cell progenitor marker	1:800	P7679, Sigma	I
anti- human calponin (clone hCP)	mouse	contractile smooth muscle cell marker	1:800	C2687, Sigma	I
anti- human collagen IV	Rabbit	basement membrane marker	1:500	ab6586, abcam	IV
anti- human CD140b-PE (Clone 28D4)	mouse	pericytic and smooth muscle cell progenitor marker	1:24	558821, BD Biosciences	IV
anti- human CD144-FITC (Clone 55-7H1)	mouse	Vascular adherens junctions	1:24	560411, BD Biosciences	IV
anti-rabbit IgG tetramethylrhodamine (TRITC)	goat		1:50	T6778, Sigma	I, III, IV
anti-rabbit IgG A568	goat		1: 400	A11011, Invitrogen	I
anti-mouse IgG fluorescein isothiocyanate (FITC)	goat		1:100	F4143, Sigma	I, III, IV

4.7 Analysis methods of the vascular network

4.7.1 Quantitative analysis of vascular-like network formation (I, III and IV)

Immunostained vascular networks were imaged using Cell-IQ with a 10x objective and 5x5 grid. Images were stitched together and converted to 8-bit gray scale, with

background subtracted and finally the binary threshold function was adjusted to obtain the best contrast of the vascular-like network against background. With these settings, the total area of the vascular-like network was calculated as the total number of pixels in images with a set threshold. For the analysis of tubules in vascularized adipose tissue, the protocol was optimized for size restriction and circularity exclusion to exclude the fluorescent adipocytes.

4.7.2 Electron microscopy of vascular structures (I)

The Angiogenesis model was cultured in 24-well UpCell plates for transmission electron microscopy (TEM) and on glass cover slips coated with 0.1% gelatin for scanning electron microscopy (SEM). Cells were washed twice with PBS prior to fixation. SEM specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer. TEM specimens were detached from the temperature sensitive plate and transferred to fixative with a 1% glutaraldehyde, 4% formaldehyde mixture in 0.1 M phosphate buffer for 10 min. The cell sheet was immersed in 2% agarose in distilled water. Details of the SEM and TEM imaging procedures can be found in Original publication I.

4.8 Analysis methods of Adipocytes

4.8.1 Triglyceride accumulation (II, III and IV)

The triglyceride accumulation was determined using Adipored. The accumulation of triglycerides was analyzed from the same wells from which the WST-1 analysis was done by using Adipored according to the manufacturer's instructions. Briefly, at the end point of cultivation, the culture was washed with DPBS. AdipoRed reagent diluted with PBS was added to the plate and after 10 min incubation at RT, fluorescence was measured as a multipoint measurement using the excitation wavelength of 485 nm and measuring emission at 572 nm. In order to quantify the amount of accumulated triglycerides per cell, Adipored values were divided by WST-1 values.

4.8.2 Analysis of secreted proteins (III, IV)

Media samples were analysed for their concentration of adiponectin, leptin and FABP4 secreted by adipocytes. Commercial ELISA kits were utilized according to manufacturer's instructions. Absorbance was measured at 450nm and at 540nm (background signal). Sample concentrations were calculated from standard curves. To normalize results, the relative cell number i.e. WST-1 results were utilized.

In the combination study Protocol 1 and Protocol 6 were compared to different controls due to the cytokine-rich composition of ATE, which is present in the medium of Protocol 6 at time point analysis. Protocol 1 was compared to medium sample of the angiogenesis model and Protocol 6 to the previous adipocyte protocol also containing ATE at day 14.

4.8.3 Glucose uptake test (II, IV)

To analyze glucose uptake in response to insulin, the following protocol was utilized. At day 14, DMEM/F12 was added to cells and incubated for 2h at 37 °C in 5 % CO. Cells were then exposed to 100 nM or 500 nM insulin and incubated at 37 °C in 5 % CO for 30 min, and [^{3}H]-2-deoxy-D-glucose at an amount of 0.2 μCi/well added for another 20 min. The cells were washed twice with ice-cold PBS and then lysed in 0.1 % sodium dodecyl sulfate (SDS). The radioactivity of samples was measured. If results were normalized total protein content was utilized.

4.8.4 Inhibition of lipolysis (II, IV)

For analysis of lipolysis inhibition of adipocytes in response to insulin, released glycerols were measured. At day 14, medium was removed, cells washed with PBS, and DMEM/F12 added to cells. After 1-2 hours, 2 μM Isoproterenol (Sigma) was added and incubated for 1.5-2h after which 100 nM or 500 nM insulin (Sigma-Aldrich) was added. After 15 min, 30 min or 45 min incubation, medium was collected. The amount of glycerol released was assessed with an EnzyChrom Adipolysis Assay Kit according to manufacturer's instructions. The fluorescence was measured at 530 nm and 585 nm. If results were normalized with total protein content, cells were lysed with 0.1 % SDS for the measurement of total protein content.

4.9 Statistical analysis (I, II, III, IV)

Statistical analyses were performed and graphs processed with GraphPad Prism. The results were subjected to one-way or two-way ANOVA followed by Dunnett's post-test, Tukey's post-test, Sidak's multiple comparison test or Fisher's LSD test depending on the materials in question. The results were reported as mean ± SD and differences were considered significant when $p < 0.05$*, $p < 0.01$** and $p < 0.001$***.

5 RESULTS

The outline of the study is presented in Figure 1. The figure depicts the aims of the study, the models developed and the features utilized in the final vascularized adipose tissue model.

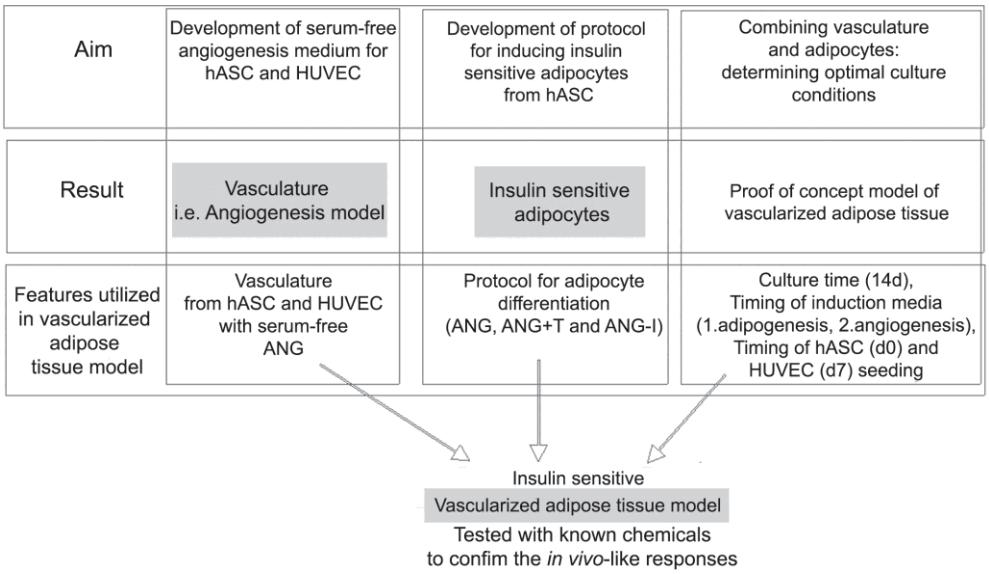

Figure 1. Outline of the study; aims, developed models and their features utilized in the vascularized adipose tissue. Blocks shaded with grey are *in vitro* models utilizable for e.g. biomedical and toxicological research.

5.1 Cell quality control (I)

Phenotypic characterization of hASC and HUVEC isolated from donor tissue samples was performed by flow cytometric analysis. The specific percentages of markers can be found in Original manuscript I, Table 2.

hASC were shown to express mesenchymal stem cell markers (CD73, CD90 and CD105) and pericyte marker (PDGFR-β i.e. CD140b). The hASC population showed low expression of endothelial markers (CD144 and CD31) and

macrophage/monocyte markers (CD68 and CD14) and moderate expression of hematopoietic markers CD34 and CD45. According to minimal criteria for mesenchymal stem cells, they must 1) be plastic-adherent, 2) differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* and 3) express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules (Dominici et al. 2006). The population obtained with the isolation method used in this study fulfills the first and second criteria as shown in earlier studies. The results of this study show positivity for CD73, CD90 and CD105, confirming presence of mesenchymal stem cells, but also shows the heterogeneous nature of the population as hematopoietic markers were higher than in a pure stem cell population. The isolated cell population was the whole stromal vascular fraction of the adipose tissue hence the presence of different stromal cell types is expected and desirable.

To ensure the presence of stem cells in the cell population, quality criteria were set for the isolated hASC populations. Based on the results of flow cytometry, only batches expressing >50% of the positive markers CD73, CD90 and CD105 were accepted for use.

The characterization of HUVEC showed the endothelial marker CD31 and specialized vascular endothelial marker CD144 were expressed at high levels. Also markers CD105 and CD73 were moderately expressed in HUVEC indicating the presence of precursor cells. The marker for macrophages/monocytes (CD68) and mural cells (NG2) were expressed at low levels.

As vascular formation ability was the most important feature of HUVEC in this study, flow cytometric analysis was omitted from the quality control analysis of isolated HUVEC. The vascular formation ability of HUVEC batches was tested according to (Sarkanen et al. 2011).

5.2 Angiogenesis model with serum-free stimulation medium (I)

In original publication I, several different angiogenesis stimulation media were tested to find the one inducing the most optimal vascular formation in the hASC and HUVEC co-culture. The chosen medium was serum-free defined medium, which could be modified to a xeno-free composition if necessary.

The developed angiogenesis medium contained AA, HE and hydrocortisone (HY) in addition to the angiogenic growth factors VEGF and FGF-2. The concentrations of VEGF and FGF-2 were optimized in the previous study (Sarkanen

et al. 2011). The concentrations of AA, HE and HY were optimized (See original publication I, Fig. 2) and a dose dependent curve was obtained for AA, which stimulated angiogenesis at the lower concentrations and caused inhibition at higher concentrations (Fig. 2). The optimal concentrations for AA, HE and HY separately were AA 100 μg/ml, HE 50 μg/ml, and HY 0.2 μg/ml. After combining, the final optimized AA, HE, HY concentrations were AA 200 μg/ml, HE 500 ng/ml and HY 2 μg/ml. The angiogenesis medium (ANG) produced a dense uniformly distributed vascular network with connected branches, and intact endothelial and basement membrane layers (Fig. 2).

Comparison of vascular structures induced by EGM-2 and ANG at the mRNA level showed up-regulation of Angpt1, F3, FIGF, IGF-1, Leptin, MDK, MMP2, MMP9, PGF, and down-regulation of CCL11, CXCL9, FN1, IL-6, IL-8, SERPINE1, transforming growth factor B2, THBS2, TIMP1 in ANG.

The electron microscopy images showed that the vascular structures were three dimensional (3D) tubules, contain lumen and are surrounded by extracellular matrix (ECM) fibers (Fig. 2). In addition, the presence of junctions, pericytes and other contractile vascular muscle cells was shown in the immunostaining results (Fig. 3). Immunostaining and electron microscopy analyses also showed that there is on-going angiogenesis as the vascular structures were in different stages of maturation (original publication I).

The ECM components seen in the electron microscopy were produced by the vascular structures thus creating a natural 3D scaffold. The interaction between the ECM and vascular network directs vessel growth (Hoying et al., 2014; Du et al., 2014). This angiogenesis model provides an *in vivo*-like test system without added artificial scaffolding that could interfere with cell-cell interactions or affect toxicological applications of the model.

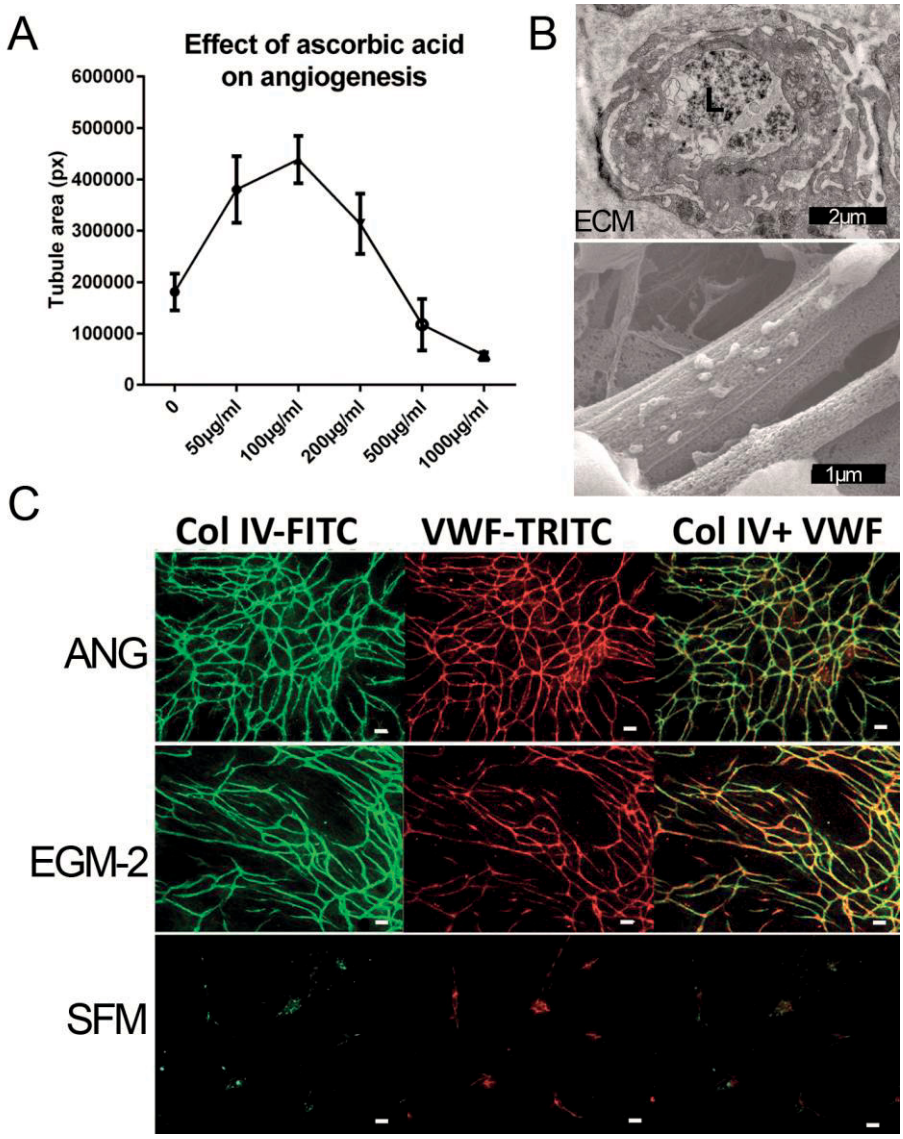

Figure 2. Results from the development of serum-free angiogenesis medium. A) The effect of different ascorbic acid concentrations on angiogenesis depicted as area of vascular structures in pixels. B) Electron microscopy images of vasculature. ECM=extracellular matrix, L= lumen) Scale bar in transmission image 2µm and in scanning image 1µm. C) Morphology of vascular structures in new Angiogenesis medium (ANG), commercial EGM-2 and in basal serum-free medium (SFM). Antibodies used are Collagen IV (FITC, green), vWf (TRITC, red) and the co-localization seen in the merged image. Scale bars 100 µm in each figure. Figure modified from Original publication I.

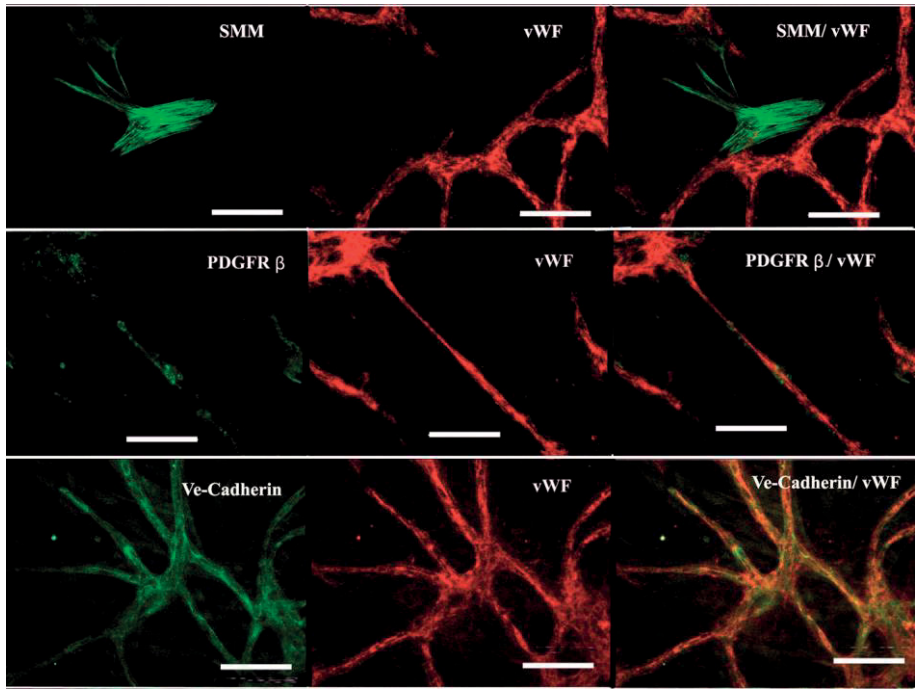

Figure 3. Angiogenesis model induced with novel angiogenesis medium contains vascular smooth muscle cells (top row), pericytes (middle row) and adherens junctions (bottom row) around and inbetween endothelial cells stained with vWf. Scale bar 100 µm in each image. SMM= Smooth muscle myosin heavy chain, PDGFR-β= Platelet derived growth factor receptor β, ve-cadherin=vascular endothelial cadherin, vWf=von Willebrand factor. Figure modified from Original publication I.

5.3 Insulin sensitive adipocytes (II)

Several medium compositions were tested to find the optimal differentiation protocol for producing insulin sensitive adipocytes from a hASC monoculture *in vitro*. The most promising protocol (protocol 7 in original publication II), induced good lipid accumulation in adipocytes, which expressed mature adipocyte marker genes and responded to insulin with increased glucose uptake and inhibition of lipolysis (Fig. 4). In this protocol, hASC had a 3-day induction with ATE medium (ATEm), 6 days in serum-free medium supplemented with 1.15 µM insulin and 9.06 µM Troglitazone (ANG+T), followed by 4 days in a defined serum- and insulin-free stimulation medium (ANG-I).

The developed protocol for insulin sensitive adipocytes induced lipid accumulation in fewer cells than the previously used ATE medium, but the size of lipid storage in individual cells was greater. hASC differentiated with this protocol consistently showed the highest and most stable expression of PPARγ, Glut4, AP2 and adiponectin. Expression of leptin mRNA remained low.

Glucose uptake by undifferentiated control cells and developed protocol adipocytes was studied by employing [3H]deoxyglucose (Fig. 4), with results showing a significant insulin-induced enhancement of glucose uptake at a 100 nM concentration of insulin in insulin sensitive adipocytes when compared to the undifferentiated control. The ability of insulin to inhibit lipolysis in undifferentiated control cells and adipocytes differentiated with the new protocol was analyzed by measuring glycerol released into growth medium after exposure of cells to 0, 100 or 500 nM insulin. Inhibition of lipolysis in adipocytes was evident with 500 nM insulin, at all studied time points.

To investigate whether the differentiation protocol selectively induced a brown or white adipocyte-like phenotype, gene expression typical for brown adipocytes, PRDM16, CIDEA, UCP1, and PGC1α (Shinoda et al. 2015, Seale et al. 2007) and white adipocytes, ADCY5, RIP140 (Knigge et al. 2015, Sawada et al. 2010, Maffei et al. 1995, Zhang et al. 1994) were studied. Gene expression analysis of white and brown adipocyte specific gene markers showed no specific tendency towards either adipocyte type; of the studied genes, all were expressed except RIP140, which was only marginally expressed.

Under long-term culture, adipocytes continued to mature without additional differentiation agents. Adipocytes in long-term culture showed the typical morphology of mature adipocytes; one large lipid storage and the nucleus pushed to the side (Fig 5, unpublished data).

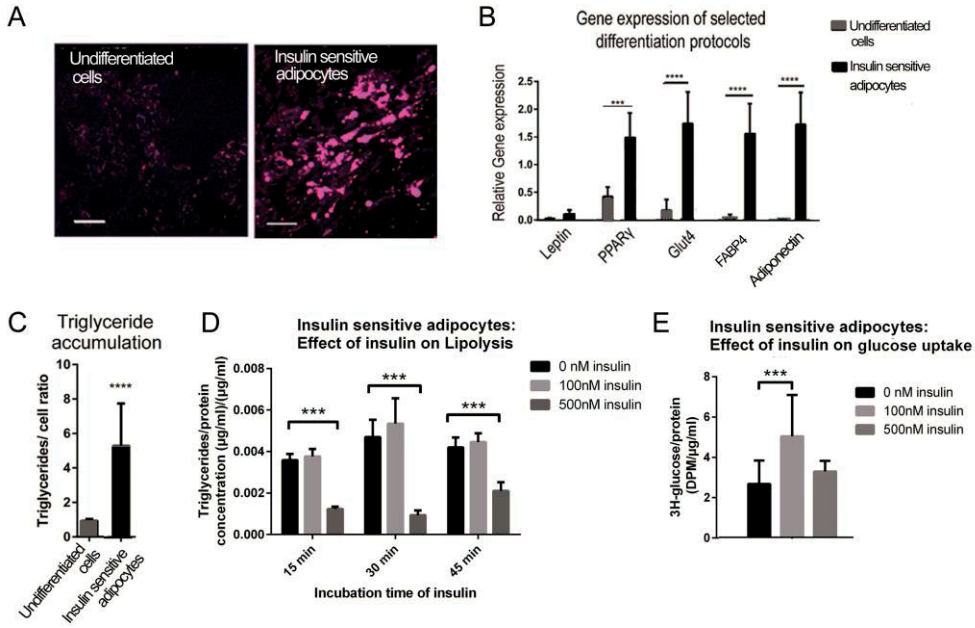

Figure 4. Characteristics of insulin sensitive adipocytes. A) Confocal image of lipid staining with adipored reagent in undifferentiated cells and insulin sensitive adipocytes, nuclei stained with DAPI, scale bar 100 µm B) The differentiated insulin sensitive adipocytes showed expression of mature adipocytes, FABP4 and Adiponectin. C) Triglyceride accumulation quantification D) Inhibition of lipolysis by 500nM was seen with 15 min, 30min and 45min in insulin sensitive adipocytes. E) Glucose uptake was increased at 100nM insulin. ***p<0.001. Figure modified from Original publication II.

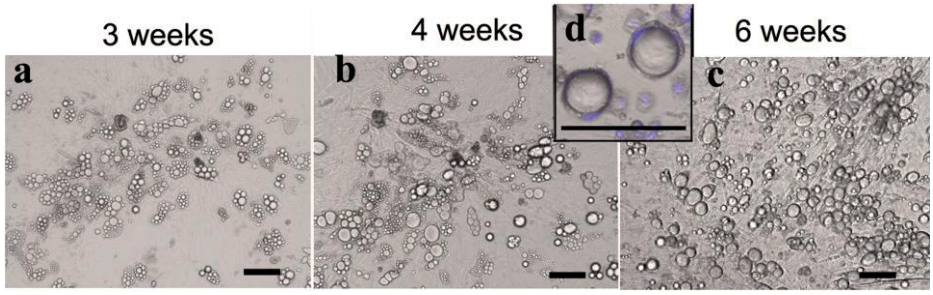

Figure 5. Phase contrast images combined with fluorescent images of long term culture of insulin sensitive adipopcytes at a) 3 weeks b) 4 weeks and c) 6 weeks. Insert of mature adipocytes seen especially from 4 weeks onwards. NucBlue was used for nuclei staining. Scale bar 100µm. Unpublished data.

5.4 Combining vasculature and adipocytes (III)

The order for induction of angiogenesis and adipogenesis, plating sequence of cells and cell numbers for the vascularized adipose tissue model were determined in experiments presented in original publication III. As a result, two protocols were selected as the best options for creating vascularized adipose tissue, although both needed additional optimization depending on the application used. Protocols P1 and P6 from study III were found to be the best options for combining vasculature and adipocytes. In protocol 1 hASC were plated on day 0 and day 7 giving a total of 40000 hASC/cm^2. HUVEC were plated on day 7. The adipogenesis was induced in the first week and angiogenesis the second week. In protocol 6, hASC and HUVEC were plated on day 0 and angiogenesis was induced during the first week of culture with adipogenesis during the second week. ATEm was used for adipogenesis induction and EGM-2 induction.

Results showed both protocols accumulated lipids, formed extensive vascular networks, and also secreted ap2 and leptin (Fig. 6). The morphology of cultures showed less branching in P1 (Fig. 6F), which was seen in the total area of vasculature (Fig. 5B). Both protocols produced cultures expressing relevant adipocyte markers (Fig. 7). P1 showed a higher expression of PPARγ, Leptin and Adiponectin whereas P6 showed a higher expression of aP2, PPARγ2 and Glut4.

Adipogenesis and angiogenesis were both successfully induced. Different cell plating and differentiation protocols had a notable effect on the resulting cell model and its technical repeatability. The results showed the presence of vasculature helped pre-adipocytes mature in a shorter time than with induction of angiogenesis post-adipocyte differentiation. However, in co-cultures where angiogenesis was induced first (P6), cells tended to detach easier from well plates than when adipogenesis was induced first (P1). This problem could be addressed with coating i.e. adding more attachment sites to culture vessels, however coating materials could potentially cause unwanted and unknown interactions between the studied cells or chemical assembly (Williams 2008).

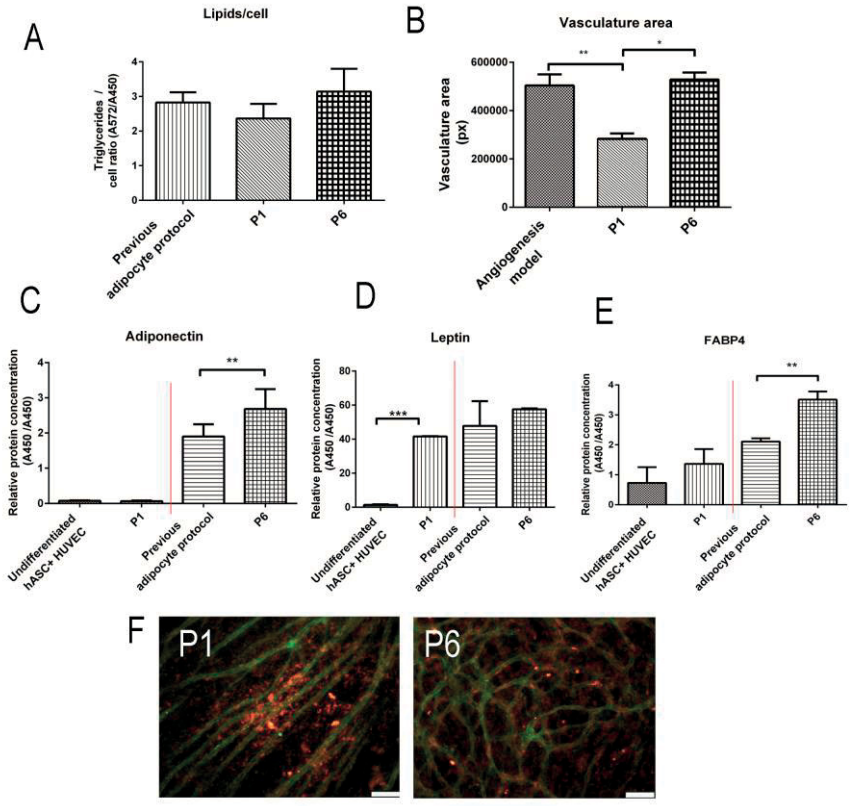

Figure 6. Characteristics of combined vascularization and adipocytes on day 14. A) Adipocyte control and both protocols P1 and P6 show lipid accumulation and B) vascular formation, although P1 produced significantly less tubules than the angiogenesis model or P6. The measurement of secreted proteins C) Adiponectin, D) leptin and E) FABP4 showed secretion of Leptin and FABP4 with both protocols and Adiponectin with P6. Secretion of P1 was compared to the serum-free medium and P6 to the Previous adipocyte control to equalize the effect of the cytokine rich ATE used in adipogenesis induction. The bars represent means with SD. *p<0.05, **p<0.01 and ***p<0.001. F) Shows the morphology of the vascularized adipose tissue with protocols P1 and P6 on day 14. Lipids are seen in orange/red, tubules were stained with anti-vWf-TRITC (red) and anti-ColIV- FITC (green). Images were obtained with a Nikon Eclipse Ti-S inverted fluorescence microscope and with 10x objective, scale bar at 100 µm. Figure modified from Original publication III.

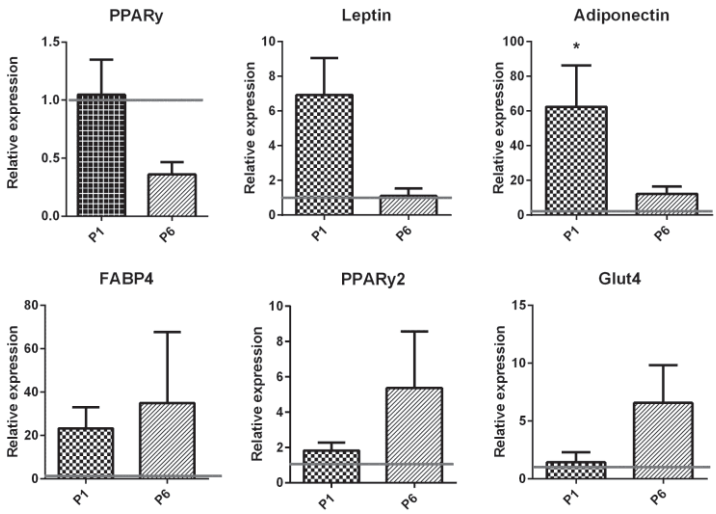

Figure 7. The expression of adipose tissue specific gene markers in the P1 and P6 protocols. Expression of the control (Previous adipocyte protocol) shown with a grey line. P1 showed higher expression of PPARγ, Leptin and Adiponectin. P6 showed higher expression of aP2, PPARγ2 and Glut4. P1 showed significantly increased expression of *adiponectin* compared to the adipocyte control. The bars represent means with SD. *p<0.05. Figure modified from Original publication III.

5.5 Vascularized adipose tissue (IV)

The serum-free ANG medium and differentiation media sequence of the Insulin sensitive adipocyte protocol were combined to create optimized vascularized adipose tissue. The culture conditions found to be optimal in P1 (14 day culture time, seeding hASC on day 0 and HUVEC on day 7, and inducing adipogenesis first followed by angiogenesis induction) were utilized here. Adipocytes were differentiated with Insulin sensitive adipocyte control with the addition of one replenishment of medium and the macromolecule ficol (Adipocyte control for vascularized adipose tissue). This same medium sequence was utilized for both angiogenesis and adipogenesis induction to produce the vascularized adipose tissue model.

Results show that adipocytes in monoculture and optimized vascularized adipose tissue share similar general characteristics. The vascularized adipose tissue showed good lipid accumulation, proper vasculature and secretion of proteins FABP4, leptin and adiponectin. The secretion of adiponectin was significantly increased in vascularized adipose tissue compared to the corresponding adipocyte control

indicating the presence of more mature adipocytes in the vascularized culture. The lipid accumulation per cell seemed to be lower in adipocytes of the vascularized culture than in adipocytes alone, but the observation of lipid storage size could not be confirmed by quantitation. The quantitative results of lipid accumulation, vascular are and protein secretion can be seen in Original manuscript IV.

The morphology of the vascularized adipose tissue can be seen in Figure 8. Vascularization formed in the vascularized adipose tissue model was analyzed by immunostaining and a panel of angiogenesis related genes, also in samples exposed to insulin. Staining showed CD144 i.e. ve-cadherin junctions located in the tubules and CD140b i.e. PDGFR-β on the surface of tubules (Fig. 8). Gene expression results show on going angiogenesis as well as mature tubules (Original publication IV, Tab. 3S). The individual genes that were up-regulated (2 fold or more compared to non-insulin treated cultures) in response to insulin were PDGF (0.67±0.52, with insulin 1.36±0.20), FGFR2 (3.34± 2.65, with insulin 7.43± 3.99) and ve-cadherin 5 (0.69±0.58, with insulin 1.39±0.25).

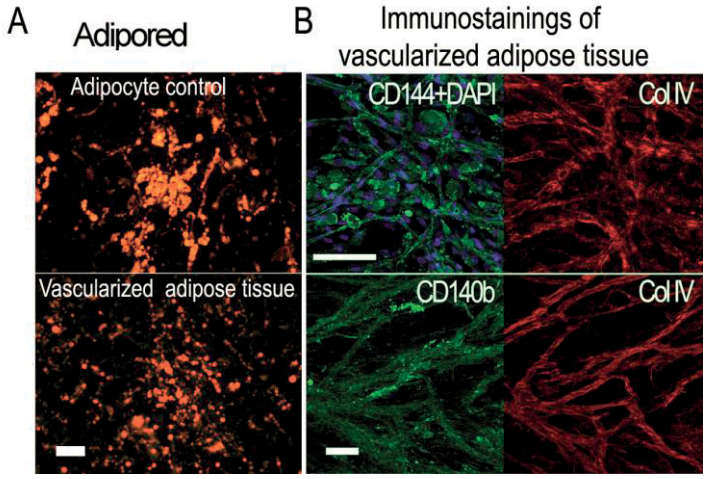

Figure 8. Morphology of the vascularized adipose tissue. A) Lipids stained with adipored (orange), both adipocyte control and vascularized adipose tissue. B) Confocal immunostaining images: CD144 (green) i.e. ve-cadherin junctions can be found in the tubules stained with Collagen IV (red). Nuclei stained with DAPI (blue). Bottom row: CD140b (green) i.e. PDGFR-β align along tubules (in red) indicating presence of pericytes. Scale bars 100µm. Figure modified from Original publication IV.

The responses to insulin were studied in both adipocytes and the vascularized adipose tissue model to see whether there was a difference in the presence of vasculature. In gene expression analysis on the effect of insulin and vascularization, Glut1 was shown to be significantly down-regulated in response to insulin (Fig. 9A).

In addition, the trend of upregulation by insulin can be seen in Glut4 in both cultures. Expression of Leptin increased in the vascularized adipose tissue model in response to insulin. When comparing unexposed cultures of vascularized adipose tissue and adipocytes, significant upregulation of Glut1 and leptin was seen in vascularized adipose tissue (Fig. 9A). However, there was no difference between the cultures for secreted leptin (Original publication IV). Inhibition of lipolysis was found to be faster in the vascularized adipose tissue model. In vascularized adipose tissue, inhibition could be seen after 15min of 500 nM insulin concentration (Fig. 9B) but in adipocytes alone, the response was seen after 30 min. Both cultures increased glucose uptake in response to insulin equally (Fig. 9C).

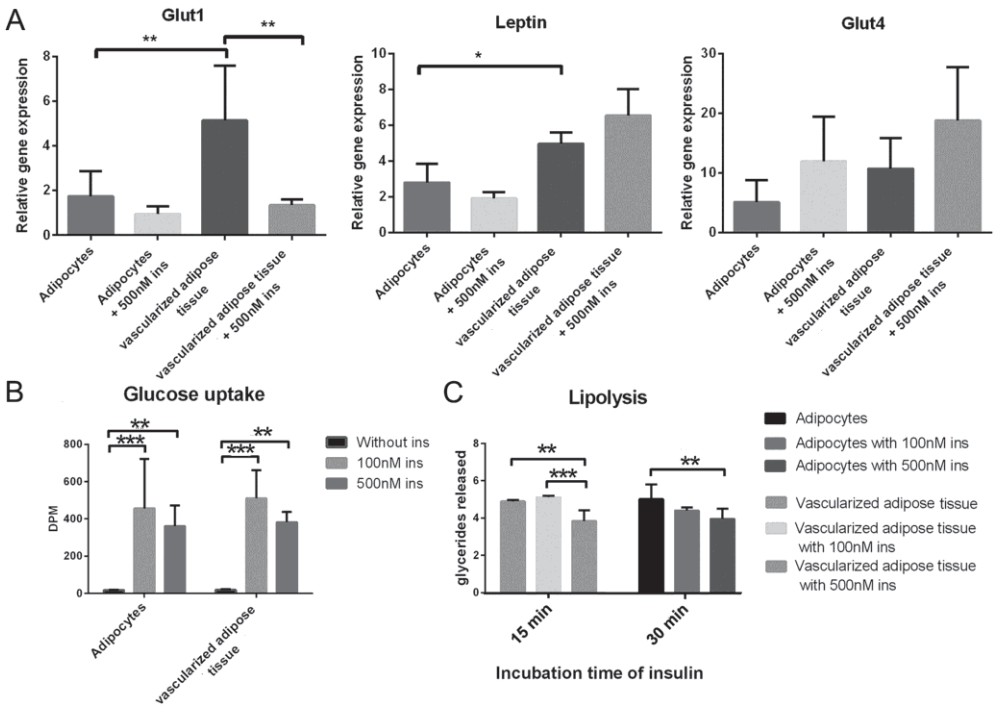

Figure 9. Insulin responses in adipocyte control and vascularized adipose tissue. A) Expression of the adipose tissue related genes Glut1, leptin and Glut4 in adipocytes with and without vasculature and with or without 500nM insulin. Glut1 and leptin are up-regulated in vascularized adipose tissue compared to adipocytes. Glut1 is significantly down-regulated in response to insulin. A trend towards upregulation by insulin can be seen for Glut4 in both cultures. B) Glucose uptake in response to insulin was equal in both cultures with 100nM and 500nM insulin. C) Significant inhibition of lipolysis was seen in the vascularized adipose tissue model after 15 min of 500nM insulin exposure whereas in adipocytes inhibition was only seen after 30 min. Figure modified from Original publication IV.

5.6 Effect of chemicals on vascularized adipose tissue (IV)

The *in vitro* human vascularized adipose tissue model was exposed to nine chemicals (Rosiglitazone, Chlorpyrifos prochloraz, Mancozeb (MANC), Butylparaben, 15-Deoxy-Δ12,14-prostaglandin J2, Bisphenol A, bis-(2-ethylhexyl) phthalate and tributyltin chloride). The exposure time was 7 days and were performed in the second week of culture. The effect of chemicals on lipid accumulation, mitochondrial activity/viability and vascularization was quantified. The results of these can be seen in Table 9. Both adipocytes and vascularized adipose tissue were exposed to three of these chemicals: rosiglitazone, prochloraz and chlorpyrifos and were also analyzed for gene expression. Exposure to 4.4μM Rosiglitazone increased expression of FABP4 and adiponectin compared to the corresponding unexposed cultures in both adipocytes and vascularized adipose tissue (Fig. 10). Glut4 was also slightly up-regulated in both cultures. In the vascularized adipose tissue, the expression of leptin was also increased. Exposure to 50μM Prochloraz increased PPARα expression significantly in the vascularized adipose tissue and expression of other studied markers was lowered. Expression of leptin was significantly higher in vascularized adipose tissue exposed with 10μM Chlorpyrifos than in adipocytes.

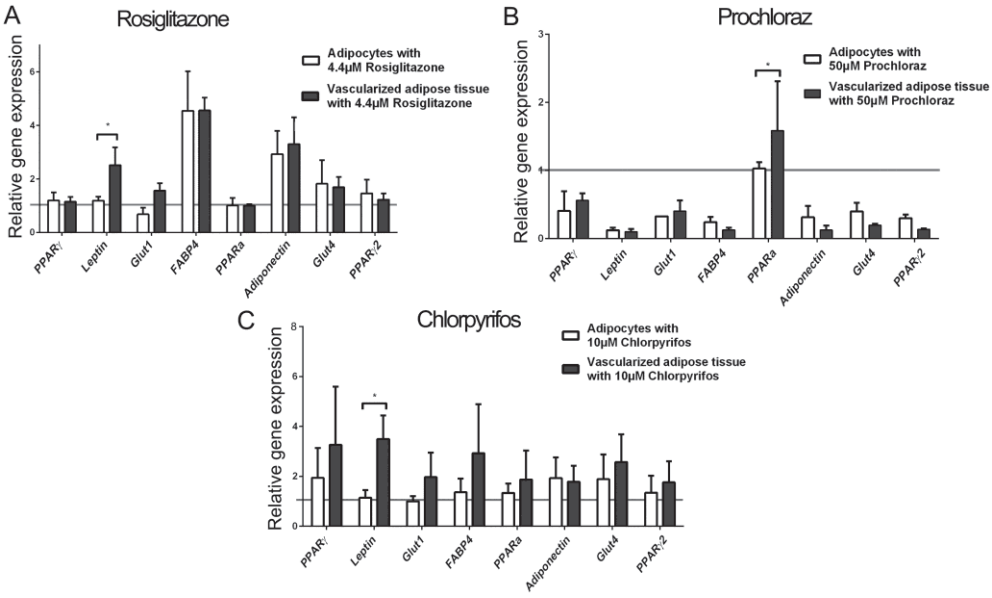

Figure 10. Adipocytes and the vascularized adipose tissue model exposed to 4.4µM rosiglitazone, 50µM prochloraz and 10µM chlorpyrifos. A) Rosiglitazone increased expression of FABP4 and adiponectin compared to the corresponding unexposed cultures in both adipocytes alone and with vascularization. Glut4 was also slightly up-regulated in both cultures. In the vascularized adipose tissue model the expression of leptin was also increased. B) Expression of PPARα increased in vascularized adipose tissue after exposure to 50µM prochloraz. Other markers were down-regulated.C) Leptin expression increased in vascularized adipose tissue in response to 10µM Chlorpyrifos. Grey lines indicate the level of unexposed corresponding cultures. Figure modified from Original publication IV.

Table 9. The results of the chemical exposures of vascularized adipose tissue.

Chemical	EC50 Mitochondria activity	Effect on mitochondria activity	EC50 lipid accumulation	Effect on adipogenesis	Effect on Angiogenesis
Rosiglitazone	-	no effect	-	weak stimulator	increase with 0-1.4µM
Prochloraz	181.6 µM	decrease	187.2 µM	decrease	no effect
Chlorpyrifos	73.51 µM	increase	-	inhibitor & increase	no effect
Mancozeb	90.24 µg/ml	decrease	84.01 µg/ml	inhibitor	increase in non-toxic concentrations
Butylparaben	433.8 µg/ml	decrease	340.8 µg/ml	inhibitor	no effect
15-Deoxy-Δ12,14-prostaglandin J2	2.675 µg/ml	increase	0.1038 µg/ml	inhibitor	increase in higher concentrations
Bisphenol A	65.3 µg/ml	decrease	0.5442 µg/ml	inhibitor	increase in higher non-toxic concentrations
Bis-(2-ethylhexyl) phthalate	0.1634 µg/ml	decrease	0.03 µg/ml and 10µg/ml	inhibitor (biphasic effect)	significant increase
Tributyltin chloride	106.2 ng/ml	increase	312.8 ng/ml	Stimulator	inhibition in lower concentrations

6 DISCUSSION

6.1 Building blocks of *in vitro* models

The development of *in vitro* cell models aims to develop as *in vivo*-like tissues as possible *in vitro*. This was the goal also in the development of the models presented in these studies (I, II, III, IV). Several aspects have to be considered in order to succeed. *In vivo* tissues get multiple signal types from their surroundings; soluble factors (nutrients, growth factors, hormones, O_2 and CO_2, Ca^{2+}, Mg^{2+}), cell-cell interactions (density, confluence, cell junctions), cell-matrix interactions (physical and chemical characteristics of a matrix, charge configuration, composition of extra cellular matrix) and cell architecture and polarity (basolateral nutrient supply) (Brunner et al. 2010, Duval et al. 2017).

In vitro models are still far from reaching the exact and optimal growth environment of cells corresponding to an *in vivo* environment, but the field is evolving and more complex culture systems are continuously developed. *In vivo* cells are in a 3D matrix and usually have cell-to-cell contact with a variety of cell types (Novakofski 2004). 3D models help to bridge the gap between animal models and 2D *in vitro* models (Turner et al. 2014, Kang et al. 2007, Wang et al. 2009). The angiogenesis model developed in study I was shown to contain 3D tubule structures. These are valuable as they can function as a natural 3D scaffold for other cell types, including adipocytes (III, IV). Although the models developed here were closer to 2D than 3D models, they contain 1) multiple stromal cell types due to the heterogeneity of hASC and hence have cell-cell interactions with relevant cell types, 2) cell-matrix interactions with the ECM produced by these cells as shown in study I, and 3) the 3D structure of vasculature showing the correct architecture in these models (I).

The fact that some disease characteristics have only been detected in 3D, but not 2D cell culture systems supports the use of 3D models (Girandon et al. 2011, Lowe et al. 2011, Turner et al. 2014, Cukierman et al. 2002). Often 3D culture models contain a complex biomaterial scaffold into which cells are seeded, but they can also be spheroid cultures with or without biomaterial. On the other hand, the less exogenous substances, such as biomaterials, added into a culture, the more

controlled the environment and thus more is known about the interactions taking place in the culture. The cultures in this study are free from exogenously added ECM proteins (I, II, III, IV). This way also additional unknown interactions between biomaterials and the studied chemicals can be avoided, also avoiding interference to cell-cell interactions. The natural scaffolding provided by vasculature brings cultures closer to an *in vivo* environment without the need for artificial scaffolds.

Although the focus of this study was high-throughput testing, the aim was still to form as *in vivo*-like a tissue as possible, keeping in mind their applicability for toxicology testing. This means all components in cultures should be defined and not cause unknown interactions with tested chemicals. By connecting different *in vitro* tissues through a blood circulation mimicking medium system, more complexity is obtained, while still maintaining high-throughput. The adipocytes (II) and vasculature (I) presented here can be cultured together as shown, or separately in chip chambers using the developed serum-free medium, which was shown to be suitable for angiogenesis and adipogenesis induction (III, IV). In addition, other cell and tissue types can also be added to these types of systems to create multi-tissue culture systems. One obstacle in compiling complex multi-tissue cultures has been the development of a suitable universal media to connect the different tissues. In order to combine two or more tissues in the same culture, suitability of the medium for all cell types is crucial. The serum-free medium developed in study I would be a good candidate as a universal medium, as it can also be modified easily according to the needs of specific applications.

6.2 Role of cells in tissue models

Cells are the most important components in *in vitro* cultures. Primary cells have many favorable characteristics even though they have also disadvantages. When working with primary cells one can be sure of the right characteristics of that cell type compared to *in vivo*. However, primary cells can quickly lose their characteristics when cultured *ex vivo*. These cells can secrete an extracellular matrix *in vivo*, but this ability decreases according to number of cell passages. Moreover, primary cells suffer from batch to-batch variations, causing discrepancy in results. For these reasons, the same cell passage number should always be used in experiments and relevant quality controls like expression of key markers and functions relevant to cell type are needed to ensure reproducibility.

Primary HUVEC were utilized in these studies as the endothelial cell population (I, II, III, IV). In primary HUVEC, along with an increase in passage number, the expression of eNOS is decreased (Shi et al. 2004) and migration ability decreases (Liao et al. 2014), hence the passage number used in this study was fixed at 4 (I, II, III and IV). An alternative for primary cells would have been the use of endothelial cell lines. However, endothelial cell lines for example have been seen to respond poorly to cytokines (Lidington et al. 1999), express integrins differently compared to primary endothelial cells (Baranska et al. 2005), have different regulation of cell proliferation and apoptosis (Boerma et al. 2006), and immortalized endothelial cells can also be incapable of migration and tube formation *in vitro* (van Beijnum et al. 2008). Although the use of HUVEC, large vein endothelial cells, has been criticized in small vessel models, they are well characterized and an easily obtainable primary cell type. The use of tissue specific endothelial cell sources should also be considered for more relevant *in vitro* vasculature.

In study I, phenotypic analysis of the building blocks of the vascular model, i.e., hASC and HUVEC, was performed using flow cytometry. High expression of the endothelial markers CD144 and CD31 was shown to be present in the HUVEC population. We showed that HUVEC have a strong expression of CD105 (endoglin), which has been shown to have a significant role in VEGF-induced angiogenesis (Liu et al. 2014). The level of CD73, which suppresses pro-inflammatory responses in human endothelial cells (Grunewald et al. 2010) was high in our HUVEC and the population did not show presence of macrophages/monocytes (CD68). Pericyte marker NG2 was also low, indicating the absence of mural cells in the HUVEC population. Based on these marker expressions, the endothelial population lacked immune and mural cells.

The use of adipose stem cells was easily justified through the aim of creating vascularized adipose tissue. In addition, the use of HUVEC and hASC in angiogenesis models was reported earlier (Traktuev et al. 2008, Merfeld-Clauss et al. 2010, Sarkanen et al. 2012a). hASC have been shown to secrete angiogenic and antiapoptotic factors (Rehman et al. 2004). Study I confirms that the stromal-vascular fraction extracted from adipose tissue is heterogenic. The expression profile of hASC indicated the presence of cells with multilineage differentiation potential (CD90, CD105 and CD73) (Dominici et al. 2006) and pericytes (CD140b), and a lack of mature endothelial cells (CD31 and CD144), macrophages and monocytes (CD68 and CD14). There was a moderate expression of CD34, which has been shown to be expressed in endothelial progenitors (Yang et al. 2011). A hASC population of CD90+/CD34+ cells was shown to be capable of differentiating into

endothelial cells and form capillary-like structures (De Francesco et al. 2009). Interestingly, there was also a report suggesting adipose stromal cells may enhance endothelial differentiation of progenitor cells (Rubina et al. 2009). These further defend the use of hASC in *in vitro* formation of vasculature. As CD142 has been shown to negatively influence the adipogenesis potential of hASC (Schwalie et al. 2018), this marker should be added to routine quality testing panels of hASC populations to be used for adipogenesis studies. The use of heterogeneous cell populations however, is one way to add complexity and *in vivo*-likeness to cultures and hence it might not be beneficial to sort unwanted cell types, but rather note the differences in different batches of cells. The heterogeneity of the hASC population adds multiple vascular stroma cell types including contractile cells and fibroblasts to the angiogenesis model (I), thus allowing the model to depict vascularization and its surroundings better than a homogenous stem cell population.

6.3 Induction of angiogenesis

To achieve relevant biological aspects and good experimental reproducibility, the composition of the cell culture medium is essential. The development of angiogenesis stimulation medium in study I was conducted by testing multiple stimulation media compositions of which the most optimal was chosen to be used in the angiogenesis model for the induction of vascular formation. The criteria for the most optimal medium were that it induced extensive reproducible vascular network formation and that the vascular structures showed *in vivo* vascular markers and morphology; an intact endothelial layer, lumen, basement membrane, 3D tubule structure, junctions and pericytes. Exclusion criterion for stimulation medium candidates was over induction of endothelial cells i.e. the formation of cell clumps, which contain an extremely dense vascular network. These cannot be analyzed reliably and hence the stimulation medium should not produce these cell aggregates.

The development of new induction medium for angiogenesis showed that serum-free media produced an extensive vascular network in the hASC and HUVEC co-culture system (I). When creating a defined medium, the replacement of serum is the first step as serum contains unknown concentrations of unknown molecules, which bind different chemicals differently (Shen et al. 2013). For the testing of chemicals *in vitro* model media has to be defined to know all possible interactions between chemical and media components. Also, guidelines for good cell culture practice (GCCP) and the ECVAM Scientific Advisory Committee (ESAC) recommend the

use of serum-free media (Coecke et al. 2005, ESAC 2008). A serum-free medium database has also been set up to ease the transition away from the use of animal serum (Brunner et al. 2010). In the clinical application of cells, regulations have to be followed carefully and the use of a defined medium is especially important to avoid the risk of unknown components causing unknown reactions including infections and severe immune reactions in the recipient. Successful efforts have been made to develop serum-free media and freezing protocols to ensure the safety of cells used in cell therapies (Patrikoski et al. 2013, Heathman et al. 2015, Oikonomopoulos et al. 2015, Holm et al. 2010).

In study I, serum was replaced with supplements found in blood *in vivo* including albumin and transferrin. All these are relevant supplements with specific purposes in human blood. Albumin has multiple physiological effects; it carries vitamins, fat (fatty acids, cholesterol), drugs and hormones, regulates colloid osmotic pressure, antioxidant properties, nitric oxide modulation and has buffer capabilities (Vincent 2009, Vincent et al. 2014). Transferrin carries iron in the blood and delivers it to cells (Luck et al. 2012).

In order to create an *in vivo* mimicking human *in vitro* model, the medium used in the model should be xeno-free. The medium developed in study I contains bovine serum albumin and heparin of bovine origin, but these can be replaced with human albumin or recombinant human protein. Only after these replacements could the produced vasculature be considered for clinical use in humans in for example implants.

The components of the final defined serum-free angiogenesis induction were general angiogenic factors and vasculature related supplements VEGF, FGF-2, HY, HE and AA (I). VEGF and FGF are well known angiogenesis and vasculogenesis inducing growth factors and hence also targets of therapies aiming at influencing angiogenesis (Keifer et al. 2014, Goel et al. 2011, Li et al. 2014, Chae et al. 2017). Although hydrocortisone is not pro-angiogenic itself, it has a beneficial effect on angiogenesis (Goding 2009), but in excess can cause health effects like those seen in Cushing's syndrome with impairment of microvascular reactivity (Prazny et al. 2008). HE has been shown to induce angiogenesis (Rema et al. 2012), likely due to its effect on $VEGF_{165}$ (Ashikari-Hada et al. 2005). The importance of AA (vitamin C) on human health has been known for a long time (Hodges et al. 1971). AA is an essential nutrient for human endothelial cells as it is needed for their effective migration as well as for the synthesis of collagen type IV, an important component of basement membrane of blood vessels (Telang et al. 2007, Hodges et al. 1971). The angiogenesis process requires this deposition of mature type IV collagen in the basement

membrane. The correct folding of type IV collagen is dependent on the hydroxylation of proline by the iron-dependent enzyme prolyl hydroxylase, which requires AA to reduce iron to its ferrous state for reactivation of the enzyme (Myllyla et al. 1978). Hence AA is important for the formation of vascular structures.

Along with the optimization of supplements, results showed that AA caused a dose dependent induction and inhibition of the vasculature (I). High concentrations of AA have been shown to inhibit angiogenesis (Mikirova et al. 2008). This inhibition of angiogenesis by AA has been shown to also inhibit tumor growth, with the use of high concentrations of AA proposed as a possible therapeutic strategy for treating cancer (Yeom et al. 2009). Due to the importance of AA on basement membrane formation, the depletion of AA has also been suggested as a cancer treatment strategy through angiogenesis (Telang et al. 2007).

T3 was also a component of the novel serum-free medium developed in study I. It is a hormone produced by the thyroid gland, which stimulates the metabolic breakdown of glucose, fats, and proteins by inducing an increase in levels of various metabolic enzymes, including liver glucose 6-phosphatase, hexokinase, and mitochondrial enzymes for oxidative phosphorylation (Michienzi et al. 2007). T3 has been shown to be especially important in muscle cell differentiation (Muscat et al. 1994) and protection against calcification of vascular smooth muscle cells (Chang et al. 2016). Based on these effects on muscle cells, T3 likely enables the presence of contractile cells around vascular structures.

6.4 *In vitro* angiogenesis and tissue engineered vascular structures

Due to the importance of vasculature, tissue engineered solutions have been created *in vitro* and *in vivo* for clinical needs and research purposes. As the current study is focused on *in vitro* angiogenesis research, *in vitro* angiogenesis models and vasculature are discussed further.

The *in vitro* tissue engineered vessels, seen in Table 1, are usually cultured using macrovascular or microvascular endothelial cells isolated from human, murine, or bovine vessels. Endothelial cells can be grown alone on biomaterial or with another cell type (fibroblast, stromal cell) present, with or without biomaterial. In these studies we chose the macrovascular endothelial cell HUVEC as the endothelial cell type along with hASC to enable the presence of the relevant stroma cell population (I, III, IV).

Surprisingly many vascular constructs are still grown in the presence of serum from animal sources, although serum could be replaced as discussed above. Cryopreserved human microvascular structures, cell spheroids, and vascularized organ chips are 3D and more complex alternatives for vascular research. In recent years the development of tissue engineered vessels has shifted in developing these more complex vascular cultures utilizing 3D printing, lithographic microfabrication, microfluidistics and biomaterial scaffolds.

The vasculature formed in study I had the morphology of *in vivo* vessels including an intact endothelial layer, basement membrane, 3D tubules with lumen, ECM proteins and surrounding pericytes. Endothelial shape changes and the resulting lumen formation is critical for the transformation of pre-vascular cords into a perfusable vascular system (Strilic et al. 2009). Lumen development is triggered by apical-basal polarity of endothelial cells in which CD144 plays a critical role in promoting the localization of polarity markers (Charpentier et al. 2014). Adipose stromal cells secrete factors that induce endothelial cell sprouting and lumen formation (Traktuev et al. 2008, Rubina et al. 2009). The presence of supporting stromal cells also enhances vascular basement membrane and lumen formation (Merfeld-Clauss et al. 2010, Newman et al. 2013, Stratman et al. 2009). Changes in ECM stroma and the interaction between stroma and vasculature are important in directing vessel growth (Hoying et al. 2014, Du et al. 2014).

In study I, the vascular network was shown to be reproducible and extensive. The comparison of vascular structures induced by EGM-2 and ANG at an mRNA level showed some differences in the expression of angiogenic marker genes (I). These changes did not show a clear shift of angiogenesis towards early or late phases in either medium (I). However the expression of angpt-1, a marker for mature tubules, was significantly higher in ANG compared to EGM-2 medium indicating the maturity of vasculature formed in the ANG medium (I). Interestingly, the expression of IGF-1 was up-regulated in ANG (I). *In vitro* IGF-1 has been shown to stimulate the migration and tube-forming activity of endothelial cells (Shigematsu et al. 1999, Nakao-Hayashi et al. 1992). Leptin was also found to be up-regulated in ANG induced vasculature. Leptin signaling acts as a link between adipocytes and the vasculature (Sierra-Honigmann et al. 1998) and it increases the production of VEGF and enhances expression of MMP-2 and MMP-9 in HUVEC (Park et al. 2001). This enhancement was also seen in the results as an up-regulation of MMP-2 and MMP-9 enzymes.

The 3D vascular structures produced by the serum-free induction medium showed all relevant markers of angiogenesis and mature vasculature (I). This *in vitro*

angogenesis protocol has now been shown to also be a relevant test for screening chemical teratogenic effects (Toimela et al. 2017). As the vasculature network combines different organs of the body, it is of great interest in tissue engineering. Combining vasculature in different cell cultures *in vitro* has been shown to be beneficial in inducing the relevant *in vivo* morphology and functions not obtained without a vascular component (Vuorenpaa et al. 2014, Vuorenpaa et al. 2017).

6.5 Induction of adipogenesis

The induction of adipogenesis is most commonly induced by a chemical mixture of IBMX, synthetic glucocorticoid dexamethasone, insulin and glitazones (Kang et al. 2009, Verseijden et al. 2009, Lequeux et al. 2009, Sarkanen et al. 2012b, Ghoniem et al. 2015, Foley et al. 2015, Rubin et al. 1978). The standard adipogenic cocktail has been criticized for only differentiating a small percentage of cells in culture. Other adipogenic factors, such as indomethacin and triiodothyronine, are also commonly used (Armani et al. 2010, Verseijden et al. 2009, Lequeux et al. 2009). To facilitate lipogenesis from glucose, biotin and pantothenate have also been utilized (Kang et al. 2009). Improved versions of induction medium have been developed for enhanced adipogenesis and one of these is adipogenic differentiation medium, which contained rosiglitazone (part of the TZD class), insulin, hydrocortisone, T3, IBMX and dexamethasone, but not contain indomethacin (Lequeux et al. 2009). The adipogenesis/angiogenesis medium (ANG+T) developed in study II shares similarities with this optimized chemical medium as it contains insulin, hydrocortisone, T3 and 9.06 μM TZD troglitazone. Insulin mimics the role of IGF-1 and activates MAPK (Qiu et al. 2001). IGF-1 mediates proliferation and differentiation of perivascular adipocytes (Liu et al. 2015).

The majority of adipogenic protocols utilize fetal bovine serum (FBS) (Ruiz-Ojeda et al. 2016) even though FBS is known to induce proliferation rather than aiding differentiation (Lee et al. 2012) and should be avoided for ethical reasons as well as for its unknown and highly variable mixture of components (Brunner et al. 2010). However, new serum-free and human serum options have been developed, including the method developed in this study (II, IV, Volz et al. 2018b). We utilized human serum in ATE medium for the first three days of culture (II, IV). A completely serum-free medium containing DMEM/F12 supplemented with HEPES, $NaHCO_3$, biotin, d-pantothenate, human transferrin, gentamicin, T3, insulin, hydrocortisone and IBMX or troglitazone for the initial 3 days has been

developed by others (Lee et al. 2014, Hauner et al. 2001). This completely serum-free medium is similar to the one developed in study II but contains IBMX and hence will likely cause unwanted negative effects on HUVEC as discussed below.

The criteria in study II for the most optimal adipogenesis medium was that it induced reproducible lipid accumulation and mature adipocyte gene expression while maintaining insulin sensitivity of adipocytes. The protocol that fulfilled these criteria i.e. induced insulin sensitive adipocyte differentiation included a culture of hASC cultured for 3 days in ATE medium (ATEm), followed by 6 days in serum-free adipogenesis/angiogenesis medium (ANG +T) containing 1,15 µM insulin and 9,06 µM Troglitazone, a thiazolidinedione (TZD) compound, then the next 4 days in serum-free angiogenesis medium w/o insulin (ANG-I) (II). The ANG medium was first developed for inducing angiogenesis (I). This serum-free medium is now used for adipogenesis induction and adipocyte maturation together with ATE (II, IV). ATE contains growth factors, cytokines and chemokines obtained from mature adipose tissue, e.g., leptin, adiponectin, FGF-2, IL-6, IGF-1, VEGF (-A) and angiogenin, and has been shown to induce natural adipogenesis *in vitro* (Sarkanen et al. 2012b). ATE is produced from mature human adipose tissue and does not inhibit cell proliferation (Sarkanen et al. 2012b, Sarkanen et al. 2012c). FGF-2 found in ANG medium is also shown to support the proliferation of multipotent cells and maintaining their differentiation ability (Tsutsumi et al. 2001).

The new adipogenesis protocol included incubation of cells with troglitazone and T3 (II, IV). TZDs are PPAR agonists, which stimulate adipogenesis and the redistribution of lipids from liver and muscle into adipose tissue (Greenberg et al. 2006) and activate AMP-activated protein kinase (AMPK), resulting in enhanced glucose uptake into adipose tissues and muscle (Fryer et al. 2002). Previous *in vitro* studies suggest that a combination of T3 and TZD signaling enhances the adipogenic differentiation potential (Ortega et al. 2009, Gerhold et al. 2002). On its own, T3 has been shown to interact with the two primary thyroid receptor isoforms expressed in fat tissue: thyroid receptor a1 (TRa1) and its antagonist receptor a2 (TRa2). The binding of T3 to TRa1 induces adipogenesis while TRa2 negatively regulates the activity of T3 (Ortega et al. 2009). This mechanism and the synergistic effect with TZDs are likely the reason for the beneficial effect seen with T3 on adipogenesis.

The glucocorticoid hydrocortisone used in ANG has been shown to increase the secretion of leptin by cultured rat adipose tissue (Mick et al. 2000). Glucocorticoid excesses in clinical cases has caused increased fat mass and obesity through the accumulation of white adipocytes (Hochberg et al. 2015, Barclay et al. 2015). Based

on these, hydrocortisone is also a relevant compound for adipogenesis and adipocyte maturation.

6.6 *In vitro* adipocyte differentiation

Many protocols for creating *in vitro* adipocytes have been developed over the years. A lot has been learnt about the mechanisms of adipogenesis from studies of adipogenic cell lines, such as mouse 3T3-L1 cells and mouse 3T3-F442A cells (Cristancho et al. 2011). However, these cell lines differ from primary cells in that the triglycerides are stored in many droplets instead of one, and in 3T3-L1 and 3T3-F442A cells the expression of leptin is much lower than in primary adipocytes (Cristancho et al. 2011). Hence better cell sources and adipogenesis methods are needed, and have already been developed. New human cell-based methods offer biologically relevant and accurate tools to study human adipose tissue related diseases.

The mouse 3T3-L1 cell line is the most commonly used cell type in *in vitro* adipose tissue studies (Ruiz-Ojeda et al. 2016), but the use of human mesenchymal cells is increasing. Among human mesenchymal cells, hASC are a feasible source for adipose tissue engineering as they possess high proliferative and differentiation capacities. Primary hASC were utilized in this thesis' studies (I, II, III, IV) and many other studies developing *in vitro* adipogenesis models (Verseijden et al. 2009, Lequeux et al. 2009, Sarkanen et al. 2012b, Ghoniem et al. 2015, Foley et al. 2015). hASC can be obtained from different fat depots, which enables the study of differences between depots (Armani et al. 2010, Walker et al. 2014). The source of adipose precursor cells has been shown to have an impact on their replication speed, need for confluency prior to differentiation and adipogenic differentiation ability; with faster replicating cells having a better adipogenesis potential (Tchkonia et al. 2005). However, due to the fact that hASC are primary cells, heterogeneity of hASC may be a problem as well as the cultures having a limited life span, which restrains the time window for experimental procedures (Scroyen et al. 2013). When developing *in vitro* tissues for mimicking human adipocytes however, human adipose tissue derived cells are as relevant a cell source as possible.

The protocol developed in study II induced reproducible lipid accumulation and expression of mature adipocyte genes (adiponectin, Glut4, AP2 and PPARγ) while maintaining the insulin sensitivity of adipocytes. The protocol did not selectively induce either brown or white adipocyte phenotypes (II). The cells showed no

regression of adipocyte morphology and remained viable and attached to substratum for at least 10 weeks without replenishment of adipogenic medium components (Fig. 4). The developed protocol contained human serum for the first 3 days, which could be criticized. The fully serum-free adipogenic medium developed by others has been shown to produce a high percentage of differentiated adipocytes and are insulin responsive (Lee et al. 2012). However, serum-free differentiation protocols do not seem to produce mature adipocyte morphology i.e. nuclei pushed to the side of the cell by one large lipid store, even at a 35 day time point (Lee et al. 2014), unlike our protocol (Fig. 4). Also, serum contains components that promote cell adherence including fibronection and laminin, which can be beneficial at the beginning of the culture.

Hormone responses are one major criteria for differentiated adipocytes depicting healthy adipose tissue. In adipose tissue, adipocytes respond to insulin by inhibiting lipolysis and increasing glucose uptake, which initiates signaling via effectors like phosphatidylinositol-3 kinase and AKT1/2 with central roles in adipogenesis (Rutkowski et al. 2015, Watson et al. 2007, Kim et al. 2004, Garofalo et al. 2003, Choi et al. 2010b). Adipocytes should also respond to beta-adrenergic agonists through activation of lipolysis. The adipocytes developed in study II responded to the beta-adrenergic agonist isoproterenol by increasing lipolysis, which was then inhibited by insulin (Fig. 3). Insulin was also shown to increase glucose uptake in the differentiated adipocytes (II).

6.7 Tissue engineered *in vitro* vascularized adipose tissue

As adipose tissue is a highly vascularized tissue (Hausman et al. 2004), the interaction between vasculature and adipocytes is an important feature to have in *in vitro* cultures. In study III, the vascularized adipose tissue model was developed utilizing bottom-up methods by first optimizing the building blocks of the model: *in vitro* vascularization and the differentiation method of adipocytes and combining the optimal components to form the vascularized adipose model. Study III showed that a higher number of cells was beneficial when adipogenesis was induced first, followed by angiogenesis induction, as in protocol P1. More mature adipocytes were obtained with P1 by first inducing adipogenesis rather than angiogenesis, although the vascular structures were not quite as branched as in P6, in which angiogenesis was induced first (III). However, if angiogenesis was induced first, as in P6, there was no need for a high confluency of hASC and the vascular network was densely

branched, although mature adipocyte markers were not as strongly expressed as in P1 (III). Two different triggers have been identified for angiogenesis in *in vivo* adipose tissue expansion: 1) angiogenesis is a response to hypoxia, which is caused by the enlargement of adipocytes and/or proliferation of adipocytes; and 2) angiogenesis is the result of developmental and/or metabolic signals in adipose tissue (Corvera et al. 2014). In the first mechanism, blood vessels are developed after adipocytes are depicted in P1, and in the second the vessels are developed during the development of adipose tissue depicted in P6. Both protocols are scaffold-free and are simpler and demand shorter culture times than other existing vascularized adipose tissue models (Kang et al. 2009, Borges et al. 2007, Borges et al. 2003, Sorrell et al. 2011).

The protocol where adipogenesis was induced first, P1 (III), was further optimized by combining with the modified protocol of insulin-sensitive adipocytes (IV). This vascularized adipose tissue contained vasculature that was well branched and adipocytes showed expression of adipocyte genes and secreted relevant proteins for adipose tissue (IV). Vascular structures had pericytes present around tubules and adherence junctions were found between endothelial cells (IV). Adipocytes remained insulin sensitive and responded faster to insulin than adipocytes cultured alone (IV). The counteractive effect of vasculature on obesity and insulin-resistance in adipose tissue has also been shown in mouse models by others (Robciuc et al. 2016, Rupnick et al. 2002). In a study where angiogenesis in adipose tissue was induced by transduction of VEGFB in adipose tissue, diet-induced obesity and related metabolic complications were prevented in obese insulin-resistant mice (Robciuc et al. 2016). Activated endothelial cells in angiogenic vessels produce various cytokines and growth factors that promote adipose tissue growth and expansion (Cao 2007). Due to cell-cell interactions and their impact on cell mechanisms, co-cultures are essential for better understanding of the connections between adipocytes and surrounding cells in both healthy and diseased situations.

The achievement of mature vascular structures in the *in vitro* adipocyte culture in study III and IV is noteworthy. Commonly used adipogenic cocktail treatments containing insulin, IBMX, biotin, pantothenate, DEX and thiazolidinediones, have been proposed to have a negative effect on endothelial cells and delay the growth of HUVEC (Kang et al. 2009, Foley et al. 2015). As a natural adipogenesis inducer ATE was utilized in study III and IV for priming of the initial induction of adipogenesis, thus there was no need to use the chemical cocktail commonly utilized by many others. ATE enables the proliferation of endothelial cells and has the potential to induce angiogenesis (Sarkanen et al. 2012b), with its effect on endothelial cells in culture opposite to components in a chemical induction cocktail.

The role of ECM produced by hASC and formed vascular structures (I) in culture is one important part of the model as it offers attachment places and support for the differentiation of adipocytes (Sorrell et al. 2011). The angiogenesis model developed here was shown to produce ECM and Collagen IV containing basement membrane (I), which also contributes to the *in vivo*-likeness of the growth environment of adipocytes, as collagen IV among others is also produced by adipocytes in *in vivo* adipose tissue (Sorrell et al. 2011, Aratani et al. 1988).

Only a few vascularized adipose tissue models have been developed for *in vitro* use (Table 2) but their execution varies compared to the quite homologous composition of different adipogenesis induction media. Additional components such as biomaterial scaffolds may interfere with the cell–cell interactions or cause unwanted and unknown interactions between biomaterials and the studied cells or chemicals (Norotte et al. 2009, Higgins et al. 2003, Williams 2008). While these *in vitro* models mimic vascularized adipose tissue, they are complex, require long culture times and are laborious to transfer into routine use. Depending on the application, culture times might vary significantly. Implantable tissues are cultured significantly longer than *in vitro* test systems.

The newly developed vascularized adipose tissue model from Volz et al., utilizing microvascular endothelial cells and serum-free medium might also be relevant to *in vitro* chemical testing, although they did not show insulin responses or chemical exposures (Volz et al. 2018a). They have a relevant endothelial source, microvascular, and the culture does not contain FBS. The collagen I coating they use is likely necessary in the absence of serum in the plating phase of cells. The vascularization model of Volz et al. (Volz et al. 2018a) seems however to be modest compared to the vascularized adipose tissue model protocol developed in this study (IV), likely due to their usage of a chemical adipogenesis cocktail. In the protocol developed in study IV, total culture time on a 48 well plate was 14 days from which hASC were cultured and differentiated alone for 7 days compared, to the 14 days of adipogenic pre-differentiation on a 24 well plate by Volz et al. (Volz et al. 2018a). Smaller well size has economic advantages as less cells and reagents are needed, and in addition, enables more high-throughput work.

6.8 Effect of chemicals on vasculature and adipose tissue

Obesity and changes in adipose tissue function are associated with multiple diseases. The treatments of these diseases and other chemicals are known to effect adipose

tissue, and also its vasculature. In cancer treatments, inhibition of angiogenesis is used as a method to restrict the growth of tumors. Many drugs have been developed for cancer angiogenesis inhibition. Bevacizumab, a humanized monoclonal antibody against all isoforms of VEGF-A, is one of these drugs. It has been approved for treatment of colorectal, lung, glioblastoma and renal cell carcinoma (Hsu et al. 2009). Eye conditions including proliferative diabetic retinopathy, age-related macular degeneration and retinal vein occlusion can also be treated by inhibiting angiogenesis with anti-VEGF (van der Giet et al. 2015). Hemangiomas, psoriasis, rheumatoid arthritis, endometriosis, atherosclerosis can be treated with anti-angiogenic agents as well (Yoo et al. 2013). Stimulation of angiogenesis has been proposed as a treatment for conditions caused by a lack of sufficient vascularization. Induction of collateral vessel formation in ischemia e.g. myocardial ischemia benefits from angiogenesis induction (Markkanen et al. 2005). Wound care has also been shown to benefit from the induction of angiogenesis, especially in diseases that affect vasculature, such as diabetes. Topical use of VEGF has also shown significant healing in wounds in diabetic mice compared to controls (Galiano et al. 2004). Reconstructive surgery benefits from angiogenesis stimulation as well (Yoo et al. 2013).

Environmental chemicals have been shown to influence angiogenesis (Toimela et al. 2017, Sarkanen et al. 2011). To date, it is not mandatory to test new chemicals for their angiogenic effects, however there might be substances in our environment that influence angiogenesis and health. Unexpected effects can also be caused by drug treatments. Ibuprofen and acetylic salicylic acid are pain killers that have been shown to cause teratogenicity. These and other teratogenic agents have been seen to inhibit angiogenesis in the angiogenesis model developed in study I, which indicates angiogenesis inhibition might be behind teratogenicity in many cases (Toimela et al. 2017). The amount of drugs and chemicals with angiogenic effects proves the need for reliable *in vitro* models of angiogenesis like the one developed in study I.

Although obesity is a condition targeted with drug treatments, many strategies primarily target organs other than adipose tissue. Some drug candidates influence the brain and suppress appetite, others influence the gut to limit free fatty acid formation and inhibit their adsorption (Pilch et al. 2006). Insulin sensitizers like rosiglitazone and pioglitazone are used to control insulin responsiveness in diabetes (Pilch et al. 2006). These are PPARγ agonists and as they induce adipogenesis, weight gain has been linked to the treatment (Evans et al. 2004). Obesity and related comorbidities could be managed by influencing the vasculature in adipose tissue or adipocytes, as discussed above (Robciuc et al. 2016, Elias et al. 2012).

Adipose tissue is affected by environmental chemicals. New obesogens, chemicals increasing adipose tissue are continuously found. Many of these obesogens are also endocrine disruptors, which is not surprising as adipose tissue is an endocrine organ. From the chemicals used in original publication IV, mancozeb, butylparaben bisphenol a, bis-(2-ethylhexyl) phthalate and tributyltin chloride, are classified as endocrine disruptors by EU categorization (Groshart et al. 2000). Exposure to known chemicals were performed to confirm responses of the developed vascularized adipose tissue model were correct and suitable for testing chemical effects. The vascularized adipose tissue model developed in this study showed various responses to chemicals; changes in lipid accumulation, amount of vasculature, and in gene expression levels (IV). Lipid accumulation was not quite as visible as expected, although the gene expression results showed an increased expression of adipocyte markers when lipid accumulation increased, as was expected (IV). To further improve the protocol for vascularized adipose tissue, a longer exposure time (now 7 days) or adjustments to energy levels in the protocol could be considered.

Both adipocytes and vascularized adipose tissue were exposed to three of the studied chemicals; rosiglitazone, prochloraz and chlorpyrifos, and the effects of these chemicals were also studied at a gene expression level (IV). Rosiglitazone is a TZD and insulin sensitizing antidiabetic drug. Prochloraz is a fungicide and chlorpyrifos a pesticide. The results of the vascularized adipose tissue showed good concordance with previous reports both with regards to the adipogenic and angiogeneic properties of these chemicals (IV).

In study IV, the exposure of vascularized adipose tissue to rosiglitazone caused elevated gene expression of FABP4, leptin, adiponectin and Glut4. This is in good concordance with the findings of others on the effects of rosiglitazone on adipocytes (Taxvig et al. 2012). Although we did not see the increase in lipid accumulation (IV) seen by others (Taxvig et al. 2012), the gene expression changes indicate an increase in adipogenesis (IV). Angiogenesis in the developed model increased (IV), which is consistent with an increase in adipose tissue vascularization in the *in vivo* exposure of humans (Gealekman et al. 2012), although contradictory results have been obtained *in vitro* (Sheu et al. 2006).

We found that prochloraz was an adipogenesis inhibitor with EC50 at a 164μM concentration and could be classified as an inhibitor of adipogenesis (IV). All other studied genes, except PPARα, were expressed at a lower level than unexposed cultures (IV). Prochloraz has been shown to decrease adiponectin and leptin, and a decrease in lipid accumulation of 3T3 cells already at 50μM (Taxvig et al. 2012). We

did not see prochloraz impacting angiogenesis (IV), which is in concordance with the results of others (Kleinstreuer et al. 2011). Exposure to 50μM prochloraz increased expression of PPARα in vascularized adipose tissue compared to adipocytes alone (IV). In 3T3-L1 cells the 50μM concentration of prochloraz had no effect on PPARα or PPARγ (Taxvig et al. 2012). The increased levels of PPARα in vascularized adipose tissue in study IV is an intriguing finding. This could be caused by the interaction of adipocytes and vessels and their mural cells or the result of an effect of prochloraz on endothelial cells, but further mechanistic studies would be needed to confirm the mechanism(s).

Chlorpyrifos caused an increase in viability/mitochondrial activity and seemed to inhibit lipid accumulation at lower concentrations and increase it at higher concentrations before inhibiting it again (IV). It has been reported that chlorpyrifos decreases lipid accumulation in 3T3 cells (Taxvig et al. 2012). Our results showed leptin significantly increased in expression in vascularized adipose tissue exposed with 10μM chlorpyrifos (IV) and the effect of this chemical at 10μM has been shown by others with increased secretion of the leptin protein (Taxvig et al. 2012). The angiogenic effect of chlorpyrifos was not seen in study IV nor in other reports (Jeon et al. 2016).

In order to identify the effects of chemicals and drugs on humans, relevant screening models are needed for preclinical and safety testing. These models need to depict human metabolism and cell biology to show the effects of chemicals on humans. As discussed here, the angiogenesis model (I), insulin sensitive adipocyte protocol (II) and vascularized adipose tissue (IV) developed in these studies are relevant for studying human biology and the effects of drugs and chemicals.

6.9 Future perspectives

The models presented in the current study have multiple possible applications. As mentioned above, the developed serum-free medium (I) is suitable for culturing the vasculature and adipocytes (I, II, III, IV), and could be a valid option in multi-tissue cultures including multi-organ chips utilizing microfluidics. When multiple organs are cultured in the same system, connected by a blood mimicking medium suitable for multiple tissues, the *in vitro* system can get closer to really depicting *in vivo* biology.

The vascularized adipose tissue model could be further optimized by lowering the troglitazone concentration and concentrations of VEGF and FGF-2 to better

allow the effect of chemicals influencing angiogenesis and adipogenesis to be seen (IV). Following the final adjustments, the vascularized adipose tissue model and adipocytes can be tested on a larger set of chemicals and their effects compared to those found in the literature. Following this, the models can be validated according to OECD guidelines (Environment Directorate 2005). In validation, the data results compared have to be considered carefully to ensure the reliability of the results given by the model. Human data from clinical trials is one relevant result with which the results given by a model should correlate, as they are obtained from humans. Validation ensures the utilization of these *in vitro* models in GLP facilities, which allows them to be used in chemical regulatory toxicity studies. The developed angiogenesis model (I) has already been intra-laboratory validated (Toimela et al. 2017) and has been offered to OECD as an *in vitro* test.

In the future, the macrovascular cells, HUVEC, could be replaced with microvascular endothelial cells to further optimize the vascularized adipose tissue model. This would further increase the *in vivo*-likeness of this model. The angiogenesis model could also be modified to depict lymphangiogenesis by replacing HUVEC with lymphatic endothelial cells. This would broaden the variety of research fields for this model.

7 CONCLUSIONS

The vascularized adipose tissue model created in this study is a promising tool for adipose tissue research and chemical testing. The components, vasculature and adipocytes, can easily be utilized by themselves or together, to study the effects each organ system has on the other.

In the first study, the angiogenesis model developed earlier, based on a co-culture of hASC and HUVEC, was improved by developing a serum-free defined medium. In culture medium serum is a component with unknown composition and hence it causes unwanted and unknown effects on chemicals, including effects on the actual free concentration in the test and hence it reduces the reliability of test results. This developed serum-free medium produced an extensive vascular network with morphology resembling vessels *in vivo* including the intact endothelial layer, basement membrane, 3D tubules with lumen, extracellular matrix proteins and surrounding pericytes. Angiogenesis and vasculogenesis are extremely important developmental events. Hence, it is important that chemicals, especially drugs, are tested for their impact on these events and for that, reliable test platforms are needed.

The second study focused on the development of an adipocyte differentiation protocol for differentiating hASC into adipocytes. The developed protocol produced *in vivo*-like insulin sensitive adipocytes. Insulin response is a critical feature in adipocytes. The lack of this response is present in diseases and hence there is interest in knowing more about it. In order to study the development of these diseases, models that accurately mimic human biology in healthy tissue are needed, as these can be used to induce a diseased state. The protocol for producing insulin sensitive adipocytes developed here, could also be applied to diseased adipocytes lacking a response. These could be used in the mechanistic studies of insulin response and its failures.

The third study combining the vascular component and adipocytes together produced two protocols (P1 and P6) for creating a proof of concept model of vascularized adipose tissue. Both of these protocols produced vascularized adipose tissue models containing properly formed vasculature among lipid accumulating adipocytes. The culture conditions; culture time, timing of seeding of hASC and HUVEC, timing of differentiation media found best for producing mature adipocyte

(P1) were then utilized in the fourth study, which combined the adipocyte protocol developed in the second study with the *in vitro* vasculature developed in the first study to create the vascularized adipose tissue model. This vascularized adipose tissue model was shown to be insulin responsive. In addition, the vascularized adipose tissue model was shown to be relevant for testing chemicals due to the fact that the responses to known chemicals correlated to responses reported in the literature. The models and analysis methods depicted in this study are simple and robust enough to be used in high-throughput chemical studies.

The models developed in this study will be validated according to OECD guidelines in a GLP laboratory. This way they can be utilized also for regulatory purposes.

The complexity of adipose tissue makes the development of a truly *in vivo*-like adipose tissue a difficult task. With increasing understanding of adipose tissue biology and the adipogenesis process, the development of a relevant model is more and more feasible. More work is still to be done to compose an adipose tissue model that mimicks all phases of adipogenesis in an environment that fully depicts the *in vivo* environment of adipocytes. However, *in vitro* models carefully developed and thoroughly characterized, like the ones developed here, are already relevant in the development of effective therapies against obesity and metabolic disease, and for understanding the effects of different cellular components on adipose tissue and its functional failures.

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ORIGINAL PUBLICATIONS

PUBLICATION I

Human Vascular Model with Defined Stimulation Medium – A Characterization Study

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Research Article

Human Vascular Model with Defined Stimulation Medium – A Characterization Study

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Summary

The formation of blood vessels is a vital process in embryonic development and in normal physiology. Current vascular modelling is mainly based on animal biology leading to species-to-species variation when extrapolating the results to humans. Although there are a few human cell based vascular models available, these assays are insufficiently characterized in terms of culture conditions and developmental stage of vascular structures. Therefore, well characterized vascular models with human relevance are needed for basic research, embryotoxicity testing, development of therapeutic strategies and for tissue engineering.

We have previously shown that the *in vitro* vascular model based on co-culture of human adipose stromal cells (hASC) and human umbilical vein endothelial cells (HUVEC) is able to induce an extensive vascular-like network with high reproducibility. In this work we developed a defined serum-free vascular stimulation medium (VSM) and performed further characterization in terms of cell identity, maturation and structure to obtain a thoroughly characterized *in vitro* vascular model to replace or reduce corresponding animal experiments.

The results showed that the novel vascular stimulation medium induced an intact and evenly distributed vascular-like network with morphology of mature vessels. Electron microscopic analysis assured the three-dimensional microstructure of the network containing lumen. Additionally, elevated expression levels of the main human angiogenesis-related genes were detected.

In conclusion, with the newly defined medium the vascular model can be utilized as a characterized test system for chemical testing as well as in creating vascularized tissue models.

Keywords: serum-free media, angiogenesis, mesenchymal stromal cells, coculture techniques

1 Introduction[#]

The formation of the blood vessel network is a vital process in growth and organ development (Carmeliet and Jain, 2011; Carmeliet, 2005). In the embryo, endothelial precursor cells form new vessels that differentiate into a primitive vascular network (vasculogenesis) (Carmeliet and Jain, 2011). Subsequent vessel sprouting (angiogenesis) creates a network of arteries and veins as well as capillaries that facilitate the exchange of gases and metabolites (Carmeliet and Jain, 2011; Adams and Alitalo, 2007). To reach this level of complex organization, the immature vascular network must mature at the level of the vessel wall

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[#]Abbreviations

AA, ascorbic acid; APC, allophycocyanin; BSA, bovine serum albumin; CD144, vascular endothelial cadherin; EGF, epidermal growth factor; EGM-2, endothelial cell growth medium-2; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FGF-2, basic fibroblast growth factor; hASC, human adipose stromal cells; HE, heparin sodium salt; HS, human serum; HUVEC, human umbilical vein endothelial cells; HY, hydrocortisone (cortisol); IGF-I, insulin-like growth factor I; PBS, phosphate buffered saline; RT, room temperature; PDGFRβ, platelet derived growth factor beta; PE, phycoerythrin; PE-CY7, phycoerythrin-cyanine; SFM, basal serum-free medium; αSMA, alpha smooth muscle actin; TRITC, tetramethyl rhodamine isothiocyanate; VEGF, vascular endothelial growth factor; VSM, vascular stimulation medium; vWf, von Willebrand factor

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morphology and as a whole network. Morphological maturation involves recruitment of mural cells, deposition of extracellular matrix and organ-specific specialization of cells, such as inter-endothelial junctions and surface receptors. Maturation of the network involves branching and expanding the network to meet local, tissue-specific demands (Jain, 2003).

Since the formation and maintenance of the vascular network is a complex process, problems related to its regulation are common (Ucuzian and Greisler, 2007). Inadequate vessel maintenance or growth causes ischemia in myocardial infarction and neurodegenerative or obesity-associated disorders, whereas excessive vascular growth or abnormal remodeling promotes cancer, inflammatory disorders and eye diseases (Potente et al., 2011). Moreover, genetic studies have shown that perturbing embryonic vascular development can have adverse consequences from benign vascular malformation to embryolethality and congenital defects (Knudsen and Kleinstreuer, 2011).

Angiogenesis models are important tools for studying the mechanisms of angiogenesis and the therapeutic strategies to modulate neovascularization (Ucuzian and Greisler, 2007). Due to an increasing amount of compounds affecting the vascular system, accurate vasculogenesis and angiogenesis models are needed for chemical safety testing and for drug development (Sarkanen et al., 2011; Bishop et al., 1999). Currently, preclinical animal models are dominantly used for angiogenesis testing although they are not considered optimal in efficacy or relevance to humans. The most commonly used *in vivo* angiogenesis assays include the chick chorioallantoic membrane (CAM) assay, Matrigel plug assay, zebrafish embryo system, corneal micropocket assay, rat/mouse hind limb ischemia model and rat aortic ring assay (Norrby, 2006; Auerbach et al., 2003). Despite the advantage of providing more information on complex cellular interactions compared to *in vitro* models, animal models are burdened by several disadvantages, such as variability, animal-specificity and ethical concerns (Norrby, 2006). Human cell based models have the potential to be valuable tools in predicting effects in man. However, the human relevance of these *in vitro* models needs to be confirmed in terms of cell identity, physiological architecture and functionality (Bale et al., 2014; Hartung, 2011). In addition, the developmental stage of the model system and a defined medium composition are critical, especially when toxicological applications are considered. Serum-free medium with xeno-free and defined supplements is considered essential for *in vitro* models to decrease the variation between experiments due to unknown components in the medium, and further, unknown binding properties of these components (Shen et al., 2013; Brunner et al., 2010; van der Valk et al., 2010). Currently, culture media are still commonly supplemented with serum, although it has a highly uncharacterized composition, including various cytokines and growth factors, as well as a lot-to-lot variability (Lindroos et al., 2011; Brunner et al., 2010).

We and others have shown that adipose stromal cells and umbilical vein endothelial cells are capable of self-assembling into a dense, three-dimensional vascular-like network (Sarkanen et al., 2012; Merfeld-Clauss et al., 2010; Verseijden et al., 2010). While adipose stromal cells secrete factors that induce endothelial cell (EC) sprouting and lumen formation (Rubina et al., 2009; Traktuev et al., 2008; Rehman et al., 2004; Kilroy et al., 2007; Bishop et al., 1999), the supporting stromal cells also enhance vascular basement membrane and lumen formation (Merfeld-Clauss et al., 2010; Newman et al., 2013; Stratman et al., 2009).

The aim of this study was to develop a defined medium and further characterize the *in vitro* vascular model developed by us, which is composed of human adipose stromal cells (hASC) and human umbilical vein endothelial cells (HUVEC) (Sarkanen et al., 2012). Our results showed that the new vascular stimulation media (VSM) developed in this study produces an extensive vascular-like network with mature properties and provides a valid alternative to commercial EGM-2 medium when comparing vascular-like network formation capacity. This vascular model has the potential to be used in the safety and efficacy assessment of angiogenic compounds. In addition, the vascular-like network combined with target cells, such as cardiomyocytes (Vuorenpää et al., 2014), can be used as a tissue engineering platform to create vascularized tissue models.

2 Materials and methods

This study conforms to the principles outlined in the Declaration of Helsinki. The human adipose tissue samples were obtained from surgical operations and human umbilical cords were received from caesarean sections with individual written informed consent at Tampere University Hospital, Tampere, Finland. The use of hASC and HUVEC were approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland with permit numbers R03058 and R08028, respectively.

Isolation and culture of human adipose stromal cells

hASC were isolated from human adipose tissue by using a mechanical and enzymatic procedure described previously (Sarkanen et al., 2012). Briefly, human adipose tissue specimens were mechanically cut into small pieces and enzymatically digested with 0.15% collagenase I (Invitrogen, Paisley, Scotland, UK) in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM/F12, Gibco, Invitrogen, Carlsbad, CA, USA). hASC were cultured in DMEM/F12 supplemented with 10% human serum (HS, Lonza Group Ltd, Basel, Switzerland) and 1% L-Glutamine (Gibco). The cells were tested for mycoplasma contamination (MycoAlert® Mycoplasma Detection Kit, Lonza Group Ltd) before experimental use.

Isolation and culture of human umbilical vein endothelial cells

HUVEC were isolated from human umbilical cord veins using 0.05% collagenase I as described previously (Sarkanen et al., 2011). The cells were cultured in EGM™-2 Endothelial Cell Growth Medium-2 (EGM-2, Lonza Group Ltd). Before use the cells were tested for mycoplasma contamination (MycoAlert® Mycoplasma Detection Kit, Lonza).

Establishment of the co-culture forming the vascular-like network

Co-culture of hASC and HUVEC was established as described previously (Sarkanen et al., 2012). Briefly, hASC were seeded in

Tab. 1: Stimulation media tested in vascular model

Acronym	Basal medium	Serum	Growth factors	Supplementation
EGM-2	EBM-2	2% FBS	VEGF, FGF-2, IGF-I, epidermal growth factor	hydrocortisone, ascorbic acid, heparin, GA-1000
EGM-2/HS	EBM-2	2% HS	VEGF, FGF-2, IGF-I, epidermal growth factor	hydrocortisone, ascorbic acid, heparin, GA-1000
basal SFM	DMEM/F12	–		ITS, BSA, NaP, L-glutamine, T3,
VSM	DMEM/F12	–	VEGF, FGF-2	ITS, BSA, NaP, L-glutamine, T3, hydrocortisone, ascorbic acid, heparin sodium salt

0.1% ITS (insulin-transferrin-sodium selenite media supplement), 1.28 mM L-glutamine, 1% BSA (bovine serum albumin), 2.8 mM NaP (sodium pyruvate), 100 IU/ml P/0.1 mg/ml S, 0.1 nM T3 (3,3',5-triiodo-L-thyronine sodium salt), HS (human serum), FBS (fetal bovine serum), VEGF (vascular endothelial growth factor A), FGF-2 (fibroblast growth factor 2), IGF-I (insulin-like growth factor I), GA-1000 (30 μg/ml gentamicin and 15 ng/ml amphotericin), VSM (vascular stimulation medium), SFM (serum free medium)

EGM-2 (Lonza) at 20,000 cells/cm^2. After 1-3 h HUVEC were seeded on top of hASC at 4,000 cells/cm^2. The hASC were used at passage 2 and HUVEC at passage 4 in the co-culture (passage number increased at seeding). The day after plating, the stimulation media were applied to the co-culture (see Tab. 1): 1) EGM-2 containing epidermal growth factor, vascular endothelial growth factor A (VEGF), fibroblast growth factor 2 (FGF-2), insulin-like growth factor I (IGF-I), ascorbic acid (AA), heparin, hydrocortisone (HY), antibiotic mix: 30 μg/ml gentamicin and 15 ng/ml amphotericin and 2% fetal bovine serum (FBS); 2) EGM-2/HS, where 2% FBS was replaced with 2% HS; 3) serum free basal medium (SFM); 4) Vascular stimulation medium (VSM).

In this first media comparison study, the AA, HY and heparin in VSM were taken from the EGM-2 kit and used according to the manufacturer's protocol (concentrations of these are not publicly available). After that, concentrations of AA, heparin sodium salt from bovine intestinal mucosa (HE) and HY purchased from Sigma were optimized for VSM. In the concentration optimization study the tested concentrations of AA were 0, 50, 100, 200, 500, 1000 and 2000 μg/ml; of HY 0, 20, 200, 1000, and 2000 ng/ml and of HE 0, 50, 500, 10,000 and 50,000 ng/ml. The concentrations of 10 ng/ml VEGF and 1 ng/ml FGF-2, used for VSM, had been optimized in our previous study (Sarkanen et al., 2012). The co-cultures were grown for 6 days prior to immunocytochemistry or quantitative real-time PCR (qPCR) processing. Stimulation medium was changed once during the 6 day culture.

Quantitative real-time PCR

Genes activated in co-culture of hASC and HUVEC in EGM-2 medium versus co-culture in VSM were analyzed by qPCR. Total RNA was extracted at day 6 using the RNAeasy minikit (Qiagen) following the manufacturer's protocol. A step to eliminate genomic DNA contamination was included in the isolation and performed with RNase-free DNase set (Qiagen). Reverse transcription of total RNA to cDNA was performed using RT^2 First Strand Kit (Qiagen) following the manufacturer's instructions.

Human Angiogenesis RT^2 *Profiler™* PCR Array (Qiagen, Valencia, California, USA) was used to profile the expression of 84 key angiogenesis-related genes (the comprehensive list of genes included in the array can be found in http://www.sabiosciences.com). The array was performed according to the manufacturer's protocol using BioRad CFX96 Real Time System (BioRad Laboratories, USA). The array contained five housekeeping genes and controls, including genomic DNA control, Reverse Transcription Control and Positive PCR controls. The co-culture of hASC and HUVEC grown in EGM-2 medium was used as a control. Three independent experiments with the array were performed using the same cells.

Immunocytochemistry

To analyze the vascular-like network formation and different cell types present in hASC and HUVEC co-culture, immunocytochemical staining was performed. In primary antibody staining, endothelial cell specific antibody for rabbit anti-human von Willebrand factor IgG (anti-VWF, 1:100, F3520, Sigma) with common pericytic marker α-human smooth muscle actin (monoclonal anti-SMA clone 1A4, 1:200, M0851, DAKO), vascular smooth muscle cell marker smooth muscle myosin heavy chain (anti-SMMHC, clone hSM-V, 1:800, M7786, Sigma), contractile smooth muscle cell marker calponin (anti-calponin, clone hCP, 1:800, C2687, Sigma), pericytic and smooth muscle cell progenitor marker platelet derived growth factor receptor-β (anti-PDGFRβ, clone PDGFR-B2, 1:800, P7679, Sigma), vascular endothelial cadherin (CD144,Clone 55-7H1, 1:50, 555661, BD Pharmingen), monoclonal occludin (clone 1G7, 1:300, WH0004950M1, Sigma) or basement membrane marker collagen IV (anti-COLIV, clone COL-94, 1:500, C1926, Sigma), all anti-human and all produced in mouse, except vWf, were used. Co-culture was fixed with 70% ethanol at day 6. After fixation, the cells were permeabilized with 0.5% Triton-X100 (MP Biochemicals, Ohio, USA) and non-specific binding sites were blocked with 10% bovine serum albumin (BSA, Roche Diagnostics Corporation, Indianapolis, USA). Primary antibody in 1% BSA was applied to the cells. Secondary antibodies used were polyclonal goat anti-rabbit IgG tetramethylrhodamine (TRITC, 1:50, T6778 Sigma), anti-rabbit IgG A568 (1: 400, A11011, Invitrogen) and anti-mouse IgG fluorescein isothiocyanate (FITC, 1:100, F4143, Sigma). After immunocytochemical staining the

vascular-like network was analyzed and photographed with Nikon Eclipse TS100 inverted fluorescence microscope (Nikon, Tokyo, Japan) and Nikon digital sight DS-U2 camera (Nikon). Images were further processed with NIS Elements (Nikon, Tokyo, Japan) and Adobe Photoshop CS3-software (Adobe Systems Incorporated, San Jose, CA, United States).

Flow cytometric surface marker expression analysis of HUVEC and hASC

hASC were cultured in hASC medium (passage 1) for 6-7 days and HUVEC were cultured in EGM-2 medium (passage 3) for 3 days prior to surface marker expression analysis using a BD FACSCanto II flow cytometer (BD Biosciences, Erembodegem, Belgium). For the flow cytometry analysis cells were divided into 5 ml polystyrene round bottom FACS tubes (BD, New Jersey, USA) at 250,000 cells per tube. The cells were washed once with warm staining buffer (1% BSA in PBS) and centrifuged at 131 x g for 5 min, after which they were stained either for surface markers or for intracellular markers.

Fixation and permeabilization were only performed for staining of intracellular markers. The fixation was conducted by incubating the samples for 30 min in 2% paraformaldehyde in PBS at room temperature (RT). The cells were then centrifuged at 500 x g for 5 min. Permeabilization of the cells was performed by 10 min incubation in 0.1% Triton-X100 in PBS at RT after which the cells were centrifuged at 500 x g for 5 min and washed once or twice with staining buffer before addition of antibodies.

The labelled mouse anti-human antibodies used were intracellular vWf-A2-allophycocyanin IgG2b (APC, #IC27641A) and eNOS- phycoerythrin IgG1 (PE, #560103), and surface markers CD144-FITC IgG1 (#560411), CD73-Phycoerythrin-Cyanine IgG1 (PE-CY7, #561258), CD309-PE IgG1 (#560872), CD68-FITC IgG2b (#562117), NG2-PE IgG1 (#FAB2585P), CD90-FITC IgG1 (#561969), CD105-V450 IgG1 (#561447), CD34-APC IgG1 (#561209), CD140b-PE IgG2a (#558821), CD31-V450 IgG1 (#561653), CD45-PE IgG1 (#560975), CD14-FITC IgG1 (#561712). Isotype controls mouse IgG2b-APC (#IC0041A), Mouse IgG1-PE-CY7 (#557872), mouse IgG1-PE (#559320), mouse IgG1-FITC (#555748), mouse IgG2b-FITC (#556655), mouse IgG1-V450-(#642268), mouse IgG1-APC (#550854), mouse IgG2a-PE (#551438). All antibodies and their corresponding isotype controls used in the flow cytometry analysis were purchased from BD except NG2, vWf and IgG2b-APC, which were purchased from R&D Systems.

Labelled antibodies were added into cell suspension in cold staining buffer and incubated on ice for 30 min in the dark. After incubation the cells were washed once with staining buffer and twice with PBS. Surface marker stained cells were centrifuged at 200 x g for 5 min and intracellular marker stained cells were centrifuged for 5 min at 500 x g.

Flow cytometry analysis was performed with cells suspended in ice cold PBS and 5,000 events were analyzed per sample. Compensation was done with compensation particles, i.e., BD™ CompBeads (BD) according to the manufacturer's instructions. The results were analyzed with BD FACSDiva™ Software (BD). The positive expression was obtained by gating 98% of the events isotype control results and then inverting the gate to obtain a percentage of positively stained cells in the samples. The results were calculated as percentages with SD.

Electron microscopy

hASC and HUVEC co-culture was performed in 24-well UpCell plates (ThermoFisher) for transmission electron microscopy (TEM) and on glass cover slips coated with 0.1% gelatin for scanning electron microscopy (SEM). Co-culture was maintained for 6 days and washed twice with PBS prior to fixation.

SEM specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer after which the SEM samples were dehydrated in alcohol and dried in Baltec critical point dryer (Baltec, CPD030, Balzers, Liechtenstein). A layer of platinum was sputtered onto the specimens with a sputter coater (Agar Scientific, Stansted, England). Specimens were examined in a Zeiss Ultra Plus scanning electron microscope, (Carl Zeiss MT – Nanotechnology System Division, Carl Zeiss NTS GmbH, Oberkochen, Germany) using 5 kV as an accelerating voltage.

TEM specimens were detached from the temperature sensitive 24-well UpCell plate and transferred to fixative with 1% glutaraldehyde, 4% formaldehyde mixture in 0.1 M phosphate buffer for 10 min. The cell sheet was immersed in 2% agarose in distilled water and postfixed in 1% osmiumtetroxide, dehydrated in acetone and embedded in Epon LX 112 (Ladd Research Industries, Vermont, USA). Thin sections were cut with Leica Ultracut UCT ultramicrotome, stained in uranyl acetate and lead citrate and examined in a Philips CM100 transmission electron microscope. Images were captured by a Morada CCD camera (Olympus Soft Imaging Solutions GMBH, Munster Germany).

Quantitative analysis of vascular-like network formation

Vascular-like networks, i.e., vWf-positive tubule structures formed in different stimulation media, were imaged using CellIQ (Chipman tech., Tampere, Finland) with 10x objective and 5x5 grid. The quantitation of the area of the vascular-like network was performed using ImageJ software (National institutes of health, NIH, Maryland, USA) for the image analysis. Images were first converted to 8-bit gray scale, then background was subtracted and finally the binary threshold function was adjusted to obtain the best contrast of the vascular-like network against the background. With these settings, the total area of vascular-like network was calculated as the total number of pixels in images with set threshold.

Statistical analysis

Statistical analyses were performed and graphs processed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). The results concerning vascular-like network formation were subjected to one-way ANOVA followed by Dunnett's post-test when applicable. The results were reported as total area ± SD and differences were considered significant when $p < 0.05$*, $p < 0.01$** and $p < 0.001$***.

PCR results were analyzed with the PCR Array Data Analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php). The following formula was used to calculate the relative amount of the transcripts of the co-culture of hASC and

Tab. 2: Phenotypic characterization of human adipose stromal cells (hASC p1) and human umbilical vein endothelial cells (HUVEC p3)
The expression of surface and intracellular markers was analysed by flow cytometry (5000 events per sample). Results are shown as percentage of positive cells.

Cells	Protein	Antigen	Positive cells %	SD	*n*
hASC	vWf	von Willebrand factor	7.5	4.3	11
	CD144	vascular endothelial cadherin	1.4	2.9	9
	eNOS	endothelial nitric oxide synthase	0.7	1.1	8
	CD140b	PDGF Receptor b	52.8	16.0	6
	CD45	leukocyte common antigen	16.4	14.9	6
	CD14	expressed on monocytes/macrophages	0.7	0.8	7
	CD68	expressed on macrophages and monocytes	1.1	1.4	8
	CD309	VEGF receptor 2	1.2	3.5	6
	CD31	platelet endothelial cell adhesion molecule	0.9	3.1	6
	NG2	chondroitin proteoglycan	31.8	29.1	8
	CD90	Thy-1	82.9	15.5	10
	CD105	endoglin	64.1	27.1	7
	CD73	ecto-5'-nucleotidase	88.8	6.7	12
	CD34	sialomucin-like adhesion molecule	40.9	34.3	10
HUVEC	vWf	von Willebrand Factor	45.6	13.2	13
	CD144	vascular endothelial cadherin	70.6	14.7	17
	CD309	VEGF receptor2	31.4	17.0	10
	CD73	ecto-5'-nucleotidase	68.7	17.1	14
	CD68	expressed on macrophages and monocytes	1.9	10.1	11
	eNOS	endothelial nitric oxide synthase	48.4	18.4	13
	CD105	endoglin	57.4	26.6	9
	CD34	sialomucin-like adhesion molecule	43.1	18.6	9
	CD31	platelet endothelial cell adhesion molecule	81.3	13.7	8
	CD140b	PDGF Receptor b	7.2	8.7	8
	NG2	chondroitin proteoglycan	2.0	1.1	7

HUVEC in the VSM compared to transcripts of the co-culture in EGM-2 medium: $\Delta\Delta CT = \Delta CT$ (VSM) – ΔCT (EGM-2). A two-fold change compared to housekeeping gene *GAPDH* was considered significant.

3 Results

3.1 Phenotypic characterization

Phenotypic characterization of the cells used in the vascular model was performed by analyzing the surface and intracellular markers of hASC and HUVEC separately (Tab. 2). The mesenchymal stem cell markers CD73, CD90 and CD105 were highly expressed (> 64%) in hASC. Also, pericyte marker PDGFR-β (CD140b) was strongly expressed (> 52%) in the hASC population. Hematopoietic marker CD34 was moderately expressed (< 40%), whereas the expression levels of endothelial markers CD144 and CD31 and macrophage/monocyte markers CD68 and CD14 were very low (< 1.4%).

HUVEC were shown to express endothelial marker CD31 and a specialized vascular endothelial marker CD144 at a high level (> 70%). Angiogenic endothelial marker CD105 and cell surface enzyme CD73 were found in the HUVEC population (57-68%). Markers for macrophages/monocytes (CD68) and mural cells (NG2) were low (≤ 2%).

3.2 VSM induced optimal vascular-like network formation

Of the different stimulation media (Tab. 1), VSM induced an optimal vascular-like network formation (Fig. 1). The imaging analysis showed morphological differences in the vascular-like network in VSM compared to EGM-2 medium. VSM produced a uniformly distributed vascular-like network with connected branches and fewer cell aggregates. Low serum (2%) and se-

rum-free medium enabled stable cell attachment and induced a dense, connected vascular-like network with intact tubule walls. Human serum was found to induce a denser and more connected vascular-like network than FBS (Fig. 1).

In the supplement optimization several different concentrations of ascorbic acid, heparin sodium salt and hydrocortisone were tested and compared to vascular-like network formed in EGM-2 medium. An optimal vascular-like network formation was obtained with the AA concentration 100 μg/ml (Fig. 2A), HE concentration 0-500 ng/ml (Fig. 2B) and HY concentration 0.2 μg/ml (Fig. 2C). The results showed that a vascular-like network was formed in the absence of HE supplement with minor morphological differences. However, low concentrations of HE (50-500 ng/ml) increased the total area of the vascular-like network significantly compared to EGM-2 medium (Fig. 2B).

3.3 Expression of angiogenesis-related genes in VSM compared to EMG-2

Expression of 84 angiogenesis-related genes was analyzed from a vascular-like network formed in co-culture of hASC and HUVEC in EGM-2 medium (control) and in VSM. Moderate differences in the expression of angiogenesis-related genes were detected between EGM-2 and VSM. In VSM, nine genes were up-regulated (*Angpt1, F3, FIGF, IGF-I, LEP, MDK, MMP2, MMP9, PGF*) and nine (*CCL11, CXCL9, FN1, IL6, IL8, SERPINE1, TGFB2, THBS2, TIMP1*) were down-regulated in comparison to EGM-2 medium. Fold changes and statistical significances of the up- and down-regulated angiogenesis-related genes are shown in Table 3.

3.4 Maturation stage, extracellular matrix production and 3D properties of the vascular-like network formed in VSM

Immunocytochemical staining of the vascular-like network formed in VSM showed the presence of platelet derived growth factor receptor-β (PDGFRβ) positive cells (Fig. 3D). Also, smooth muscle actin (SMA), smooth muscle myosin heavy chain (SMMHC) and contractile smooth muscle calponin positive cells surrounded the tubule structures (Fig. 3A-C). CD144 and occludin positive staining indicated the presence of adherence junctions between endothelial cells (Fig. 3E-F).

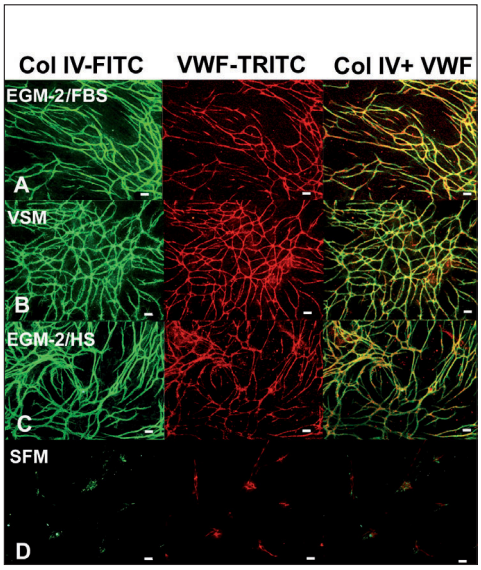

Fig. 1: Morphology of vascular-like network in different stimulation media with different serum concentrations

Vascular-like networks were stained against Col IV (FITC, green) and vWf (TRITC, red). Co-localization is shown in the merged image. Vascular-like network formed in (A) commercial EGM-2 medium with 2% FBS, (B) serum-free VSM, (C) EGM-2 with 2% HS and (D) basal SFM. Scale bars 100 μm in each figure. Col IV= collagen type IV, VWF= von Willebrand factor, VSM= vascular stimulation medium, FBS= fetal bovine serum, HS= human serum, SFM=basal serum free medium.

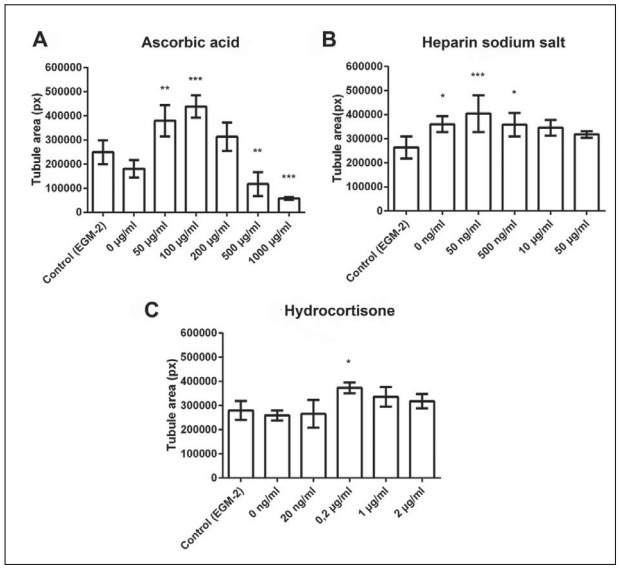

Fig. 2: Vascular-like network formation (area in pixels) in different concentrations of (A) ascorbic acid, (B) heparin sodium salt and (C) hydrocortisone in VSM

Vascular-like network formed in VSM supplemented with different concentrations of ascorbic acid, heparin sodium salt or hydrocortisone were compared to EGM-2 medium and differences presented as follows: $p < 0.05$*, $p < 0.01$** and $p < 0.001$***. An optimal vascular-like network formation was obtained with 100 μg/ml ascorbic acid and 0.2 μg/ml hydrocortisone. Heparin sodium salt could be used in concentrations 0-500ng/ml. Results are depicted as total area with standard deviations, n = 5 in each supplement and concentration.

Tab. 3: Fold changes and statistical significance of expression of angiogenesis-related genes in vascular-like network formed in VSM compared to EGM-2 medium

Genes	Target	Fold change 2^(- ΔΔCT)	Statistical significance
Up-regulated genes			
ANGPT1	angiopoietin 1	2.964	*
F3	coagulation factor III	3.255	*
FIGF	vascular endothelial growth factor D	3.043	ns
IGF-I	insulin-like growth factor I	16.555	ns
LEP	leptin	2.932	ns
MDK	neurite growth-promoting factor 2	2.06	ns
MMP2	matrix metallopeptidase 2	2.011	*
MMP9	matrix metallopeptidase 9	3.919	*
PGF	placental growth factor	2.319	*
ANGPT2	angiopoietin 2	1.769	**
Down-regulated genes			
CCL11	chemokine ligand 11	2.613	ns
CXCL9	chemokine ligand 9	2.197	ns
FN1	fibronectin 1	2.295	*
IL6	interleukin 6	2.331	ns
IL8	interleukin 8	2.546	ns
SERPINE1	serpin peptidase inhibitor	3.465	**
TGFB2	transforming growth factor beta 2	2.080	ns
THBS2	thrombospondin 2	2.085	**
TIMP1	TIMP metallopeptidase inhibitor 1	5.01	**
VEFG-A	vascular endothelial growth factor A	1.804	ns
TGFβ1	transforming growth factor beta 1	1.807	*
FGF-2	fibroblast growth factor 2	1.917	ns

Electron microscopic analysis confirmed the microstructure of the tubules formed in VSM. Tubules were shown to have a lumen, basement membrane and junctions (Fig. 4A-C). The three dimensional structure of the vascular-like network and shape of the tubules can be seen in the close-up image Figure 4D.

4 Discussion

Present chemical safety testing and non-clinical drug development rely mainly on animal biology although relevant safety testing data should be of human origin. Further, *in vitro* test systems should be thoroughly characterized in their human relevance and functionality. In this study, we developed a novel vascular stimulation medium (VSM) and further characterized the *in vitro* human vascular model developed by us (Sarkanen et al., 2012). This defined *in vitro* vascular model has the potential to be used for safety assessment of compounds and for tissue engineering applications.

The phenotypic analysis of the building blocks of the vascular model, i.e., the hASC and HUVEC, was performed using flow cytometry. HUVEC showed a strong expression of CD105 (endoglin), suggesting an active rather than quiescent state of the cells (Bernabeu et al., 2009). Interestingly, a recent study reported a minor role of endoglin in vasculogenesis whereas VEGF-induced angiogenesis was severely impaired in an *endoglin* deficient mouse embryonic stem cell model (Liu et al., 2014). The high expression of CD144 and CD31 supports the active status of HUVEC in the formation of intercellular junctions. The level of CD73, a 5'-ectonucleotidase that normally suppresses pro-inflammatory responses in human endothelial cells (Grunewald and Ridley, 2010) was high in our HUVEC analysis. Pericyte markers NG2 and CD140b (PDGFRβ) were low, indicating the absence of mural cells in the HUVEC population.

This study confirms the earlier results shown by us (Sarkanen et al., 2012) and others (Lindroos et al., 2010; Traktuev et al., 2008) that the stromal-vascular fraction extracted from adipose tissue is heterogenic. hASC expressed mesenchymal stem cell markers CD90, CD105 and CD73 at high levels, showing the

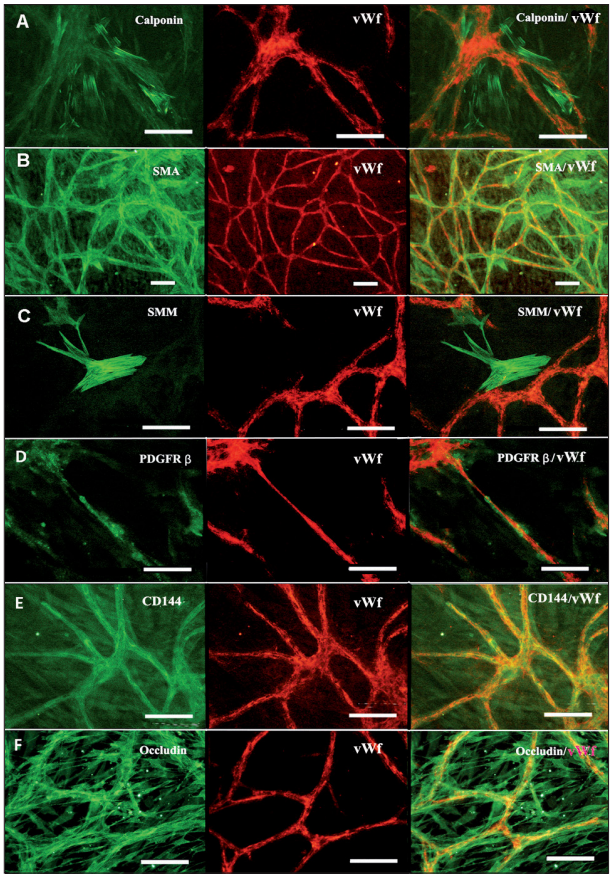

Fig. 3: Vascular-like network formed in serum-free VSM shows vascular maturation markers at day 6

α-vWf-staining is shown in red in all figures. (A) Calponin-, (B) SMA- and (C) SMM-positive cells surrounding the vascular-like network. (D) α-PDGFRβ stained pericytes are located along the tubules. (E) CD144- and (F) occludin-positive junctions can be found in the tubule structures. Scale bar 100 µm in each image. VSM= vascular stimulation medium, vWf = von Willebrand factor, SMA = smooth muscle actin, SMM = smooth muscle myosin heavy chain, PDGFRβ = platelet derived growth factor receptor β, CD144 = vascular endothelial cadherin.

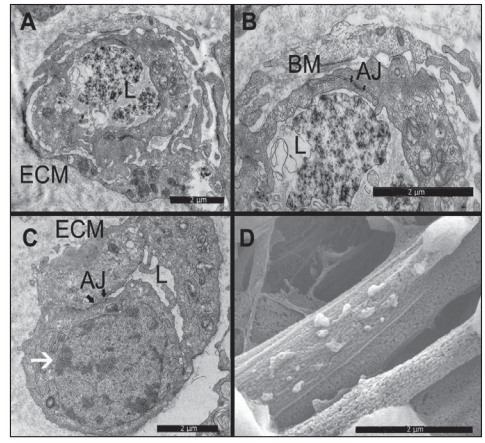

Fig. 4: Transmission (A, B, C) and scanning (D) electron microscopy images of the vascular-like network cultured in VSM

(A) Mature tubule with lumen = L and extracellular matrix = ECM. The lumen is filled with debris from the apoptotic cells. Scale bar 2 μm. (B) Close-up image with basement membrane = BM, adherence junction = AJ and lumen of the vascular-like tubules. Scale bar 2 μm. (C) Tubule in earlier stage of vascular development showing endothelial cell with large nucleus (white arrow), lumen, adherence junctions and ECM. Scale bar 2 μm. D) Scanning electron microscopy image shows the 3D structure and the tubule shape of the vascular-like network. Scale bar 2 μm. VSM = vascular stimulation medium.

presence of a cell population with multilineage differentiation potential. Pericyte markers CD140b (PDGFRβ) and NG2 were expressed at high to moderate level, supporting the finding that pericytes from the hASC population are lining the vascular-like network in our model.

We also analyzed whether the population of endothelial progenitor cells capable of vasculogenesis could be found in hASC as suggested by some earlier reports (Sarkanen et al., 2012; Miranville et al., 2004; Heydarkhan-Hagvall et al., 2008). The results showed that, in addition to the high expression of CD90, there is a moderate expression of CD34; these are markers for mesenchymal stem cells and endothelial progenitors. A hASC population of CD90+/CD34+ cells was shown to be capable of differentiating into endothelial cells and form capillary-like structures (De Francesco et al., 2009).

The expression of mature endothelial cell markers CD31 and CD144 in the hASC population was very low. Interestingly, there is a report suggesting that adipose stromal cells may enhance endothelial differentiation of progenitor cells (Rubina et al., 2009). However, another essential marker for endothelial progenitor cells, CD309 (VEGFR2) (Yoder, 2012), showed very low expression in the six hASC lines that were analyzed in this study. Therefore, the presence of an endothelial progenitor cell population in the stromal-vascular fraction needs to be studied further.

Serum-free VSM was shown to produce a reproducible, extensive mature vascular-like network and provide a suitable alternative to the commercial EGM-2 medium (previously used in the model, containing 2% FBS). Serum had a strong correlation with vascular-like network formation. At higher serum (10%) concentrations the network was shorter with broken tubule walls, and random detachment of the cell layer occurred (data not shown). In low- or serum free (0-2%) environment,

network formation increased, tubules were highly branched and tubule walls remained intact. In serum-free conditions we detected thinner and more branched tubules as also reported by Yang and Xiong (Yang and Xiong, 2012). Serum-free medium is ideal for use in drug development, since serum is a complex mixture of components with unknown composition and protein binding affinities (Shen et al., 2013). Furthermore, a tissue construct aimed for clinical therapy should be cultured in human serum or, preferably, in a serum-free environment to avoid the risk of infection and severe immune reactions in the recipient (Patrikoski et al., 2013; Lindroos et al., 2011, 2010; Holm et al., 2010).

Fetal bovine serum (FBS), although being the most widely used growth supplement, holds ambiguous, unknown effects for cell culture and also raises ethical concerns due to the number of bovine fetuses needed for serum production (Gottipamula et al., 2013; Brunner et al., 2010). In this study, microscopical analysis showed that HS was more inductive for vascular-like network formation than FBS. Replacement of FBS with HS has been reported to support equal or higher proliferation rates and differentiation capacity of adipose stromal cells (Lindroos et al., 2011; Brunner et al., 2010). Furthermore, human mesenchymal stem cells have been shown to maintain their immunophenotype and multilineage potential in serum-free medium (Patrikoski et al., 2013; Mark et al., 2013).

In this study we developed a defined vascular stimulation medium since commercial media producing companies do not necessarily publish the specific composition of their media. This might complicate the use of commercial media in toxicological studies as medium components may interact with test compounds. In addition to commonly used VEGF and FGF-2 defined by us earlier (Sarkanen et al., 2011), we found that the network formation can be further improved by addition of AA, HE and HY. Ascorbic acid (vitamin C) is an essential nutrient for human endothelial cells necessary for their effective migration and for the synthesis of collagen type IV, an important component of basement membrane (Telang et al., 2007). In this study, the formation of basement membrane around the tubules was impaired in the absence of AA (data not shown). However, AA inhibited angiogenesis at high concentrations (1000-2000 μg/ml) as reported previously (Mikirova et al., 2008). Therefore, AA is essential for collagen IV formation in basement membrane at low concentrations.

Although HE is needed for the attachment of some growth factors to their cell surface receptors (Ashikari-Hada et al., 2005) and was found to be beneficial for the network formation, it did not induce a significant advantage to the morphology or branching of the tubules. Since HE is an animal derived substance it should rather be avoided where clinical applications are concerned. The lack of need for added HE can be explained by the secretion of perlecan by HUVEC (Murikipudi et al., 2013; Schlessinger et al., 2000). Our results correlate with earlier studies (Khorana et al., 2003; Jung et al., 2001) showing that HE inhibits vascular-like network formation at high concentrations.

Hydrocortisone, although not pro-angiogenic itself, has a beneficial effect on angiogenesis (Goding, 2009). In this study, hydrocortisone increased the number of branches in the vascular-like network. However, it produced cell aggregates at high concentrations whereas a lack of hydrocortisone induced a sparse vascular-like network. Our results suggest that hydrocortisone acts as a mitogen in the vascular model. Other supplements of the VSM included ITS (insulin, transferrin, selenic acid medium supplement), BSA, sodium pyruvate, L-glutamine and T3 (3,3',5-triiodo-L-thyronine). The concentrations of these supplements were reported earlier by us (Vuorenpää et al., 2014) and were used in this study with minor modifications.

Lumen formation is critical for the transformation of cords into a perfusable vascular system (Charpentier and Conlon, 2014). Electron microscopic analysis assured the formation of 3D vascular microstructure including lumen in the novel VSM. Tubules at different stages of lumen development were detected, indicating the ongoing process of network formation at day 6. The initiation of lumen development is triggered by apical-basal polarity of the endothelial cells in which CD144 plays a critical role in promoting the localization of polarity markers (Charpentier and Conlon, 2014). The electron microscopic analysis as well as immunocytochemistry results of this study showed that hASC and HUVEC co-culture actively produces extracellular matrix (ECM) components, including fibrillins, thus creating natural 3D scaffold around them. The reciprocal interaction between ECM stroma and vascular network is important in directing vessel growth (Hoying et al., 2014; Du et al., 2014). The hASC and HUVEC co-culture gives mechanical support for other target cells, e.g., cardiomyocytes, and, additionally, the microenvironment formed by the co-culture enhances target cell viability as reported previously (Vuorenpää et al., 2014). This 3D vascular model provides a more *in vivo*-like test system without an artificial scaffold that may interfere with the cell-cell interactions or affect the toxicological applications of the model.

In the genotypic analysis 84 human angiogenesis-related genes were studied. Nine genes were down-regulated and nine genes up-regulated in VSM compared to EGM-2 medium. The expression of *angiopoietin 1*, a marker for mature tubules, was significantly higher in VSM compared to EGM-2 medium, whereas *angiopoietin 2*, a marker for the early stage, i.e., sprouting angiogenesis, was slightly upregulated in VSM. *VEGF-A* and *FGF-2*, indicators of early stage angiogenesis, were moderately yet not significantly down-regulated in VSM compared to EGM-2. Since both media included VEGF and FGF-2, the expression of these growth factors was apparently unnecessary for the cells. On the contrary, *placental growth factor* (PGF) showed modest up-regulation in VSM. PGF and its receptor VEGFR-1 are minimally expressed in adult quiescent vasculature, but are markedly up-regulated during pathological conditions (Carmeliet et al., 2001). *TGFβ1*, a marker for tubule maturation, was slightly down-regulated in VSM. Vessel maturation relies partly on TGF-β signaling and TGF-β stimulates mural cell proliferation and migration and production of ECM (Potente et al., 2011). The gene expression analysis confirms the finding that VSM enhances the maturation of the vascular-like network.

Interestingly, the expression of *insulin like growth factor* (IGF-I) showed the highest up-regulation in VSM. IGF-I treatment in myocardial infarction has been shown to increase circulating angiogenic growth factors, thus providing protection

against myocardial ischemia in rats (Mathews et al., 2011). *In vitro* IGF-I stimulates migration and tube-forming activity of endothelial cells (Shigematsu et al., 1999; Nakao-Hayashi et al., 1992). Also leptin, a hormone secreted by adipocytes, was found to be up-regulated. Leptin signaling acts as a link between adipocytes and the vasculature (Sierra-Honigmann et al., 1998). It also increases the production of VEGF and enhances the expression of *MMP-2* and *MMP-9* (matrix metallopeptidases) in HUVEC (Park et al., 2001). This was seen as an up-regulation of *MMP-2* and *MMP-9* enzymes that are involved in degradation of the ECM at the early stages of angiogenesis (Kasper et al., 2007; Cornelius et al., 1998).

In vivo studies in mice have revealed that the adult quiescent vasculature becomes less dependent on VEGF for its maintenance (Gerber et al., 1999). However, during pathological conditions – such as ischemia, inflammation or malignancy – angiogenic endothelial cells are stimulated by increased VEGF levels (Carmeliet et al., 2001). The VEGF signaling pathways have been conclusively identified as central for the processes of vasculogenesis, angiogenesis and lymphangiogenesis. VEGF-D induces sprouting lymphangiogenesis when overexpressed in transgenic mice and also in various tumor models (Lohela et al., 2009). The up-regulation of *vascular endothelial growth factor D (FIGF)* as a marker for lymphangiogenesis suggests the versatile modifications of our vascular model. In future applications, it may be possible to replace HUVEC with lymphatic endothelial cells to form a lymphangiogenesis model.

5 Conclusion

The vascular model characterized in this study forms a vascular-like network with mature properties. The developed novel medium provides a valid alternative to commercial EGM-2 medium and benefits the use of the model in toxicological studies as well as in efficacy studies in drug development. This characterized vascular model is currently under intra-laboratory validation that is performed according to OECD guidance document No. 34 (OECD, 2005) to verify the reliability and human relevance of the test system with known reference chemicals. The validated vascular model will be used in toxicity testing and can be combined with other target cell types from different tissues to create complex, vascularized tissue models.

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Conflict of interest statement

None of the authors have any conflicts of interest.

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PUBLICATION II

Differentiation of human adipose stromal cells *in vitro* into insulin-sensitive adipocytes

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Differentiation of human adipose stromal cells in culture into insulin sensitive adipocytes

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Abstract: Adipose tissue related diseases such as obesity and type 2 diabetes are worldwide epidemics. In order to develop *in vitro* adipose tissue cultures which mimic more faithfully the physiology *in vivo*, new well characterized and publicly accepted differentiation methods of human adipose stem cells are needed. The aims of this study were (i) to improve the existing natural adipose tissue extract (ATE)-based induction method and (ii) to study the effects of differentiation method on insulin responsiveness of the resulting adipocytes. Different induction media were applied on human adipose stromal cell (hASC) monocultures to study the differentiation capacity of the induction media and the functionality of the differentiated adipocytes. Cells were differentiated for 14 days to assess triglyceride accumulation per cell and adipocyte-specific gene expression (*PPARγ, adiponectin, AP2, leptin*, *Glut4, Prdm16, CIDEA, PGC1-α, RIP140, UCP and ADCY5*). Insulin response was studied by measuring glucose uptake and inhibition of lipolysis after incubation with 100 or 500 nM insulin. The selected differentiation method included 3-day induction with adipose tissue extract, 6 days in serum-free medium supplemented with 1.15 µM insulin and 9.06 µM Troglitazone, followed by 4 days in a defined serum- and insulin-free stimulation medium. This protocol induced prominent general adipocyte gene expression, including markers for both brown and white adipocytes, and triglyceride accumulation. Moreover, the cells were sensitive to insulin as observed from increased glucose uptake and inhibition of lipolysis. This differentiation protocol developed provides a promising approach for induction of hASC adipogenesis to obtain functional and mature human adipocytes.

Key words: Adipocyte, adipogenesis, in vitro differentiation, maturity, adipose stromal cells

INTRODUCTION

The prevalence of adipose tissue related diseases, such as obesity and type 2 diabetes, have reached worldwide epidemic proportions. In addition to type 2 diabetes, obesity and overweight are associated with an increased incidence of other comorbidities including several cancer types, sleep apnea, asthma, degenerative joint disease, hypertension, renal failure, stroke, and cardiovascular disease (Switzer et al. 2013, van Baak 2013). As the prevalence of obesity has increased, also the need to study adipose tissue and find treatments to related diseases has raised. The growing interest creates the need to develop better methods to study the process of adipogenesis and adipose tissue function.

Adipose tissue is not only a depot for energy storage, but a dynamic endocrine organ secreting bioactive factors that control systemic insulin sensitivity, energy metabolism, immune responses and cardiovascular homeostasis (Gu and Xu 2013, Choi et al. 2010a). Adipose tissue can expand via adipocyte hypertrophy, where existing cells grow in size, or by hyperplasia, increase in adipocyte number, which requires progenitor cells to differentiate into adipocytes (Spalding et al., 2008). New adipocytes develop through adipogenesis, which is typically described as a two-phase process that includes commitment and terminal differentiation (Cristancho and Lazar 2011, Rosen and MacDougald 2006). In the determination phase, stem cells transform into committed preadipocytes (Rosen and MacDougald 2006). Commitment is followed by a growth arrest after which an appropriate mixture of adipogenic and mitogenic signals are needed for terminal differentiation (Gregoire 2001). In terminal differentiation, preadipocytes transform into insulin-sensitive, lipid synthesizing and transporting mature adipocytes that secrete adipocyte-specific secretory products (Rosen and MacDougald 2006, Gregoire et al. 1998). Differentiation into mature adipocyte phenotype is typically characterized by chronological changes in the expression of the early, intermediate and late mRNA/protein markers (Gregoire et al. 1998).

Insulin is an important regulator of adipocyte metabolism. It promotes the synthesis and storage of triglycerides and inhibits their catabolism (Rutkowski et al. 2015). In the high fed state, insulin binds to its receptor on adipocytes and causes the translocation of glucose transporter 4 (Glut4) from the cytosol to the cell surface, thus allowing effective glucose influx into the adipocytes (Rutkowski et al. 2015, Watson and Pessin 2007). The influx of glucose is not merely necessary for ATP production, as glucose is also needed for effective adipocyte lipid packaging (Bederman et al. 2009, Guan et al. 2002). Insulin also inhibits lipolysis by activating phosphodiesterase 3B, which inactivates the function of cAMP (Choi et al. 2010b). Thus, the downstream activation of protein kinase A (PKA), adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) is inhibited (Choi et al. 2010b).

The complex pathways of lipid metabolism are highly species specific (Bergen and Mersmann 2005). Thus, new human cell based methods offer biologically relevant tools to study human adipose tissue-related diseases. Currently available cell models include preadipocyte cell lines that are already committed to the adipocyte lineage and multipotent stem cells that are able to commit to different lineages, including adipocyte, muscle and bone (Armani et al. 2010). Multipotent stem cells used in adipogenesis assays include embryonic stem cells and adipose stromal cells (ASC) obtained from adipose tissue (Armani et al. 2010). ASC can be obtained from various species (including humans) and from different fat depots, which enables the study of differences between species and depots (Gregoire et al. 1998, Armani et al. 2010). ASC have been proved as a feasible source for adipose tissue engineering as they possess high proliferative and differentiation capacity (Girandon et al. 2011). However, due to the fact that ASC are primary cells, the heterogeneity of ASC may be a problem as well as cultures having a limited life span, which restrains their time window for experimental procedures (Gregoire et al. 1998, Scroyen et al. 2013).

In order to achieve maximal adipogenic differentiation *in vitro*, cell culture models treated with adipogenic cocktails, typically including supraphysiological concentrations of insulin, dexamethasone (DEX) and isobuthylmethylxanthine (IBMX) have been developed (Gregoire 2001, Armani et al. 2010, Ntambi and Young-Cheul 2000). Other adipogenic factors, such as indomethacin,

glucocorticoids, troglitazone and triiodothyronine, are also commonly used (Armani et al. 2010). Improved versions of induction medium have been developed for enhanced adipogenesis. Among those, Lequeux et al. (Lequeux et al. 2009) composed an improved adipogenic differentiation medium in which they replaced indomethacin by adding rosiglitazone, hydrocortisone and triiodothyronine to the basic adipogenic ingredients isobutyl-methylxanthine and dexamethasone. Also new ways of adipogenesis induction have been studied. We have previously developed a novel cell-free angiogenesis- and adipogenesis-inducing agent, adipose tissue extract (ATE), from mature human adipose tissue. ATE contains a wide number of promoters of e.g. adipogenesis and it has shown to induce dose-dependent adipogenesis and angiogenesis *in vitro* (Sarkanen et al. 2012a). ATE is produced from mature human adipose tissue without affecting cell viability, the final mixture containing a majority of growth factors, cytokines and chemokines present in mature adipose tissue (Sarkanen et al. 2012a). The advantage of ATE is that it can be used to induce natural adipogenesis in vitro and the majority of adipose stromal cells are committed towards adipocytes (Sarkanen et al. 2012a). However, due to the content of ATE, as seen in its dual role as adipogenesis and angiogenesis inducing agent, the differentiation may not be driven into fully differentiated adipocytes.

The aim of the present study was to find the most effective adipocyte *in vitro* differentiation protocol mimicking natural adipogenesis. The adipogenic effect of ATE was further studied and improved, and the additional components and combinations needed for optimal adipocyte differentiation and maturation were investigated. To assess the functionality of the differentiated adipocytes, their insulin responsiveness was monitored.

MATERIALS AND METHODS

This study conforms to the principles outlined in the Declaration of Helsinki. The human adipose tissue samples were obtained from waste material of surgical operations and human umbilical cords were received from caesarean sections with informed consents at Tampere University Hospital, Tampere, Finland. The use of human adipose stromal cells (hASC) was approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland with permit number of R03058,respectively.

Isolation and culture of human adipose stromal cells

Adipose stromal cells (hASC) were isolated from human adipose tissue by using mechanical and enzymatic procedure as described previously (Sarkanen et al. 2012b). Briefly, human adipose tissue specimens were mechanically cut into small pieces and enzymatically digested with 0.15 % collagenase I (Invitrogen, Paisley, Scotland, UK) in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM/F12, Gibco/ Invitrogen, Carlsbad, CA). The cells were tested for mycoplasma contamination (MycoAlert® Mycoplasma Detection Kit, Lonza Group Ltd, Basel, Switzerland) following the isolation and characterized for markers CD73, CD90 and CD105 (BD) with flow cytometer FACSCAnto II (BD) according to Huttala et al. 2015, before experimental use.

Adipose tissue extract

Adipose tissue extract (ATE) was produced as described previously (Sarkanen et al. 2012a) with slight modification to the ratio of medium to fat. Briefly, the human adipose tissue sample was manually cut into small pieces. The sample was then incubated in DMEM/F12 in ratio of 2:3 i.e. 2

parts manually dissected fat and 3 parts DMEM/F12, for 24 hours at 37 °C in 5 % CO_2 humified atmosphere. This was then centrifuged at 200 x g for 3 min and liquid was filtered through 0.2µm filter. To determine the protein content of the extract Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) was used according to manufacturer's instructions using BSA as a standard. Results were measured after 30 min incubation at 37 °C at 562 nm with Varioskan™ Flash Multimode Reader (Thermo Scientific). ATE was stored at -20 °C until used. Patches were not pooled.

Cell culture set up for differentiation protocols

To initiate adipogenic differentiation, hASCs were plated at passage 2 at a density of 20,000 cells/cm^2 in hASC medium (Table 1). The next day, different differentiation media (Table 1) were applied on hASCs according to scheme seen in Table 2. Troglitazone (Trogl, Sigma-Aldrich, St. Louis, MO) was used at concentration of 4 µg/ml (9.06µM). Combination of hASC medium and serum-free medium was used as the negative and ATE as positive control for adipocyte differentiation (Table 2). The culture time for all the treatments was 14 days.

Triglyceride accumulation

The triglyceride accumulation was determined as a relative amount of triglycerides per cell. This was done by first determining the relative cell number by using Cell Proliferation Reagent WST-1 (Roche Life Science, Indianapolis, IN) according to the manufacturer's instructions. Briefly, 50 µl /48-well plate well of WST-1 reagent was added in the culture medium on day 14. After 1.5h incubation at 37 °C 5 % CO_2 the absorbances were measured at 450 nm with Varioskan™ Flash Multimode Reader (Thermo Scientific). Accumulation of triglycerides was analyzed from the same wells from which the WST-1 analysis was done by using AdipoRed Assay reagent (Lonza, Basel, Switzerland) according to manufacturer's instructions. Briefly, at the end point of cultivations, the culture was washed with DPBS, Then AdipoRed reagent diluted with PBS was added on the plate and after 10 min incubation at room temperature, absorbances were measured with Varioskan™ Flash Multimode Reader (Thermo Scientific) with excitation at 485 nm and emission at 572 nm.

Morphological analysis

For the microscopic inspections, cultures were washed with DPBS and fixed using cold 70 % EtOH, left in DPBS and stored at +4 °C until imaging. Microscopic imaging was done with Nikon Eclipse Ti-S inverted fluorescence microscope (Nikon, Tokyo, Japan) and Nikon digital sight DS-U2 – camera (Nikon) and automated imaging with Cell-IQ (Chipman Tech., Tampere, Finland) with 10x objective and 5x5 grid. Confocal imaging was done with LSM710 and with Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany). Images were further processed with NIS Elements (Nikon, Tokyo, Japan), ZEN 2012 software (Carl Zeiss) and Adobe Photoshop CS3-software (Adobe Systems Incorporated, San Jose, CA).

RNA isolation and gene expression analysis

At day 14 the cell cultures where washed with PBS and total RNA isolated with PureLink® RNA Mini kit (Ambion/Life Technologies). Five-six wells/treatment where combined to form each total RNA sample. Samples were obtained from three independent cell culture experiments. Reverse transcription was carried out by using the VILO® kit (Invitrogen, Carlsbad, CA). Messenger RNAs were quantified by quantitative reverse transcriptase PCR (qPCR) with the gene-specific primer pairs for *PPARγ, adiponectin, AP2, leptin* and *Glut*, by using *36B4* (acidic ribosomal phosphoprotein) and

SDHA (succinate dehydrogenase complex, subunit A) as housekeeping references (primer sequences s. Electronic Supplementary Material, Table S1). Relative gene expression results were calculated by normalizing the Ct values with the geometric mean of the two housekeeping genes.

For the analysis of brown and white adipocyte-typical mRNAs primer pairs for *Prdm16, CIDEA, PGC1-α, UCP, RIP140, leptin and adenylate cyclase* (*ADCY5*) were used (s. Electronic Supplementary Material, Table S1). These were analyzed from two independent cell culture experiments; two cDNA syntheses and qPCR analyses (each in triplicate wells) were carried out from each (n=4). Relative gene expression levels were calculated by normalizing the Ct values with the geometric mean of the two housekeeping genes.

Assays for insulin responsiveness

Glucose uptake test

At day 14, DMEM/F12 (Gibco) was changed on the cells and incubated for 2 h at 37 °C 5 % CO_2. Cells were then exposed to 100 nM or 500 nM insulin and incubated at 37 °C 5 % CO_2 for 30 min and [^{3}H]-2-deoxy-D-glucose (0.2 µCi/well, Perkin Elmer, Waltham, MA) was added for another 20 min. The cells were washed twice with ice cold PBS and then lysed in 0.1 % sodium dodecyl sulfate (SDS). The radioactivity of the samples was measured by liquid scintillation counter Wallac1410 (Perkin Elmer) using OptiPhase HiSafe 2 oscillation liquid (Perkin Elmer). Total protein content of the samples was analyzed with Pierce™ BCA Protein Assay Kit (Thermo Scientific) as described above, and total protein content was used to normalize the results.

Inhibition of lipolysis

At day 14 the medium was removed, cells were washed with PBS (Gibco/Life Technologies) and DMEM/F12 (Gibco/Life Technologies) was added on the cells. After two hours, 2 µM Isoproterenol was added and incubated for 1.5 hours after which 100 nM or 500 nM insulin (Sigma-Aldrich) was added. After 15 min, 30 min or 45 min incubation medium was collected and the amount of glycerol released was assessed with EnzyChrom Adipolysis Assay Kit (BioAssay Systems, Hayward, CA) according to manufacturer's instructions. The fluorescence was measured with Varioskan™ Flash Multimode Reader (Thermo Scientific) at I_{ex} 530 nm and I_{em} 585 nm. Following the collection of the medium, cells were lysed with 0.1 % sodium dodecyl sulfate (SDS) and protein content was measured with BCA kit as described above. Protein content was used to normalize the results.

Statistical analysis

Statistical analyses were performed and graphs processed with GraphPadPrism 5.0 (GraphPad Software, Inc., San Diego, CA). The results concerning triglyceride accumulation were subjected to One-way ANOVA followed by Dunnett's post-test. The results concerning gene analysis, glucose uptake and lipolysis were subjected to two-way anova followed by Tukey post test. The results were reported as mean ± SD and differences were considered significant when $p<0.05$*, $p<0.01$**, $p<0.001$*** or $p<0.0001$****.

RESULTS

Effects of the differentiation protocols on triglyceride accumulation

Eleven different differentiation protocols (Table 2) for hASC were tested to find the most effective differentiation protocol for mature *in vivo* like adipocytes. The first phenotypic criterion assessed was triglyceride accumulation, as determined by AdipoRed staining at day 14. When comparing the accumulation of triglycerides per relative cell number, the cells grown by protocols 2, 6, 7 and 11 differed significantly from the undifferentiated cells (Table 2 and Fig. 1). Morphological examination of cultures stained with AdipoRed revealed that the pattern of lipid accumulation was morphologically most different between protocol 2 (ATE induction) and protocol 7 (3-day induction with ATE, 6 days in serum-free medium supplemented with 1.15 µM insulin and 9.06 µM Troglitazone, followed by 4 days in a defined serum- and insulin-free stimulation medium) (Fig. 1). ATE induced triglyceride accumulation with many small cytoplasmic lipid droplets in nearly all of the cells in the culture, but the amount of the accumulated lipid per cell was modest compared to protocol 7 cells. Protocol 7 induced accumulation of lipid in fewer cells than plain ATE induction but the size of the lipid storage in individual cell was greater. The outcome of protocol 6 resembled protocol 7, while that of protocol 11 resembled protocol 2 (Fig. 1).

Effect of differentiation protocols on gene expression

The expression of adipocyte marker mRNAs *PPARγ, adiponectin, leptin, AP2* and *Glut4* were studied in the differentiated cells at day 14. Adipose stromal cells differentiated with protocols 3, 6, 7, 10 and 11 showed the most elevated expression of the gene markers (s. Electronic Supplementary Material, Fig. S1). Based on the results of the triglyceride accumulation, three of the new protocols, protocols 6, 7 and 11, were chosen to a closer inspection at gene expression level (Fig. 2). Protocol 7 yielded consistently the highest and most even expression of *PPARγ*, *Glut4, AP2* and *adiponectin*. Expression of *leptin* mRNA in all cell specimens, except for those from protocol 11, remained low. Protocols 2 and 6 did not yield significant upregulation of the studied genes compared to undifferentiated control.

To investigate whether the tested adipocyte differentiation protocols selectively induce a brown or a white adipocyte-like phenotype, we performed qPCR analysis of mRNAs typical of brown adipocytes, *PRDM16, CIDEA, UCP1*, and *PGC1α*; and white adipocytes, *ADCY5*, *RIP140*, and *leptin* with protocols 2, 6, 7 and 11 with undifferentiated cells (protocol 1) as control (Fig. 3). The protocols 2, 6 and 7 resulted in elevated expression of the brown adipocyte markers *PRDM16*, *CIDEA*, and *UCP1*, while *PGC1α* expression was induced only under conditions 6 and 7. Of the white adipocyte markers, *ADCY5* was induced under conditions 6 and 7, while *RIP140* was only marginally affected. Of note, *leptin* was induced most prominently by protocol 11, but not by protocol 7.

Insulin responsiveness of the adipocytes

Glucose uptake

Glucose uptake by undifferentiated control cells and the most promising differentiation method, protocol 7 adipocytes was studied by employing [^{3}H]deoxyglucose (Fig.4). The cells were treated for 30 min with 100 or 500 nM insulin prior to the uptake assays. The differentiated cells showed a significant insulin-induced enhancement of glucose uptake at 100 nM concentration of insulin, when compared to the undifferentiated control.

Lipolysis

The ability of insulin to inhibit lipolysis in undifferentiated control cells and protocol 7 adipocytes was monitored by measuring glycerol released in the growth medium after exposure of the cells to 0, 100 or 500 nM insulin at time points of 15, 30 and 45 min (Fig. 4) at day 14. In the control cells, treatment with 500nM insulin enhanced, rather than inhibited, the release of glycerol, whereas a marked (50-80%) inhibition of lipolysis was evident in protocol 7 differentiated cells upon treatment with 500 nM insulin, at all the studied time points.

DISCUSSION

Differentiation of pre-adipocytes into adipocytes is defined by the acquisition of a lipid-filled morphology and appropriate hormone responsiveness, signaling pathways and metabolism (Novakofski 2004). In the last phase of adipocyte differentiation, preadipocytes transform into insulin-sensitive, lipid synthesizing and transporting mature adipocytes that secrete adipocyte-specific secretory products (Stephens 2012, Rosen and MacDougald 2006, Gregoire 2001, Gregoire et al. 1998). In order to develop a new, native-like human adipocyte *in vitro* model, eleven different *in vitro* differentiation protocols were studied using human adipose stromal cells (hASC) (Table 1 and Table 2). The outcome was analyzed by using triglyceride accumulation, morphology of lipid deposits, and adipocyte gene expression as criteria. Of the tested differentiation strategies, the most promising ('protocol 7') was chosen for further studies of insulin responsiveness. This protocol consisted of culture of the hASC (i) for three days in adipose tissue extract (ATE) medium, (ii) for six days in serum-free medium with 1,15 µM insulin and 9,06 µM Troglitazone, a thiazolidinedione (TZD) compound, and (iii) for 4 days in serum-free medium in the absence of insulin. The expression of adipocyte marker genes (*adiponectin, Glut4, AP2* and *PPARγ*) was significantly higher in the selected differentiation protocol 7 than in the other protocols tested. Moreover, the resulting adipocytes were responsive to insulin as judged from glucose uptake and inhibition of lipolysis at insulin concentrations of 100 or 500 nM, respectively. According to the criteria set for the differentiated adipocytes, protocol 7 produced mature native-like adipocytes.

In complicated 3D cell cultures, the major challenge is to create a culture environment that allows the growth and differentiation of multiple cell types. We have previously developed angiogenesis stimulation medium for creating mature vascular structures from adipose stromal cells and endothelial cells *in vitro* (Huttala et al. 2015). This stimulation medium (SM), consisting of 5.5 µM hydrocortisone, 0.1 nM 3,3',5-Triiodo-L-thyronine sodium salt (T3), 1.15 µM insulin, 1 % bovine serum albumin (BSA), 10 ng/ml vascular endothelial growth factor (VEGF) and 1 ng/ml fibroblast growth factor (FGF-2) as active components, was further used in the current study for adipogenesis induction and adipocyte maturation together with the adipose tissue extract (ATE) (Sarkanen et al. 2012a). ATE is a cell-free angiogenesis- and adipogenesis-inducing agent from human adipose tissue. ATE contains a majority of growth factors, cytokines and chemokines present in mature adipose tissue, e.g. leptin, adiponectin, FGF-2, IL-6, IGF-1, VEGF (-A), and angiogenin, among others, and has potential to induce natural adipogenesis *in vitro* (Sarkanen et al. 2012a). ATE has been shown to induce a homogenous adipogenic differentiation of hASC and to induce triglyceride accumulation comparable to treatment with commonly used adipogenic cocktail, with dexamethasone (DEX), insulin, indomethacin and isobutylmethylxanthine (IBMX) (Sarkanen et al. 2012a, Verseijden et al. 2009; Ghoniem et al. 2015, Foley et al. 2015, Lequeux et al. 2009, Rubin et al. 1978). ATE was

shown to be essential component for triggering adipogenesis, as any of the tested media did not induce adipogenesis on their own (data not shown). ATE seemed to cause cell commitment towards adipocytes, but also cell proliferation. Therefore, constant addition of ATE was not beneficial for adipogenesis. The explanation for this may come from the multiple cytokines in ATE contributing to the adipose stromal cell commitment (Sarkanen et al. 2012a). Due to the multiple factors in the plain ATE induction adipogenesis may not be the dominant event in cell culture, which was also seen in the current study as relatively small lipid droplet size and fairly low level of adipocyte marker gene expression. The balance of contradictory signals experienced by preadipocytes *in vivo* influence whether cells undergo adipogenesis (MacDougald and Mandrup 2002). It is known that e.g. macrophage-derived proinflammatory factors impair adipogenesis (MacDougald and Mandrup 2002, Lacasa et al. 2007).

Due to the improper maturation of adipocytes in plain ATE induction, this natural adipogenesis induction was modified and improved by adding 9.06 μM troglitazone in serum-free stimulation medium and depleting both insulin and troglitazone from culture for the last days of the culture period. Although the SM is serum-free, it contains albumin which acts *in vivo* as a carrier of fatty acids that is needed for their internalization into cells (Walker et al. 2014). This novel treatment, protocol 7, a combination of ATE and other adipogenesis stimulators, resulted in smaller percentage of cells accumulating triglyceride droplets than plain ATE induction, but the lipid accumulation per cell was greater, the differentiated cells resembled fully differentiated native adipocytes in morphology. Another tested protocol 11 (incubation for the entire 14 days in ATE + serum-free SM) also resulted in prominent triglyceride accumulation; however, gene expression analysis demonstrated under these conditions only induction of leptin and PPARγ, while the markers of more mature adipocytes, adiponectin and AP2, were not induced. Of note, protocol 11 produced the most homogenious lipid accumulation among cells and could thus represent a useful model for the study of the early stages of adipogenesis.

The new differentiation method ‘protocol 7’ included incubation of the cells with TZD compound Troglitazone and T3. TZDs are PPAR agonists, which stimulate adipogenesis and the redistribution of lipids from liver and muscle into adipose tissue (Greenberg and Obin 2006). Moreover, they activate AMPK, a central sensor for nutrient status, resulting in enhanced glucose uptake into adipose tissues and muscle (Fryer et al. 2002). TZDs have also been shown to decrease 11βHSD-1 and increase adiponectin (Greenberg 2003). Work on preadipocytes among human adipose derived stem cells or ones developed *in vitro* from other cell lineages suggests that integration of T3 and TZD signaling enhances the adipogenic differentiation potential (Ortega et al. 2009, Gerhold et al. 2002). T3, on its behalf, interacts with the two primary thyroid receptor isoforms, thyroid receptor a1 (TRa1) and its antagonist receptor a2 (TRa2) expressed in fat tissue. The binding of T3 to TRa1 induces adipogenesis while TRa2 negatively regulates the activity of T3 (Ortega et al. 2009). These observations provide plausible explanations for the observed beneficial effects of the Troglitazone and T3-containing media on the adipogenic differentiation in the new differentiation protocol.

The glucocorticoid hydrocortisone used in the SM has been shown to increase the secretion of leptin by cultured rat adipose tissue (Mick et al. 2000) and the synthetic glucocorticoid DEX is commonly used to induce adipogenesis as it increases the expression of c/EBP and PPARγ (Wu et al. 1996). Clinical cases of glucocorticoid excess are characterized by increased fat mass and obesity through

the accumulation of white adipocytes (Hochberg et al. 2015, Barclay et al. 2015). Hydrocortisone has also been shown to increase the secretion of leptin by cultured rat adipose tissue (Mick et al. 2000), however, this was not seen in our study. Previously Lequeux et al. (Lequeux et al. 2009) also composed an improved adipogenic differentiation medium which was further studied by Ghoniem et al (Ghoniem et al. 2015). Lequeux et al. replaced indomethacin with 1μM rosiglitazone (another TZD), 10nM hydrocortisone and 2 nM triiodothyronine (T3) and added the basic adipogenic ingredients 0.15 UI/ml insulin, IBMX and DEX. Our serum-free SM, which contains 5.5μM hydrocortisone, 0.1nM T3 and 9.06 μM TZD Troglitazone, is by parts similar to their differentiation medium. Although our concentrations are higher, we have no serum, IBMX and DEX, which could explain the need for higher concentrations of the active components we used.

Angiogenic factors are known promote also adipose tissue growth and expansion (Cao 2014). Angiogenic factors FGF2 (Xiao et al. 2010, Marra et al. 2008, Tabata et al. 2000) and VEGF (Girandon et al. 2011), among others, have been used when creating new adipocytes (Lowe et al. 2011). In addition, obesity is associated with elevated circulating levels of VEGF, particularly in the context of visceral adipose tissue expansion (Miyazawa-Hoshimoto et al. 2003, Fain et al. 2004). These findings prompted the use of VEGF and FGF2 in our SM medium. As ATE also contains several angiogenic inducers including VEGF and FGF-2 (Sarkanen et al. 2012a), it further enables the adipogenesis but could also be the reason for the lack of maturation in protocol 11 as large quantity of angiogenic factors may drive the differentiation towards angiogenesis hence preventing adipogenesis.

A number of well-studied genes are characteristically expressed during adipocyte differentiation or in mature adipocytes. Peroxisome proliferator-activated receptor γ (PPARγ) induces adipocyte differentiation by regulating several genes critical for adipogenesis, lipid uptake and lipid metabolism (Schoonjans et al. 1996, Rosen et al. 1999) and is regarded as a mid-differentiation marker (Flynn and Woodhouse 2008). Its induction was detected in most of the differentiation protocols tested in the present study, and thus proved that all the protocols were directing the cells towards adipogenesis. Expression of the glucose transporter Glut4 has been shown to rise in the terminal stage of differentiation (Gregoire et al. 1998). Glut4 is activated and built into the plasma membrane upon insulin stimulation (Berenguer et al. 2010), and effect is enhanced by TDZ rosiglitazone (Martinez et al. 2010). In the present study, a strong induction of Glut4 was observed only in protocol 7, suggesting that this protocol produces a somewhat more mature adipocyte phenotype than the related protocol 6 which lacks Troglitazone. The adipocyte hormone leptin is mainly produced and secreted by mature adipocytes (Gregoire 2001). Its main function is the regulation of the body energy balance (Coelho et al. 2013, Kershaw and Flier 2004, Gregoire 2001). Among the present adipogenesis schemes, leptin was prominently induced only in protocol 11 and mildly in protocol 2. One reason for low leptin expression could be a low energy supply in the serum-free medium at day 14. Both protocol 2 and 11 contain human serum in addition to the ATE. They may thus support the cellular energy status better than the other tested conditions, potentially resulting in an induction of leptin.

Adipocyte lipid binding protein 2 (AP2, also known as FABP4) on its behalf, is a cytoplasmic fatty acid chaperone expressed in adipocytes (Yang and Smith 2007) and considered to be a critical link between lipid metabolism, hormone action and cellular functions in adipocytes (Maeda et al. 2001). It triggers ubiquitination and subsequent proteosomal degradation of PPARγ in the terminal stage of

adipocyte differentiation (Yang and Smith 2007, Rodriguez et al. 2007, van Beek et al. 2007, Gregoire et al. 1998). AP2 was found to be elevated in the hASC based obesogen screening model (Foley et al. 2015), as well as in our new differentiation method, protocol 7. Another adipocyte marker representing terminal differentiation is adiponectin (Flynn and Woodhouse 2008). It is a hormone that decreases free fatty acids (FFA) in serum, glucose and triacylglycerol concentrations (Fruebis et al. 2001) and is secreted exclusively by adipose tissue (Kershaw and Flier 2004). Adiponectin regulates the energy balance of the body via activation of AMP-activated protein kinase (AMPK) in the hypothalamus (Coelho et al. 2013). Strong induction of both AP2 and adiponectin upon the present protocol 7 suggests that this differentiation protocol produced relatively mature, terminally differentiated adipocytes.

To investigate whether the adipocyte differentiation protocols 2, 6, 7 and 11 selectively induce a brown or a white adipocyte-like phenotype, we carried out qPCR analyses of mRNAs predominantly expressed in either one of these adipocyte types. As mRNAs abundant in brown adipocytes we employed *PRDM16, CIDEA, UCP1*, and *PGC1*α (Shinoda et al. 2015; Seale et al. 2007), and in white adipocytes *ADCY5, RIP140*, and *leptin* (Knigge et al. 2015; Sawada et al. 2010; Maffei et al. 1995; Zhang et al. 1994). The previously published ATE treatment (Sarkanen et al. 2012a) and conditions 6 and 7 resulted in elevated expression of the brown adipocyte markers *PRDM16, CIDEA*, and *UCP1*, while *PGC1α* expression was induced only under conditions 6 and 7. The induction of brown adipocyte-typical messages was most prominent with protocol 6. Of the white adipocyte-typical messages, *ADCY5* was induced under conditions 6 and 7, while RIP140 was only marginally affected. To conclude, none of the protocols tested selectively induces either the brown or the white adipocyte phenotype, but overall induction of adipocytic genes is observed.

The role of insulin in adipocyte differentiation is evident as the regulation of adipose tissue metabolism is one of the central physiologic functions of insulin (Cohen 2006). Insulin stimulates the uptake of amino acids and glucose into adipocytes and initiates signaling via effectors like phosphatidylinositol-3 kinase and AKT1/2 with central roles in adipogenesis (Kim and Chen 2004, Garofalo et al. 2003) and represses lipolysis in adipocytes (Watson and Pessin 2007, Choi et al. 2010b). It promotes the synthesis and storage of triglycerides and inhibits their catabolism (Rutkowski et al. 2015). When developing cell models for the mechanisms underlying adipose insulin sensitivity/resistance in metabolic disease, it would be essential to generate adipocyte cultures in which the insulin responsiveness characteristics of the donor tissue are preserved. Since adipocytes generally lose a lot of their insulin responsiveness in culture (Gerrits et al. 1993), this requirement forms a major challenge. In the present study we showed that ‘protocol 7’ adipocytes do respond to insulin in terms of two central parameters: glucose uptake (100 nM insulin) and inhibition of lipolysis (500 nM insulin), further supporting the view that this optimized protocol yields insulin-responsive adipocytes with patterns of lipid accumulation and gene expression resembling those of native human adipocytes. The lack of accelerated glucose uptake with 500 nM insulin (Fig. 4B) is most likely due to a negative feedback response to excessive insulin hyperstimulation (Ma et al. 2013). Further optimization of the differentiation protocol is therefore necessary. Moreover, the insulin responsiveness of adipocyte cultures could be increased by selecting well responsive hASC through a more profound characterization.

CONCLUSION

In this study, a novel adipocyte differentiation protocol mimicking natural adipogenesis was developed. The new protocol employed natural inducer of adipogenesis, adipose tissue extract, our previously developed serum-free angiogenesis stimulation medium and the thiazolidinedione Troglitazone. The results showed that the new protocol produces adipocytes which accumulate triglycerides, express general adipocyte marker mRNAs and respond to insulin as judged by glucose uptake and inhibition of lipolysis. Our qPCR analyses suggested that the protocol does not selectively induce either the brown or the white adipocyte phenotype. The cells showed no regression of adipocyte morphology and remained viable and attached to substratum for at least 10 weeks when the differentiation medium was removed.

A functional model for human adipogenesis is a desirable tool that would enormously benefit adipose tissue and obesity research. This can be achieved by use of well characterized cells of human origin and a differentiation protocol that produces mature and functional *in vivo*-like adipocytes. The new differentiation protocol developed in this study and the adipocyte model generated with it from hASC represent a crucial step towards this goal. By developing it further it will be possible to create accurate, reliable and efficient test systems for the research of obesity and metabolic disease.

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Conflict of interest statement

Patent issued in USA (WO2010026299A1), pending elsewhere.

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Figures

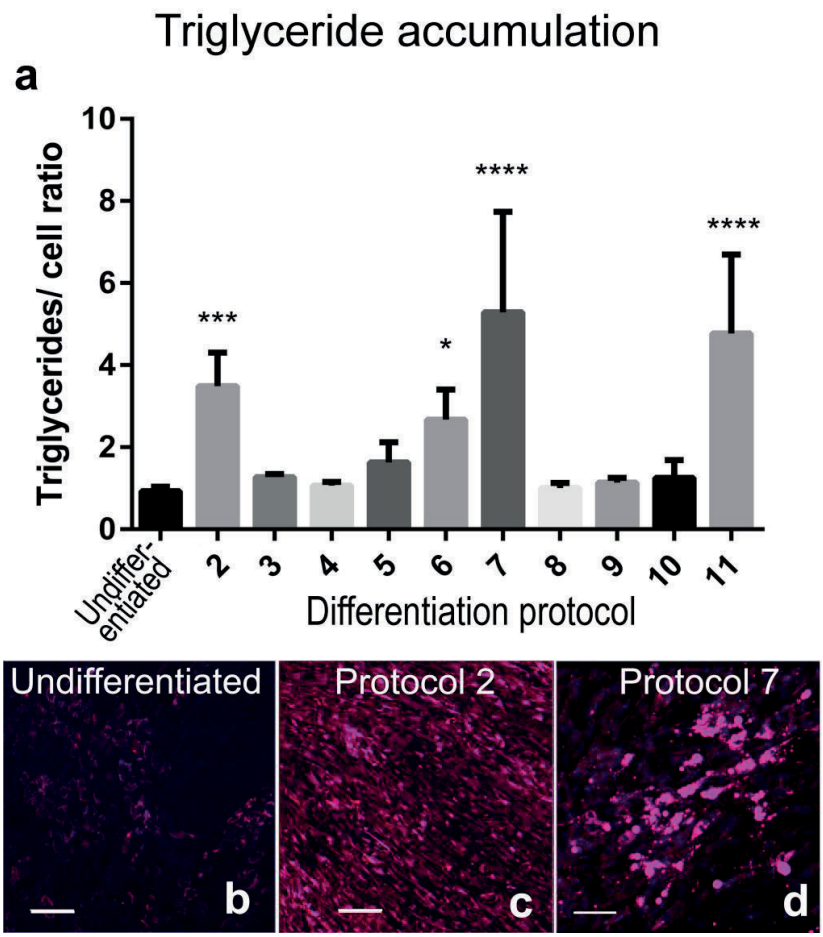


Fig. 1 Cellular lipid accumulation in the differentiation protocols tested. **a**) Bar graph shows mean ± SD for triglyceride accumulation in eleven different differentiation schemes. Protocol 2 (ATE induction), protocols 6, 7 and 11 showed a statistically significant difference when compared to the negative control (=undifferentiated cells) in one-way anova with Dunnett test, $n \geq 6$; *$p<0.05$; ***$p<0.001$; ****$p<0.0001$. In the confocal images **b**) the undifferentiated cells, **c**) adipocytes from ATE induction and **d**) protocol 7 adipocytes stained with AdipoRed and DAPI at day 14. Scale bar, 100 µm

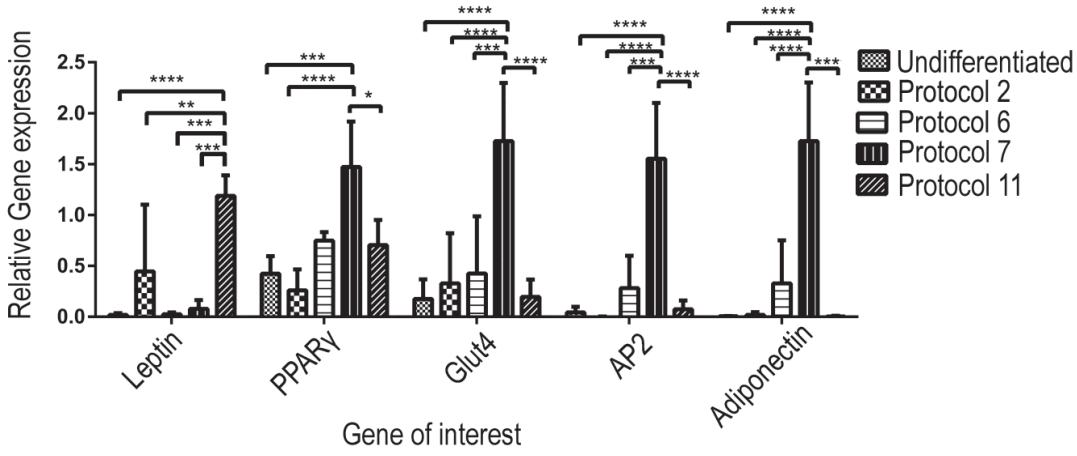


Fig. 2 Expression of *Leptin*, *PPARγ*, *Glut4*, *AP2* and *Adiponectin* mRNAs in adipocytes of selected protocols: undifferentiated control, protocol 2 (ATE induction), protocols 6, 7 and 11. Expressions of *PPARγ*, *Glut4*, *AP2* and *Adiponectin* were significantly higher in protocol 7 adipocytes than in other adipocytes. *Leptin* was expressed at the highest level in protocol 11 adipocytes. The bars represent mean ± SD. Statistics calculated with Two-way anova and Tukey test, n=5; *p<0.05;**p<0.01, ***p<0.001; ****p<0.0001

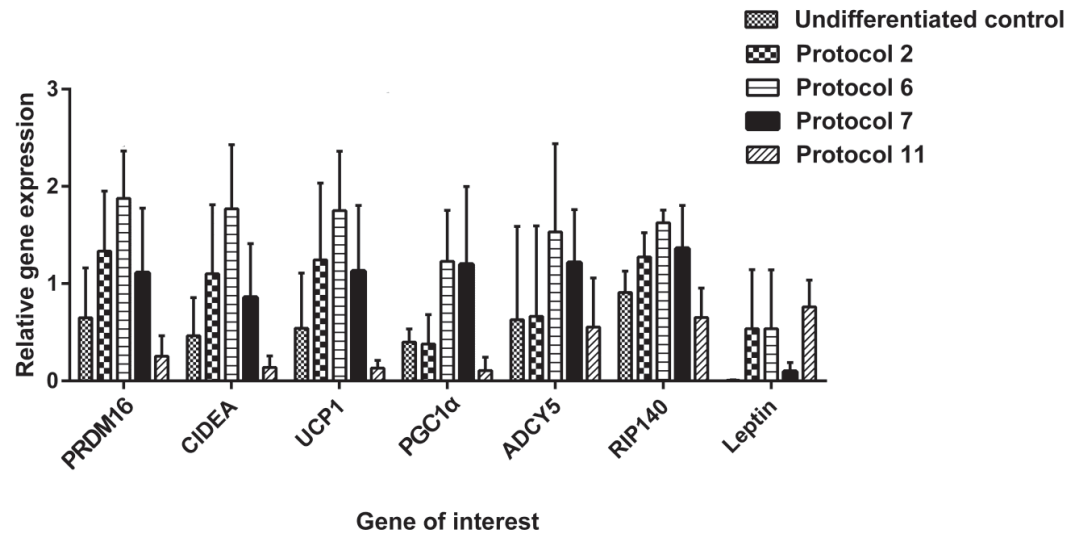


Fig. 3 Expression of brown (*PRDM16, CIDEA, UCP1, PGC1α*) or white (*ADCY5, RIP140*, and *leptin*) adipocyte-typical mRNAs in the adipocytes differentiated with protocols 2, 6, 7 and 11. The results are from two independent cell culture experiments, two cDNA syntheses and qPCR analyses (each in triplicate wells) carried out from each experiment (n=4). The bars represent mean ± SD. Statistics calculated with Two-way anova and Tukey test *p<0.05;**p<0.01

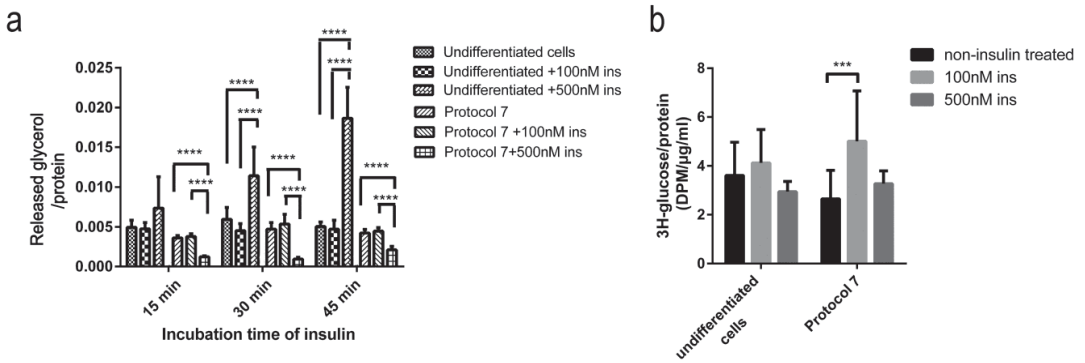
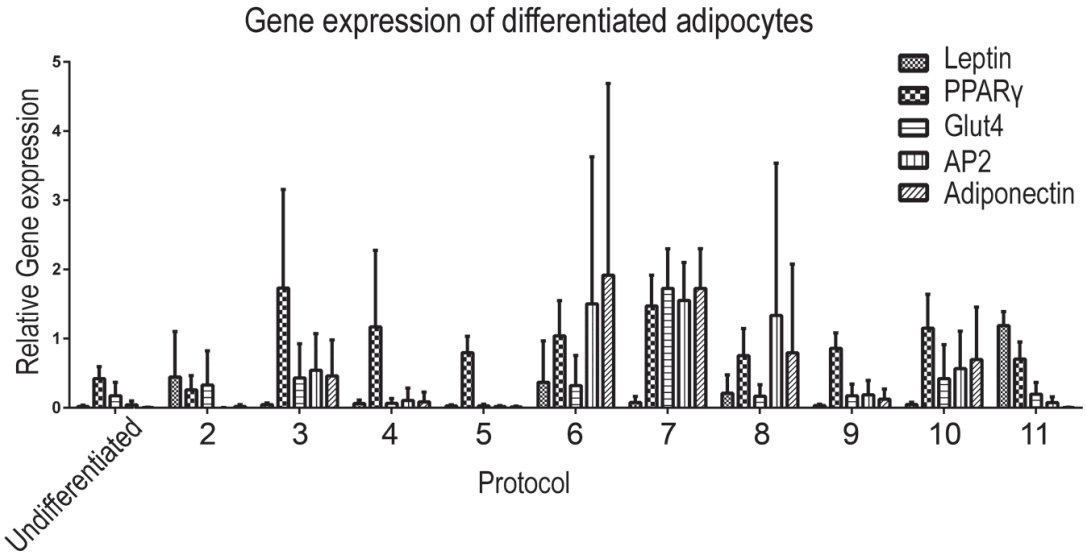


Fig. 4 Responses to 100 or 500 nM insulin at day 14 in undifferentiated control and protocol 7 adipocytes. **a**) Released glycerol per total protein after insulin exposure. The cells were incubated with or without insulin for 15, 30 or 45 min. Protocol 7 responded to insulin at all studied time points. **b**) Glucose uptake. Glucose uptake was significantly higher in 100 nM insulin treated protocol 7 adipocytes than in the adipocytes without insulin treatment. The bars represent mean ± SD. The results were analyzed by two-way anova with Tukey post test, $n \geq 6$; ***p<0.001, ****p<0.0001



Differentiation of human adipose stromal cells in culture into insulin sensitive adipocytes,
Cell and tissue research, Huttala O, Mysore R, Sarkanen JR, Heinonen T, Olkkonen VM, Ylikomi T.

Fig. S1 Expression of adipocyte marker mRNAs in the differentiated adipocytes. Expression of *adiponectin, Glut4, AP2, leptin* and *PPARγ* in adipocytes differentiated by 11 protocols in the study. The bars represent mean ± SD (n≥3). Statistical significances are not shown in the figure.

Table 1 Media used in the differentiation protocols, their abbreviations and content

	Medium	Abbreviation	Content	Manufacturer
1	hASC medium	hASC medium	DMEM/F12 10 % Human serum (HS) 2 mM L-glutamine (L-glut)	Gibco PAA Gibco
2	Serum-free medium	SFM	DMEM/F12 2,56 mM L-glut 0,1 nM 3,3',5-Triiodo-L-thyronine sodium salt (T3) ITS™ Premix: 6,65 µg/ml (1,15 µM) insulin 6,65 µg/ml Transferrin 6,65 ng/ml seleniuous acid 1 % Bovine serum albumin (BSA) 2.8 mM Sodium puryvate (NaP)	Gibco Gibco Sigma BD PAA Gibco
3	ATE medium	ATEm	1800 µg/ml Adipose tissue extract (ATE) DMEM/F12 10 % HS 2 mM L-glut 100 IU/ml Penicillin/0.1 mg/ml streptomycin	- Gibco PAA GIBCO GIBCO
4	Stimulation medium	SM	Serum free medium (See medium 2 above) supplemented with 200 µg/ml AA 0,5 µg/ml Heparin (HE) 5,5 µM: 2 µg/ml Hydrocortisone (HY) 10 ng/ml Vascular endothelial growth factor (VEGF) 1 ng/ml Fibroblast growth factor (FGF-β)	 Sigma Sigma Sigma R&D systems R&DSystems
5	Stimulation medium w/o insulin	SM -ins	Same as Stimulation medium except for ITS (BD) which has been replaced with: 6,65 µg/ml Transferrin 6,65 ng/ml Seleniuous acid	 Sigma Sigma
6	ATE+ASM	A+A	Stimulation medium ATE medium	

Table 2 Differentiation protocols; culture settings and medium changes

Protocol	Day0	Day1	Day 3-4	Day 6-7	Day 10	Day 14
1 (undifferentiated control)	Cell seeding in hASC medium on 48 well plate 20 000 cells/ cm^2	hASCm	SFM	SFM	SFM	End point analysis
2 (ATE induction)		ATEm	ATEm	ATEm	ATEm	
3		ATEm	SM	SM	SM	
4		ATEm	ATEm	SM	SM	
5		ATEm	SM	SM	SM-ins	
6		ATEm	SM -ins +9µM **Trogl**	SM -ins +9µM **Trogl**	SM -ins +9µM **Trogl**	
7		ATEm	SM +9µM **Trogl**	---- (no medium change)	SM -ins	
8		ATEm	ATE +9µM **Trogl**	SM	SM -ins	
9		ATEm	SM	SM-ins	SM -ins	
10		ATEm	SM	SM	SM -ins	
11		A+A	A+A	A+A	A+A	

Table S1 Primer sequences used in the quantitative PCR

Gene	Primer sequence
36B4	Forward- ATGCTCAACATCTCCCCCTTCTCC Reverse- GGGAAGGTGTAATCCGTCTCCACAG
ADCY5	Forward- TCTCCTGCACCAACATCGTG Reverse- CATGGCAACATGACGGGGA
Adiponectin	Forward- GGCCGTGATGGCAGAGAT Reverse- CCTTCAGCCCGGGTACT
AP2	Forward- GCTTTTGTAGGTACCTGGAAACTT Reverse- ACACTGATGATCATGTTAGGTTTGG
CIDEA	Forward- GATGCCCTCGTCATCGCTAC Reverse- GCGTGTTGTCTCCCAAGGTC
Glut4	Forward- TGGGCGGCATGATTTCCTC Reverse- GCCAGGACATTGTTGACCAC
Leptin	Forward- GCCCTATCTTTTCTATGTCC Reverse- TCTGTGGAGTAGCCTGAAG
PGC1-α	Forward- GCTTTCTGGGTGGACTCAAGT Reverse- GAGGGCAATCCGTCTTCATCC
PPARγ	Forward- GATCCAGTGGTTGCAGATTACAA Reverse- GAGGGAGTTGGAAGGCTCTTC
Prdm16	Forward- CGAGGCCCCTGTCTACATTC Reverse- GCTCCCATCCGAAGTCTGTC
RIP140	Forward- GGATCAGGTACTGCCGTTGAC Reverse- CTGGACCATTACTTTGACAGGTG
SDHA	Forward- CATGCTGCCGTGTTCCGTGTGGG Reverse- GGACAGGGTGTGCTTCCTCCAGTGCTCC
UCP1	Forward- CAATCACCGCTGTGGTAAAAAC Reverse- GTAGAGGCCGATCCTGAGAGA

PUBLICATION III

Development of versatile Human *In vitro* Vascularized Adipose Tissue Model with Serum-Free Angiogenesis and Natural Adipogenesis Induction

Huttala O, Palmroth M, Hemminki P, Toimela T, Heinonen T, Ylikomi T, Sarkanen JR

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Development of Versatile Human *In Vitro* Vascularized Adipose Tissue Model with Serum-Free Angiogenesis and Natural Adipogenesis Induction

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Abstract: Many adipose tissue-related diseases, such as obesity and type 2 diabetes, are worldwide epidemics. For studying these diseases, relevant human cell models are needed. In this study, we developed a vascularized adipose tissue model where human adipose stromal cells and human umbilical cord vein endothelial cells were cocultured with natural adipogenic and defined serum-free angiogenic media for 14 days. Several different protocols were compared to each other. The protocols varied in cell numbers and plating sequences. Lipid accumulation was studied with AdipoRed reagent, relative cell number with WST-1 reagent, gene expression of *glut4*, *leptin*, *aP2*, *adiponectin, PPARγ* and *PPARγ2* with RT-qPCR. Secretion of adiponectin, leptin and aP2 was analysed with ELISA. The immunostained vascular network was imaged with Cell-IQ and area quantified using ImageJ. In this study, both angiogenesis and adipogenesis were successfully induced. Protocols produced strong lipid accumulation, good vascular network formation and induced adipocyte-specific protein secretion and expression of studied adipocyte genes. Results showed that cell numbers and cell plating sequences are important factors when aiming at *in vitro* standardized tissue model. Presence of mature vasculature appeared leads to faster the maturation of adipocytes judged by the lipid accumulation and gene expression results. The developed vascularized adipose tissue model is simple to use, easily modifiable to suit various applications and as such, a promising new tool for adipose tissue research when, for example, studying the effect of different cell types on adipose tissue function or for mechanistic studies.

Interest in studying adipose tissue and its functions is growing along with the growing prevalence of obesity and the many adipose tissue-related diseases, such as type 2 diabetes mellitus, which is already a worldwide epidemics [1]. Many experimental animal models have been developed to study diabetes [2], but due to species-specific differences, the results from the animal models, although they increase the understanding of the mechanisms behind diseases and adverse effects, are weakly transferable to human situations [2–4]. Also, due to new regulations which promote and demand replacement of animal experiments, more human biology mimicking *in vitro* experiment models is needed. Examples of such as are Directive 2010/63/EU [5], the cosmetics regulations [6] and the EU chemicals legislation REACH [7].

More than being the energy storage, adipose tissue is an active endocrine organ, which is particular closely associated with vascular system. Enlargement of the adipose tissue can be supported by new blood vessel formation, neovascularization, or by dilating and remodelling of the already existing capillaries [8]. Mouse studies have shown that induction of vessel expansion in obese mice counteracts obesity and related metabolic complications [9] but also contrary results have been obtained as inhibition of vessel formation by an anti-angiogenic drug decreased fat pad weights of normal mice by 12–22% and decreased their bodyweights in a dose-dependent and reversible manner [10].

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Two different angiogenesis triggers in *in vivo* adipose tissue expansion have been proposed: (i) angiogenesis is a response to hypoxia, which is caused by the enlargement of adipocytes and/or proliferation of adipocytes; or (ii) angiogenesis is a result of developmental and/or metabolic signals in adipose tissue; that is, blood vessels develop in parallel or before the adipose tissue expansion [11]. There are several studies supporting the role of hypoxia [11–13]. However, in foetal development, angiogenesis/vasculogenesis precedes adipogenesis and blood vessel extracellular matrix (ECM) develops before adipose tissue ECM development [14]. The reciprocal interaction between ECM stroma and vascular network has also been shown to be important in directing vessel growth [15–17].

Current human cell *in vitro* vascularized adipose tissue models are based on (i) culturing human adipose stromal cells (hASC) and human umbilical cord vein endothelial cells (HUVEC) on a scaffold such as porous silk protein scaffold [18] or (ii) culturing human pre-adipocytes with human endothelial cells in a fibrin glue matrix on a chick chorioallantoic membrane [19,20] or (iii) by adding multiple different cell types into the culture [21]. While these models are mimicking vascularized adipose tissue, they are complex, require long culture times and are laborious to be transferred into routine use. In addition, the additional components such as biomaterial scaffolds may interfere with the cell–cell interactions or cause unwanted and unknown interactions between material and the studied cells or chemicals [22–24].

The aim of this study was to develop a robust human vascularized adipose tissue model without artificial scaffold. Such model could be utilized in basic adipose tissue research, for drug discovery and safety studies, and it could also be modified to disease model, which could be used in the studies of disease pathogenesis, development of intervention therapeutics and screening of personalized therapeutics. In the model, we used hASC and HUVEC to form coculture in which adipogenesis was induced by the novel natural adipogenesis inducer adipose tissue-derived extract (ATE) [25] and angiogenesis by our previously developed defined serum-free angiogenesis medium [26]. The defined serum-free angiogenesis medium produces mature vascular structures *in vitro* [26], and this medium together with ATE differentiates hASC to insulin-sensitive adipocytes [27]. In this work, we compared several protocols to find the most optimal for formation of vascularized adipose tissue bearing in mind the use of the model as a routine standard model. The formed vascularized adipose tissue was characterized for morphology, lipid accumulation, secretion of adiponectin, leptin and adipocyte protein 2 (aP2, also known as fatty acid-binding protein 4) and the mRNA expression of *glut4*, *leptin*, *aP2*, *adiponectin, PPARγ* and *PPARγ2*. The findings of this article present two protocols, which are easy-to-use, adjustable for different applications and relevant for culturing a vascularized adipose tissue cell model.

Materials and Methods

Development strategy. Six different protocols (table 1) were compared to each other for their capability to create vascularized adipose tissue. The studied protocols had following variables: cell number, cell plating time-point (on day 1 or on two separated days, i.e. on days 1 and 7) and the differentiation process (adipogenesis induced prior to angiogenesis or vice versa). Adipogenesis was induced with the ATE and angiogenesis with the defined serum-free angiogenesis medium. Adipogenesis is induced with ATE medium and angiogenesis with angiogenesis medium. Three types of controls were used (table 1) negative control with no induction, vasculature control in which only angiogenesis was induced and adipocyte control in which only adipogenesis was induced.

Isolation and culture of human adipose stromal cells and umbilical cord vein endothelial cells. The human adipose tissue samples and umbilical cords were obtained from Tampere University Hospital, Tampere, Finland, with individual written informed consent. The use of ATE, hASC and HUVEC was approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland, with permit numbers R03058 and R08028, respectively.

Human adipose stromal cells were isolated using collagenase I (Gibco, Carlsbad, CA, USA) as described previously [26,28]. HUVEC were isolated by cannulating the umbilical cord vein and infusing the vein with collagenase I (Gibco) and cultured in endothelial cell growth medium (EGM-2; Lonza, Basel, Switzerland) as described earlier [26,28]. Possible mycoplasma contamination was tested with MycoAlert® Detection Kit (Lonza) from both cells before cryopreservation.

Human adipose stromal cells-HUVEC coculture plating and differentiation protocols to form vascularized adipose tissue. In order to find out the optimal plating time for hASC and HUVEC with respect to the differentiation of the cells into adipocytes and blood vessels, six different protocols were compared to each other (table 1). On day 0, hASC (P1) were seeded in endothelial cell growth medium (Lonza) at 20,000 cells/cm^2 or 40,000 cm^2 on 48-well Nunclon™ Δ-Surface (Sigma-Aldrich, Saint Louis, MO, USA) plates. In some protocols, there was addition of hASC on day 7 (table 1) in 50 µl of medium without changing the medium in the well. HUVEC (p3) were seeded on top of hASC in 50 µl at 4000 cells/cm^2 either on day 0 or on day 7 depending on the plating strategy (table 1). Three different hASC-HUVEC combinations were used, and all strategies were tested with all of the hASC-HUVEC combinations.

Human adipose stromal cells-HUVEC cocultures were exposed to adipogenic ATE medium [25] or angiogenesis medium [26] on day 1. ATE was prepared as described earlier [27]. Composition of all media used in the study is listed in table 2. Vasculature control was exposed only to angiogenesis medium, adipocyte control only to ATE medium and negative control (undifferentiated cells) to serum-free medium (table 1). Differentiation media were changed on days 4, 8 and 11.

Analysis of the coculture. All analyses of the cultures were performed on day 14.

Analysis of secreted proteins. Media samples were analysed for their concentration of adiponectin, leptin and aP2 with Quantikine ELISA (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. Absorbance was measured at 450 and 540 nm.

Relative cell number. The relative cell number was defined using cell proliferation reagent WST-1 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instruction by incubating the reagent for 1 hr and measured at 450 nm by multipoint measurement.

Lipid accumulation. The lipid accumulation was determined using AdipoRed assay reagent (Lonza) according to the manufacturer's instructions from the same wells from which the WST-1 staining was performed. The fluorescence was measured by multipoint measurement with Varioskan Flash Multimode Reader (Thermo Fischer Scientific, Waltham, MA, USA) with excitation at 485 nm and emission at 572 nm.

Immunocytochemical stainings. To visualize and analyse the formed vasculature, immunocytochemical stainings were performed. For this, the same cell cultures, which were used for AdipoRed and WST-1 measurements, were stained.

Cells were fixed with 4% formaldehyde (Thermo Fisher Scientific) at RT for 20 min. and treated with 0.5% Triton X-100 (MP Biochemicals, Santa Ana, CA, USA) at RT for 15 min. and with 10% bovine serum albumin (BSA; Roche Diagnostics) at RT for 30 min. Primary antibodies, von Willebrand factor IgG (anti-vWf IgG produced in Rabbit, #F3520, Sigma-Aldrich; 1:100) and anti-collagen IV IgG (anti-ColIV IgG produced in mouse, #C1926, Sigma-Aldrich, 1:500), diluted in 1% BSA in DPBS with Ca and Mg (Lonza) were incubated for 1 hr at RT or overnight at +4°C. Cells were then incubated for 40 min. at RT with secondary antibodies diluted in 1% BSA in DPBS, FITC-labelled goat polyclonal antibody antimouse IgG (Sigma-Aldrich, 1:100) and TRITC-labelled goat polyclonal antibody anti-rabbit IgG (Sigma-Aldrich, 1:50).

Microscopic analyses. Vasculature and lipid accumulation were imaged with Nikon Eclipse Ti-S inverted fluorescence microscope (Nikon, Tokyo, Japan) and Nikon Digital Sight DS-U2 camera (Nikon). Images were processed with NIS Elements (Nikon) and Adobe Photoshop CS3 software (Adobe Systems Incorporated, San Jose, CA, USA).

Table 1.
Plating and differentiation details of different protocols tested on hASC-HUVEC cocultures.

Protocol name	hASC plating day(s)	Number of hASC/well	HUVEC plating day	Number of HUVEC/well	Medium on days 1–7	Medium on days 8–14
P1	Day 0	22,000 cells	Day 7	4400 cells	ATE medium	Angiogenesis medium
	Day 7	22,000 cells				
P2	Day 0	22,000 cells	Day 0	4400 cells	Angiogenesis medium	ATE medium
	Day 7	22,000 cells				
P3	Day 0	44,000 cells	Day 0	4400 cells	ATE medium	Angiogenesis medium
P4	Day 0	44,000 cells	Day 0	4400 cells	Angiogenesis medium	ATE medium
P5	Day 0	22,000 cells	Day 0	4400 cells	ATE medium	Angiogenesis medium
P6	Day 0	22,000 cells	Day 0	4400 cells	Angiogenesis medium	ATE medium
Negative control	Day 0	44,000 cells	Day 0	4400 cells	Serum-free Basic medium	Serum-free Basic medium
Vasculature control	Day 0	22,000 cells	Day 0	4400 cells	Angiogenesis medium	Angiogenesis medium
Adipocyte control	Day 0	22,000 cells	–	–	ATE medium	ATE medium

ATE, adipose tissue-derived extract; hASC, human adipose stromal cells; HUVEC, human umbilical cord vein endothelial cells.

Table 2.
Composition of differentiation media used in the study.

Medium	Components	Manufacturer
ATE medium	DMEM/F-12	Gibco
	1800 μg/ml ATE	
	10% Human serum	PAA Laboratories (Pasching, Austria)
	2 mM L-Glutamine	Gibco
	50 units/μl Penicillin-50 μg/μl streptomycin	Gibco
Serum-free Basic edium	DMEM/F-12	Gibco
	1% BSA	Biosera (Boussens, France)
	2.8 mM Sodium puryvate	Gibco
	2.56 mM L-Glutamine	Gibco
	ITS supplement:	BD Biosciences (NJ, USA)
	6.65 μg/ml Insulin	
	6.65 μg/ml Transferrin	
	6.65 ng/ml Selenious	
	0.1 nM 3,3,5-Triiodo-L-thyronine sodium salt	Sigma-Aldrich (MO, USA)
Angiogenesis medium	Serum-free medium	
	200 μg/ml Ascorbic acid	Sigma-Aldrich
	2 μg/ml Hydrocortisone:	Sigma-Aldrich
	1 ng/ml FGF-β	R&D Systems
	10 ng/ml VEGF	R&D Systems
	0.5 μg/ml Heparin	Sigma-Aldrich

ATE, adipose tissue-derived extract; BSA, bovine serum albumin.

Quantification of vasculature. To determine the area of vasculature, immunostained cells were imaged with Cell-IQ (CM Technologies Oy, Tampere, Finland) with 10× objective with grid of 5 × 5. Images were stitched together with Cell-IQ Analyzer (CM Technologies Oy) and further analysed with ImageJ software (The National Institutes of Health, MD, USA). Images were converted to 8-bit grey scale, and the background was subtracted. Then, binary threshold function was adjusted to separate the tubules from background staining. The total tubule area was calculated as the total number of pixels in images with a set threshold. Results were plotted in GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA). n was 12 for negative control, adipocyte control, vasculature control and P1 and P5. For protocols 2 and 6, n was 5. Results are depicted as mean ± standard deviations.

Gene expression. The cells were lysed with the lysis buffer from PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) so that parallel sample cells from five to eight wells were lysed into 600 μl of the lysis buffer and combined as one sample. Isolation was continued according to the instructions of the PureLink RNA Minikit (Life Technologies). The 260/280 purity ratios were measured with μPlate (Thermo Fischer) and Varioskan Flash Multimode Reader (Thermo Fischer). Genomic DNA contaminations were eliminated with PureLink DNase treatment (Life Technologies). The quality of RNA samples was checked using QIAxcel RNA QC kit v2.0 (Qiagen, Venlo, the Netherlands) and QIAxcel Advanced (Qiagen). cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the protocol of the manufacture. cDNAs were then preserved at −80°C until RT-qPCR.

qPCR to study mRNA expression of *PPARγ, PPARγ2, Adiponectin, Leptin, aP2* and *Glut4* was performed with CFX96 Real-Time System (Bio-Rad) and with iQ™ SYBR® Green Supermix (Bio-Rad). These gene markers were chosen according to our previous study [27] and to represent markers of different stages of adipocyte differentiation. The specificity of the primer sequences was tested with NCBI/Primer-BLAST. Primer concentration was 300 nM, and amount of template was 30 ng. Thermal cycling conditions were as follows: 95°C 3 min., 95°C 10 sec., 51–65°C (gradient) 15 sec., 72°C 30 sec., repeated 40 times. Melt curve analysis was performed at 55–95°C (0.5°C increment/10 sec.). Housekeeping genes and adipocyte marker genes used in this study are listed in table 3. The quality of amplified DNA was studied with gel electrophoresis using high sensitivity DNA analysis kit (Agilent Technologies, Santa Clara, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions.

Data handling and statistical analyses.

Quantification of lipid accumulation. To achieve a lipids/cell value, normalized AdipoRed assay absorbance values were divided with mean WST-1 assay absorbance values for the protocol in question. Normalization and AdipoRed WST-1 ratio calculations were made with Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). n was 12.

Quantification of secreted leptin, aP2 and adiponectin. From the ELISA analysis, protein concentration results were obtained by subtracting the 540 nm reading from the 450 nm absorbance values, creating the standard curve and calculating the concentrations of the studied proteins. These results were then divided by the relative cell number, that is WST-1 results. In ELISA samples, adipocyte control and P2 and P6 contain cytokine-rich ATE and hence the protein concentrations are compared to each other. Similarly, negative control, P1 and P5 were compared to each other. n was 3.

RT-qPCR result quantification. qPCR data were analysed using CFX96 Real-Time System software (Bio-Rad). The relative quantification of mRNA expression data was calculated using ΔCt method with Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) with the following equation:

$$2^{(\mathrm{Ct(mean\ of\ housekeeping\ genes\ SDHA\ and\ 36B4,\ studied\ protocol)}\ -\ \mathrm{Ct(Gene\ of\ interest,\ studied\ protocol))}} \Big/ 2^{(\mathrm{Ct(mean\ of\ housekeeping\ genesSDHA\ and\ 36B4,\ control)}\ -\ \mathrm{Ct(Gene\ of\ interest,\ control))}}$$

For the expression analysis, adipocyte control was used as control. Four biological replicates of each strategy were analysed (n = 4).

Statistics. All results were plotted, and statistical analyses were performed with GraphPad Prism (GraphPad Software Inc.). Results are depicted as mean ± standard deviation. Results from lipid accumulation, Cell-IQ image analysis and RT-qPCR were subjected to one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Results for protein secretion were subjected to one-way analysis of variance (ANOVA), followed by Sidak's multiple comparison test. Differences were considered significant when $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

Practical considerations.

Cell detachment occurred when high number of cells was plated at once on the same day, that is in protocols P3 and P4 (table 1). Due to this issue, those protocols were omitted from further analyses even though both produced dense tubule network and evenly distributed AdipoRed staining. All other protocols (P1, P2, P5 and P6) were analysed further. When the same number of cells was plated on two separate days (P1 and P2), that is half on day 1 and other half on day 7, no detachment was observed from the culture vessel during the 14-day culture. However, P2 and P6, in which angiogenesis was induced prior to adipogenesis, were found to detach from the culture vessel easier than P1 and P5 during handling the cultures.

Lipid accumulation.

Cultures were stained with AdipoRed lipid dye on day 14. The lipid accumulation was strong and evenly distributed throughout the culture in all studied protocols (P1, P2, P5 and P6), including the adipocyte control (fig. 1). Negative control and vasculature control showed only slight staining with AdipoRed (fig. 1).

Lipid accumulation was quantified by calculating the ratio between the normalized AdipoRed values and WST-1 values giving relative lipids/cell value (fig. 2). Adipocyte control ($p = 0.0021$) and protocol P6 ($p = 0.0008$) accumulated significantly more lipids than negative control. P5 accumulated significantly less lipids than adipocyte control ($p = 0.0253$). P6 accumulated significantly more lipids than P2 ($p = 0.0455$) and P5 ($p = 0.0091$).

Formation of vasculature.

Vasculature was visualized with immunocytochemical stainings and imaged, and the area covered by tubules was quantified. Figure 1 presents the morphology of vasculature for protocols P1, P2, P5 and P6. Branched vascular networks were obtained in vasculature control as well as in all protocols but were not observed in the negative control or in the adipocyte control. The networks were more dense and more branched when they had 14 days to mature (in vasculature control, as well as in P2 and P6) compared to those in P1 and P5, which had 7 days to differentiate (table 1). The stainings showed intact outer basement membrane (collagen IV) and inner endothelial layer (vWf) in all of the samples (fig. 1). The width of the formed vascular structures in tubule control and in different strategies did not differ markedly from each other as observed with microscope.

Table 3.

Studied genes and the primer sequences used.

Gene name	Abbreviation	Primer sequence (5′–3′)	Function
Succinate dehydrogenase complex, subunit A	SDHA	F: CATGCTGCCGTGTTCCGTGTGGG R: GGACAGGGTGTGCTTCCTCCAGTGCTCC	Used as a housekeeping gene in this study
Acidic ribosomal phosphoprotein P0	36B4	F: ATGCTCAACATCTCCCCCTTCTCC R: GGGAAGGTGTAATCCGTCTCCACAG	Used as a housekeeping gene in this study
Leptin	–	F: GCCCTATCTTTTCTATGTCC R: TCTGTGGAGTAGCCTGAAG	Adipose tissue secretory product [37]
Adiponectin	–	F: GGCCGTGATGGCAGAGAT R: CCTTCAGCCCGGGTACT	Adipose tissue secretory product [37]
Adipocyte protein 2 (fatty acid-binding protein 4)	AP2	F: GCTTTTGTAGGTACCTGGAAACTT R: ACACTGATGATCATGTTAGGTTTGG	Carrier protein for fatty acids [38]
Glucose transporter type 4	Glut4	F: TGGGCGGCATGATTTCCTC R: GCCAGGACATTGTTGACCAC	Insulin-responsive glucose transporter [47]
Peroxisome proliferator-activated receptor γ	PPARγ	F: GATCCAGTGGTTGCAGATTACAA R: GAGGGAGTTGGAAGGCTCTTC	Transcription factor in adipogenesis [48]
Peroxisome proliferator-activated receptor γ variant 2	PPARγ2	F: CAGTGTGAATTACAGCAAACC R: ACAGTGTATCAGTGAAGGAAT	Transcription factor in adipogenesis, adipocyte-specific variant [49]

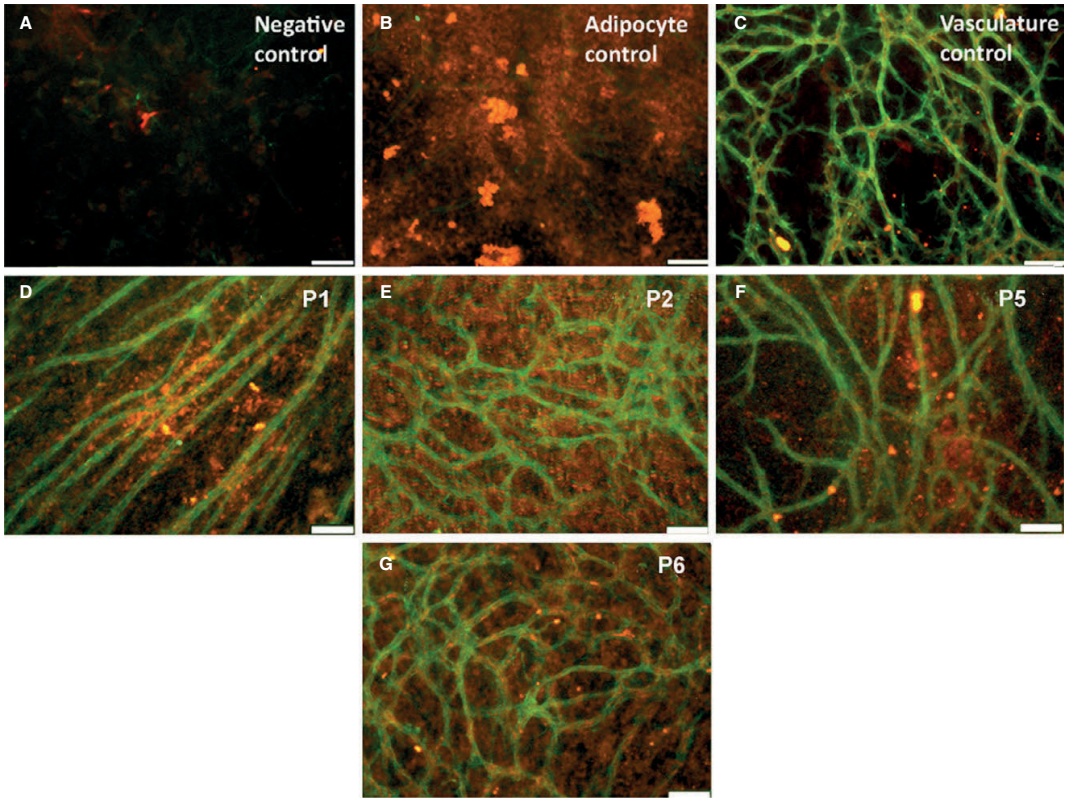

Fig. 1. Morphology of the vascularized adipose tissue models on day 14. Lipids are seen in orange/red; tubules are stained with anti-vWF-TRITC (red) and anti-ColIV-FITC (green). (A) Negative control. (B) Adipocyte control with strong AdipoRed staining. (C) Vasculature control with strong tubule network. (D) P1 produced tubules but only few branches. AdipoRed staining showed strong lipid staining within the cells. (E) P2 produced well-branched tubule network and evenly spread AdipoRed staining. (F) P5 produced dense tubule network, but it was less dense than with (G) the protocol 6. Both P5 and P6 show good AdipoRed staining. Images are obtained with Nikon Eclipse Ti-S inverted fluorescence microscope and with 10× objective, scale bar is 100 μm.

The area of the vascular networks was quantified from Cell-IQ images with ImageJ (fig. 2). Out of the studied protocols (P1, P2, P5 and P6; table 1), P1 was the only studied protocol which produced significantly less tubules than vasculature control ($p = 0.0012$). However, P1 also contained adequate vascular network as shown in fig. 1, although not as extensive as in other protocols.

Secretion of adipocyte-specific proteins.

ELISA was performed for the quantification of adipocyte-specific proteins in the samples collected from the culture media on day 14 (fig. 3). Medium of adipocyte control, P2 and P6 contained cytokine-rich ATE, and hence, these medium samples were compared to each other. Samples from P1 and P5 did not contain ATE, and they were compared to the negative control, which contained the same basal medium as the protocols did.

Secretion of adiponectin was significantly increased in P6 compared to adipocyte control and P2 ($p = 0.0062$ and $p = 0.0011$, respectively). Also aP2 secretion was significantly increased in protocol P6 compared to adipocyte control and P2 ($p = 0.0024$ and $p = 0.0002$, respectively). Significantly more leptin was secreted in P1 ($p = 0.0001$) and P5 ($p < 0.0001$) than in the negative control. Leptin secretion was also significantly higher in protocol P6 than in P2 ($p = 0.0150$).

The adipocyte-specific mRNA expression in the formed adipose tissue.

Expression of marker genes *PPARγ, PPARγ2, aP2, adiponectin*, *leptin* and *Glut4* was investigated on day 14. *PPARγ, PPARγ2, leptin, aP2* and *glut4* were shown to be expressed in P1, P2, P5 and P6, and there was no significant difference in the expression between the studied protocols and adipocyte control (fig. 4). *Adiponectin* expression was significantly higher in P1 adipocytes when compared to adipocyte control ($p = 0.0115$) or P5 ($p = 0.0357$). The expression of *PPARγ* and *PPARγ2* showed a trend that when general *PPARγ* was expressed at high level, *PPARγ2* was expressed at low level

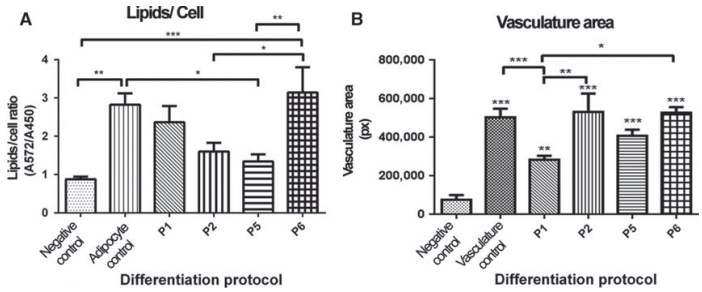

Fig. 2. Lipid accumulation and area of vasculature in the vascularized adipose tissue models on day 14. (A) Adipocyte control and all protocols show lipid accumulation; P5 was the only one, which accumulated significantly less lipids than the adipocyte control. (B) All protocols produced dense tubular network except P1, which formed significantly less tubules compared to the vasculature control, P2 and P6. The bars represent mean ± S.D. n was 12 except for P5 and P6 for which the n was 5 due to detachment. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

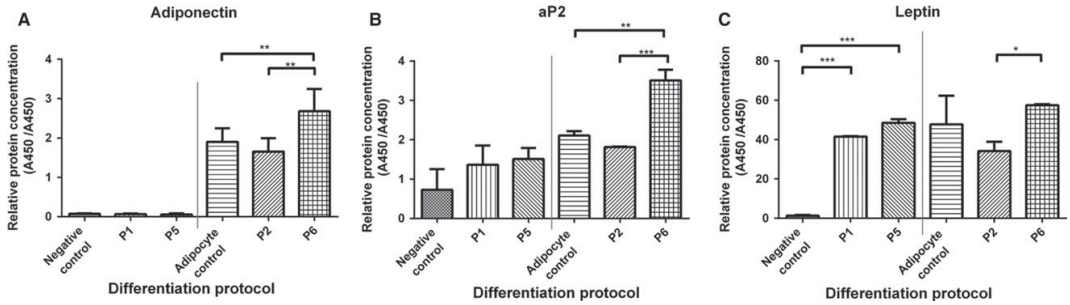

Fig. 3. Protein secretion of adipocytes in the studied protocols. Adipocyte control and P2 and P6 contain cytokine-rich adipose tissue-derived extract, and hence, their results were compared to each another whereas negative control, P1 and P5 were compared to each other. (A) Secretion of adiponectin was significantly increased in P6 compared to adipocyte control and P2. (B) aP2 was secreted significantly more in P6 than in the adipocyte control and P2. (C) Secretion of leptin was significantly higher in P1 and P5 than in the negative control, and also higher in P6 than P2. The bars represent mean ± S.D. For lipid accumulation, n was 15, and for ELISA, n was 3. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

and vice versa. The gene expression results (fig. 4) are given as relative expression using adipocyte control as control.

Discussion

In this study, we developed a human relevant vascularized adipose tissue model aiming at standardized artificial scaffold-free test for routine use. The used human ATE induces adipogenesis in hASC culture [25], and the vascular network was constructed as published earlier by Huttala *et al.* [26]. ATE has been found to be a superior inducer of adipogenesis over the commonly used chemical induction cocktail [25]. The vascular network, induced by serum-free angiogenesis medium, has also been characterized earlier and found to produce optimal, *in vivo* like vascular structures [26]. Six different protocols were studied and characterized in this study. They were evaluated by their reproducibility and their ability to induce adipocyte-specific marker expression in the cultures. Simultaneously, the effect of the vasculature on the adipogenesis was assessed.

Adipogenesis and angiogenesis were both successfully induced, although differences were seen between the protocols in triglyceride accumulation and in density of vascular networks. The results showed that dense vasculature which existed before start of adipogenesis induction helped the pre-adipocytes mature faster. Different cell plating and differentiation protocols had a notable effect on the resulting cell model and its technical repeatability. The most optimal protocols based on triglyceride accumulated, sufficient vascular network formation and relevant adipocyte-specific genes and protein expression for building the vascularized adipose tissue model were P1 and P6 (table 1). These protocols are scaffold-free, and they are simpler and demand shorter culture time than other existing vascularized adipose tissue models [18–21]. Due to the simplicity of these two protocols, two cells and two induction medias, they can also be easily modified to suit the various applications, for example effect of third cell type on adipose tissue metabolism.

Vasculature formation in different protocols.

Dense vascular networks were induced with all protocols, and the vasculature has been shown to be mature in 7 days [26]. Apart from the angiogenesis induced with the stimulation

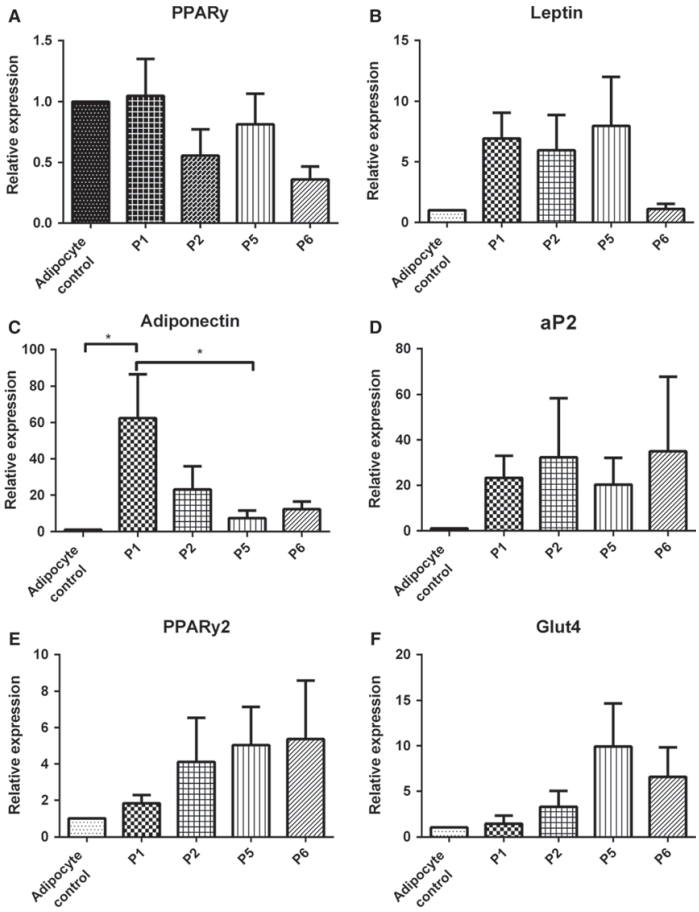

Fig. 4. The expression of adipose tissue specific gene markers in the studied protocols. (A) *PPARγ,* (B) *leptin,* (C) *adiponectin,* (D) *aP2,* (E) *PPARγ2,* (F) *Glut4*. P1 showed significantly increased the expression of *adiponectin* compared to adipocyte control and P5. The bars represent mean with S.D. n was 4, $*p < 0.05$.

medium, the adipogenesis inductor, ATE, has also been shown to induce angiogenesis [25]. Thus, the ATE treatment allows further maturation of blood vessels while inducing adipogenesis in the model. In other published vascularized adipose tissue models, adipogenic cocktail treatments, for example insulin, IBMX, biotin, pantothenate, DEX and thiazolidinediones, have been proposed to have a negative effect on endothelial cells and delay the growth of HUVEC [18,29].

In this study, the cocultures where angiogenesis structure was induced first (P2 and P6) tended to detach easier than the ones where adipogenesis was induced first (P1 and P5). This problem could be addressed by coating the culture vessels. However, the addition of external materials can cause unwanted and unknown interactions between the studied cells or chemicals assembly [23].

Effect of vasculature on adipocyte differentiation.

Based on the commonly used adipogenesis markers *PPARγ, PPARγ2, leptin, adiponectin, Glut4* and *aP2* [27,30,31], adipogenic differentiation of hASC occurred in all protocols. In addition, the gene expression results suggested that vasculature has a positive effect in adipocyte differentiation. There was a clear trend of higher expression of all the studied gene markers in the vascularized adipose model compared to the adipocyte control even though statistically significant difference was found only in adiponectin expression in protocol P1. The role of ECM produced by the hASC and the formed vascular structures in the culture is one important part of the model as it offers attachment places and support for differentiation of adipocytes [21]. The hASC and HUVEC vascular network has already been shown to produce ECM and collagen IV

containing basement membrane [26], and its role in adipogenesis should be studied further.

PPARγ expression is considered a general marker for ongoing adipogenesis and thus should be found in the adipocyte models [29,32]. *PPARγ2* is more adipocyte-specific isoform of *PPARγ* [33,34], and it is transcriptionally regulated by nutrition [33,35]. *PPARγ2* has been found to be important for the storage of lipids in adipocytes in adipose tissue instead of other organs, and hence, its proper expression is highly important in obesity [36]. Our results support this and the finding of Robciuc *et al.* [9] because the adipocytes in P6, which had extensive vasculature prior to adipogenesis induction, had strong expression of *PPARγ2* (fig. 4), and also the size of the lipid storage per cell was largest of the studied protocols (fig. 2). This is an interesting finding and should be studied further.

Leptin and adiponectin, the most abundant secretory products of adipose tissue [37], are typically investigated when studying the secretory properties of the formed adipose tissue [30]. In our models, the expression of *leptin* was elevated implying that functional adipose tissue was formed. P1 adipocytes, which showed strong lipid accumulation, were also secreting leptin, indicating the saturated state of the adipocytes.

aP2 (also known as fatty acid-binding protein 4) regulates the lipid trafficking and response in cells [38]. Along with *aP2,* the glucose transporter Glut4 has been connected to the terminal stage of differentiation in 3T3-L1 cells [32]. As angiogenesis medium includes insulin, which increases glucose influx through Glut4 and represses lipolysis in adipocytes [39,40], it is possible that the combination of ATE and angiogenesis media treatments enhances adipose tissue maturation more than just ATE treatment in adipocyte control. Activated endothelial cells in angiogenic vessels are known to produce various cytokines and growth factors that promote adipose tissue growth and expansion [41].

According to our findings, 1 week of adipogenesis induction was enough to produce mature adipocytes if there was a dense and mature blood vessel network present prior to induction of adipogenesis; that is, vasculature was enabling a faster differentiation of adipocytes. This was seen from the gene expression results, which did not differ significantly regardless of whether the adipocytes had 2 weeks (P1 and P5) or 1 week (P2 and P6) to mature in the culture. The positive effect of vasculature on adipose tissue has also been shown in mouse models by others [9,10], and hence, it should be studied further to reveal the underlying mechanisms of the interaction of vasculature and adipocytes. This model provides a useful tool for studying these mechanisms and the role of vasculature on the adipocyte differentiation.

Technical aspects in model development.

When developing a multicellular tissue model with relatively thick structures, gaining adequate and reproducible cell adherence might be difficult. This essential feature largely defines the applicability and repeatability of the otherwise functional model. According to our results, plating density had the strongest impact on the adherence of the cell model.

Foetal calf serum is the standard component of the present test models although it might have unpredicted effects during the culture. In addition to the lot-to-lot variation, proteins in foetal serum may bind studied compounds and affect the results obtained [42]. Therefore, the ultimate goal is to use serum-free medium whenever possible. In our model, the defined serum-free medium is present for 7 days in the culture. This makes the model less vulnerable to batch variation and other unknown protein interactions.

Cells of human origin should be used when studying human effects especially when metabolism of test substances might play a role [43–45]. However, only a few human cell-based adipose tissue models have been developed so far [46]. Primary human cells utilized in this model might contain lot-to-lot variation and thus cause difficulties in standardization. However, the variation can be minimized by preset quality control procedures which includes verification of sufficient expression of prominent markers for each cell type used, for example CD73, CD90 and CD105 for hASC [26] and morphological monitoring of the cells.

Conclusions

As obesity is a severe and growing global problem, there is an urgent need for well-characterized, functional and relevant human *in vitro* models, which could be used in safety testing of chemicals, in drug development as well as in biomedical research of healthy and disease modelling tissue. Also new legislations, such as REACH, are increasing the demand of *in vitro* models. This study provides new information on combining two components, vasculature and adipocytes, in culture and led to the development of novel *in vitro* model for adipose tissue studies. Both angiogenesis and adipogenesis were successfully induced in the coculture with the protocols used in this study. Presence of mature vasculature showed to be beneficial; it appears to lead to further maturation of pre-adipocytes and adipocytes. The underlying mechanisms of this interaction should be studied further as they might provide a target for therapeutic strategies.

The next step is to show with a set of reference chemicals the applicability of the model for chemical testing on adipogenesis, but even now, the model is ready to be used for adipose tissue research when, for example, studying the effect of different cell types on adipose tissue function or for mechanistic studies.

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Conflict of Interest

Sarkanen JR and Ylikomi T have a patent issued in USA (US 20110151005 A1) and pending application (WO20 10026299A1). Other authors have no conflict of interest to declare.

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PUBLICATION IV

Presence of vasculature results in faster insulin response in adipocytes in vascularized adipose tissue model

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