Adipose Tissue Engineering

Development of Volume-Stable 3-Dimensional Constructs and Approaches Towards Effective Vascularization

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In Dankbarkeit an meine Familie.

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Introduction

1.1 BACKGROUND

Upon temporary illness or injury, the human organism can draw to a wide-ranging endogenous repair system constituted by cells and bioactive components. This system is overstrained when whole organs or tissues fail. Even today, the therapeutic options are scarce owing to the shortage of transplantable organs and replacement tissues [1], as well as the limited reparative capacity of prosthetics or implant materials [2-4]. A vast supply of vital organ and tissue substitutes would thus revolutionize clinical treatment.

Early in the tissue engineering era, this vision dominated both scientific discourse and press releases, praising advances in the field as 'the greatest scientific achievements of the 20th century' [5,6]. As an interdisciplinary research area, tissue engineering combines fundamentals of engineering and biosciences towards the development of biological substitutes that restore, maintain and improve organ and tissue function [2,7,8]. While considerable progress has been made, the impact of tissue engineering in the clinic has been moderate until today [4], and the envisioned off-the-shelf substitute body parts and engineered organs [9,10] have and will in the near future not become reality. Instead, traditional tissue engineering has merged with the field of regenerative medicine, focusing on tissue- and organ-specific regeneration and repair rather than whole organ replacement.

Especially for tissue regeneration, advances in stem cell biology and material sciences fuel on-going research and provide valuable therapeutic impulses. As a subfield, adipose tissue engineering aims at the development of adipose tissue substitutes to cure soft tissue defects resulting from trauma, injury or disease. To provide innovative solutions for the successful restoration of such ailments, adipose tissue engineering approaches integrate in-depth knowledge on the structural and functional traits of fat tissue, mechanisms of cellular response and crosstalk, bioinduction and growth factors, as well as biomaterial design and cell-substrate interactions.

1.2 ADIPOSE TISSUE

Adipose tissue regulates metabolism and energy homeostasis in the human body [11,12]. It is an abundant loose connective tissue, which can, in response to nutritional status, store excess energy by accumulation of triglycerides (TGs) as well as release free fatty acids (FFAs) in time of caloric need to ensure muscle, liver and other tissue function [13-15].

Fat tissue is ubiquitously distributed throughout the body to mechanically protect inner organs and limbs, and to prevent heat loss via the skin surface [16]. Subcutaneous depots are found primarily in abdominal and gluteal areas, whereas intraabdominal reservoirs include omental, retroperitoneal and visceral fat [15,17-19]. In adults, the total body fat mass ranges from 2 to 3% in trained athletes to up to 70% in obese individuals [20]. The average amount of adipose tissue mass is gender-dependent, with healthy males having between 10 and 20%, and females approximately 20 to 30% of body fat [18]. Apart from localization, adipose tissue depots are distinguishable by differences in metabolic activity, for example lipolysis and fatty acid synthesis, as well as secretory profile [15,21]. These depot-specific variations in tissue function are consistent with different degrees of metabolic disease risk associated with tissue expansion at the respective sites [22].

Adipose tissue exists in two major forms: white adipose tissue (WAT), which is commonly referred to as ‘adipose tissue’, and brown adipose tissue (BAT) [23,24]. BAT is characterized by its profound vascularity and high density of mitochondria, and functions as a heat source to the body via mitochondrial uncoupling [13,25]. Larger BAT depots are restricted to neonates, as with age, they are continuously replaced by WAT [25,26]. More recent work, however, has identified distinct BAT deposits in adults [27-30] and its metabolic superiority to WAT has raised interest in the reconversion of WAT into BAT as potential therapeutic intervention in obesity [31-34]. Despite this renaissance of BAT research, the present work attends solely to WAT, which remains the primary focus of current investigations on adipose tissue and tissue engineering approaches thereof.

1.2.1 Adipose tissue structure

Mature adipocytes, which store triglycerides in a unilocular lipid vacuole that takes up most of the cells' cytoplasm, are the main constituent of adipose tissue volume [24,35]. The stromal compartment hosts endothelial cells, fibroblasts, smooth muscle cells, macrophages, pericytes, committed preadipocytes and multipotent mesenchymal stem cells (MSCs) [13,35-39]. Histologically, the tissue unit is composed of densely packed adipocytes alongside with stromal cells, nerve tissue and a dense microvascular system, which are structurally and mechanically supported by a tissue-specific extracellular matrix (ECM) [23,40] (Figure 1).

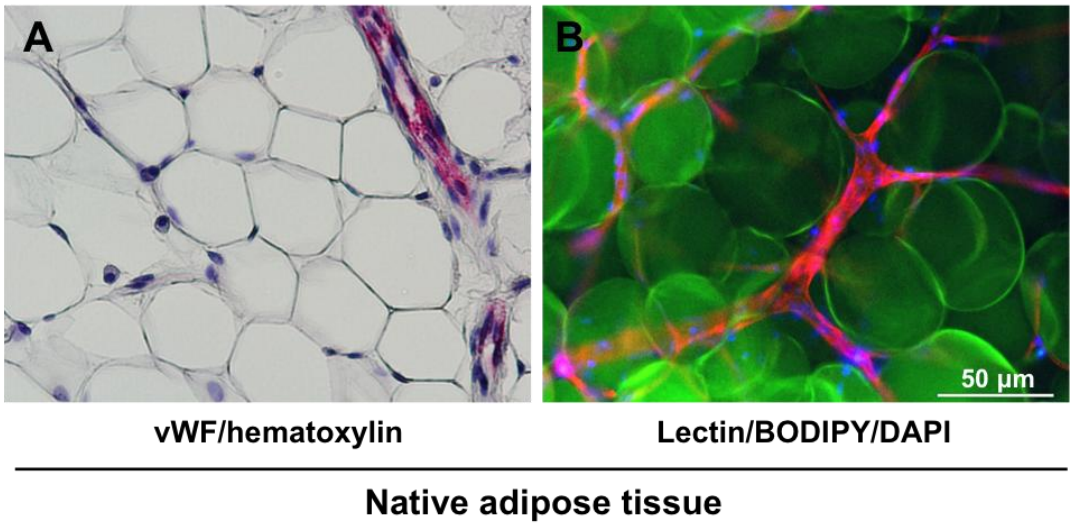

Figure 1. Histological appearance of adipose tissue. Cross-sectioned adipose tissue showing the characteristic hexagonal structure of mature adipocytes and arterioles stained against human von Willebrand factor (vWF; red); nuclei are stained blue with hematoxylin (A). 3D whole mount staining of adipocytes (BODIPY; green), capillaries (lectin; red), and nuclei (DAPI; blue) (B). Scale bar represents 50 µm.

Adipose tissue exhibits a profound functional plasticity with the ability to dynamically expand and regress throughout the entire life span [15,41,42]. Prolonged positive energy balance results in hypertrophic expansion of mature adipocytes and eventually, reaching the obese state, in hyperplastic growth of preadipocytes, which can be recruited from the stroma [12,18,43]. Upon maturation, fibroblastic preadipocytes aquire the characteristic, rounded signet ring morphology resulting from the formation of the lipid vacuole [35]. Cellular dimensions therewith change dramatically, mirrored by an increase in size from an initial diameter of 20 µm to maximally 200 µm [18]. In periods of excess lipid storage, adipocytes

expand to up to four times their normal size, whereas rapid energy turnover during starvation can almost deplete the body's fat reserves [18].

This plasticity is closely interlinked with dynamic changes of the adipose tissue vasculature [42,44] and the underlying ECM [45,46]. Almost each adipocyte is in direct contact with a capillary [15,42,47-50], implying that expansion of adipose tissue is accompanied by that of its vasculature, whereas weight loss is associated with the regression of blood vessels [15]. Angiogenesis, as the sprouting or branching of capillaries from existing blood vessels [51,52], is thus centrally involved in adipose tissue growth [15,21,50,53,54]. The crucial relevance of an adequate blood supply to nurture adipose tissue is also mirrored by the fact that during development, adipogenesis and angiogenesis are temporally and spatially coupled [42,54]. Similarly, the adipose ECM is subject to constant dynamic remodeling and changes in composition. This involves the on-going assembly and disassembly of major ECM components including collagen fibers, laminins and fibronectin [46,55-57], as well as their reorganization by a series of constructive and degrading mechanisms, of which many are mediated by ECM-processing enzymes such as matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) or fibrinolytic substances [46,58,59]. Accordingly, having long been solely attributed a scaffolding function, the ECM has evolved as a pivotal tissue component associated with the control of cell morphology, proliferation and differentiation, as well as global tissue homeostasis and function [60].

1.2.2 Adipose tissue function

Adipose tissue is not only a global regulator of energy reserves, but also of far-reaching physiological processes [61-63]. To fulfill this role, it is involved in a wide range of autocrine, paracrine and endocrine mechanisms and the production of soluble effectors thereof [11,61,64].

The discovery of leptin in 1994 first hinted towards the endocrine role of adipose tissue and implicated its participation in signaling pathways and feedback loops along the hypothalamus-pituitary-axis [65]. Since then, adipose has increasingly been acknowledged a secretory and endocrine organ [16,40,66] with central systemic relevance in glucose balance, lipid metabolism, inflammation, immune response, angiogenesis and vascular function as well as reproductive processes [20,63,67-70]. This is reflected by the secretion

of a wide array of specific bioactive proteins, so-called adipokines, the production of steroid hormones, prostaglandins and prostacyclins, complement factors and other soluble mediators [40,66,71]. Leptin and adiponectin are the most prominent adipokines, likewise adipose tissue secretes among others resistin, visfatin, apelin, tumor necrosis factor alpha (TNF-α), acylation stimulating protein (ASP), plasminogen activator inhibitor 1 (PAI-1) and a variety of matrix components as well as matrix remodeling proteins [18,61,72].

On account of the multifaceted physiological processes guided by adipose tissue, its dysfunction is directly related with the pathogenesis of obesity and type 2 diabetes mellitus [11,14,40,73]. Obesity in particular, is often accompanied by a cluster of comorbidities like hyperglycemia, dyslipidemia, hypertension and insulin resistance, known as the metabolic syndrome [74,75]. Other pathological states related to dysfunctional adipose tissue are chronic inflammation, atherosclerosis and cardiovascular complications [11,14,73,76,77].

Through leptin for example, adipocytes are involved in the regulation of insulin signaling and food intake, thus disruption of leptin signaling is regarded a key event in the development of obesity [40,78,79]. Leptin exerts its impact on energy homeostasis primarily via hypothalamic pathways [78,80] and its secretion corresponds with the proportion of adipose tissue mass [40,44]. Accordingly, obesity is characterized by elevated circulating levels of leptin [40,81,82]. Besides its metabolic effects as a satiety homone, leptin influences steroid production and insulin action in the liver, and is involved in immune function, hematopoiesis, bone development and notably, angiogenesis [40,83,84].

In contrast to leptin, adiponectin secretion correlates negatively with body weight and nutritional status, and is attributed anti-diabetic, anti-inflammatory and anti-artherogenic effects [40,85]. An inverse association between adiponectin levels and both insulin resistance and inflammatory states has been established [86-88], accordingly, low adiponectin levels in obesity enhance and amplify related comorbidities [44]. Physiologically, adiponectin enhances insulin sensitivity in the liver and is involved in glucose use and fatty acid oxidation in muscle tissue [40,88].

Other adipose-associated factors include TNF-α as potent mediator of inflammation, angiogenesis and adipogenesis [15], and further, angiotensinogen and PAI-1, which mainly occupy vasoregulatory functions [89-91]. TNF-α is secreted by both, adipocytes and

stromal-vascular cells [92] and affects insulin resistance in diabetes and obesity via inhibition of insulin receptor signaling [61,76,93-96]. Also being attributed a role in obesity, angiotensin II increases lipogenesis in adipocytes and thereby regulates lipid synthesis and storage [97], whereas high levels of PAI-1, a protein associated with the fibrinolytic system, are presumably involved in the development of obesity-related cardiovascular pathologies [15,98,99]. For a detailed description of angiogenesis-related actions of PAI-1 and various other prominent angiogenic modulators, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), the reader is referred to section 1.2.4 Adipose tissue angiogenesis.

Overall, since adipose tissue evidently links between energy homeostasis and other major physiological systems, it is relevant to acquire a comprehensive understanding of this endocrine organ as a cohesive unit. Thus, to fully restore adipose tissue, not only basic structure should be considered but also the interaction between the individual cellular and molecular components that centrally determine adipose function.

1.2.3 Adipocyte differentiation

Stem cell commitment to the adipose lineage and subsequent differentiation occur during embryonic development as well as in adult adipose tissue [100,101]. Despite the substantial progress in defining the sequence of events during adipogenesis, as of yet, there is only ambiguous information regarding the regenerative niche from which adipose MSCs and progenitor cells are recruited *in vivo* [31].

Originially found in bone marrow stroma, MSCs exist in a variety of adult tissues, and can, in response to appropriate extrinsic and intrinsic developmental cues, adopt lineage-specific properties of diverse cell types [102,103]. Accordingly, adipose tissue contains a population of multipotent stem cells that are biologically similar but not identical to bone marrow-derived MSCs (BM-MSCs). Termed adipose-derived stem cells (ASCs), these cells feature self-renewal capacity and multilineage differentiation potential [24,47,104]. Lineage-specific stimulation of ASCs gives rise to adipocytes, osteoblasts, chondrocytes, myocytes and various other cell types [105,106]. This multidifferentiation capacity together with the ability to self-renew and proliferate, relative abundancy and the potential for autologous transplantation, render ASCs profoundly advantageous for applications in tissue engineering and the treatment of human degenerative diseases [107-110].

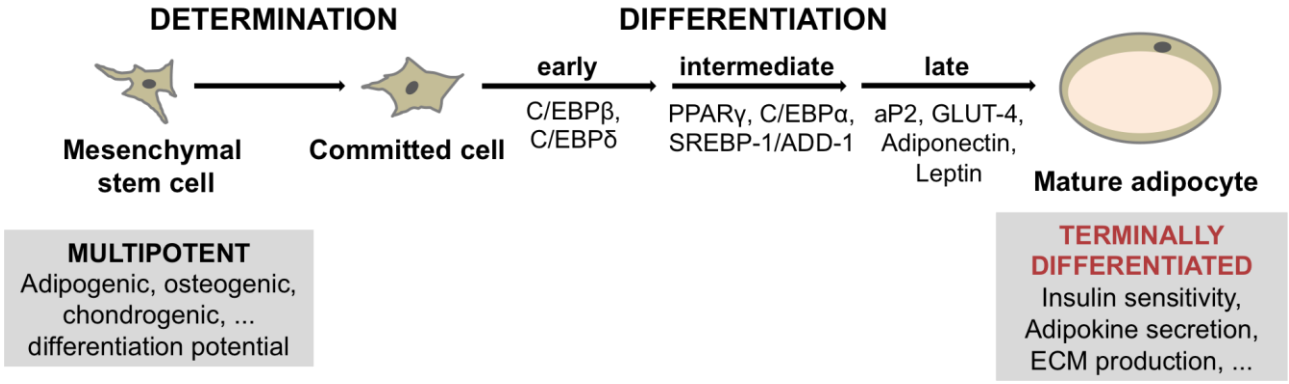

Figure 2. Adipogenic development. During the determination step, multipotent stem cells commit to the adipocyte lineage. To subsequently reach the terminally differentiated state, the committed cells proceed through early, intermediate and late differentiation, characterized by serial activation of a network of transcription factors and adipocyte-specific genes. (PPARγ: peroxisome proliferator-activated receptor gamma; C/EBPα/β/δ: CCAAT-enhancer-binding protein alpha/beta/delta; SREBP-1/ADD-1: sterol regulatory element binding protein 1/adipocyte determination and differentiation-dependent factor 1; aP2: adipocyte protein 2; GLUT-4: glucose transporter 4; Adapted in part from [12,111,112].)

Microenvironmental cues comprised of integrated stimulatory and inhibitory signals from soluble factors, cell-cell and cell-matrix interactions as well as pH and local oxygen tension have the ability to initiate and direct adipogenesis [24,61,113]. On the molecular level, adipogenesis is controlled via the concerted activation of a series of complex signaling cascades and the coordinated transcription of adipocyte-specific factors [14,113-116].

The adipogenic differentiation program is divided into two main steps (Figure 2). First, during determination, multipotent MSCs become restricted to the adipogenic lineage [14] and lose the capacity to differentiate into other cell types [24,68,113,117]. Accumulating evidence suggests that the process of determination is regulated by a network of signaling pathways, targeting the promoters of lineage-specific transcription factors [68,115,116]. Here, members of the bone morphogenic protein (BMP) family, such as BMP-2 and BMP-4 [118-120], as well as Wnt [121,122] and Hedgehog (Hh) signaling [123,124] have been attributed modulatory function. The second, terminal differentiation step involves the expression of specific genes responsible for the development of functional and phenotypic properties of mature adipocytes [113,117]. This comprises the acquisition of insulin and hormone sensitivity, the formation of a unilocular lipid vacuole by coalescence of pre-formed smaller droplets, the production of tissue-specific ECM, adipokine production and secretory function [111-113].

Detailed knowledge on the signaling events that underlie adipogenesis, especially during terminal differentiation, is derived from studies using established cell lines or primary preadipocytes [61,113,114]. Central regulators have been identified *in vitro*, mainly employing the two most commonly used cell lines, 3T3-L1 and 3T3-F422A, which are derived from murine Swiss 3T3 fibroblasts [61,103,114,125].

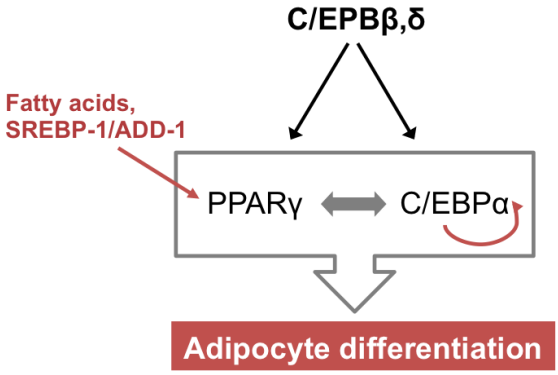

Figure 3. Transcriptional events underlying adipogenesis. C/EBPβ and C/EBPδ stimulate C/EBPα and the master regulator of adipogenesis PPARγ. Fatty acids and other endogenous ligands also transactivate PPARγ. Full differentiation is maintained by a positive feedback loop between PPARγ and C/EBPα, the concomitant expression of SREBP-1/ADD-1 and by autoactivation of C/EBPα. (Adapted in part from [12,61].)

At the center of transcriptional events underlying adipocyte differentiation (Figure 3) stand members of the CCAAT-enhancer-binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) families of transcription factors, which act cooperatively to promote and maintain adipogenesis [61,67,114,126]. C/EBPβ and δ guide early differentiation processes and mediate C/EBPα and PPARγ induction, before their expression decreases towards the terminal phase [113,127-129]. Once activated, PPARγ and C/EBPα drive the expression of adipocyte-specific and lipogenic genes, including among others adipocyte protein 2 (aP2), glycerol-3-phosphate dehydrogenase (GPDH), lipoprotein lipase (LPL), leptin, adiponectin and the glucose transporter GLUT-4 [14,61,130,131]. Many of these genes, for example leptin, feature C/EBP binding sites in their proximal promoters for transactivation [61,132]. Via such a site, C/EBPα self-activation upholds high expression levels even in the mature state [61]. Throughout terminal differentiation and beyond, C/EBPα and PPARγ reciprocally maintain the expression of one another, resulting in the tissue-specific gene expression pattern [14,61,113,115,130]. Apart from PPARγ and C/EBPα, sterol regulatory element binding protein 1/adipocyte determination and

differentiation-dependent factor 1 (SREBP-1/ADD-1) and endogenous ligands (e.g. fatty acids) are similarly involved in the final differentiation phase, especially promoting the expression of lipogenic genes [14,61]. In addition to these factors, Krox20, Krueppel-like factors (KLFs) and early B cell factors (EBFs) have been reported to support differentiation, while others, such as GATA binding protein 2/3, hairy and enhancer of split-1 (HES-1) and T-cell factor/lymphoid enhancer factor (TCF/LEF) inhibit adipogenesis [17,133-135].

Whereas adipogenesis *in vivo* is triggered by integrated microenvironmental signals, various components such as insulin and insulin-like growth factor 1 (IGF-1), glucocorticoids, tryonine (T3), as well as cyclic adenosine monophosphate (cAMP), have been identified to positively influence adipogenesis *in vitro* [61]. Accordingly, pharmacological inducers often applied to stimulate adipogenic differentiation include insulin or IGF-1, a glucocorticoid (typically dexamethasone), 3-isobutyl-1-methylxanthine (IBMX) and indomethacin [23,24,104]. Here, insulin or IGF-1 act primarily via IGF-1 receptor signaling resulting in the upregulation of adipogenic transcription factors [136-138], dexamethasone activates glucocorticoid receptors [104], IBMX increases intracellular cAMP levels and by this activates protein kinase pathways [100,139], and indomethacin is added as a direct PPARγ agonist to sustain differentiation [24,140,141]. Since adipogenesis *in vitro* closely recapitulates the sequence of developmental events *in vivo* [104], the hormonal induction cocktail has effectively been established for adipogenic induction in 2D and 3D models [24].

1.2.4 Adipose tissue angiogenesis

Already in late prenatal and early postnatal life, adipogenesis and microvessel growth are temporally and spatially coupled, with arteriolar differentiation and blood vessel ECM deposition preceeding adipocyte differentiation [49,54,142,143]. Likewise, in the adult tissue, adipogenesis and angiogenesis are interlinked by the functional communication between capillary endothelial cells and adipoyctes via paracrine signaling, interaction with extracellular components and direct cell-cell contact [42,48,54,144]. This close interrelationship is rooted in the essential need for oxygen and nutrient supply as a prerequisite for the physiological functioning of adipose tissue, which is, when normalized to cytoplasmatic volume, among the tissues with the highest metabolic turnover in the human body [24,47,111].

To ensure the high degree of plasticity inherent to adipose tissue, adipocytes mediate vascularity primarily by secretion of VEGF and HGF, as well as other angiogenesis-related factors like leptin, IGF-1, PAI-1, TNF-α, placental growth factor (PlGF), resistin, tissue factor (TF), neuropeptide Y (NPY) and angiopoietins, among others. ASCs specifically produce high levels of VEGF, HGF, granulocyte macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor 2 (FGF-2) and transforming growth factor beta (TGF-β) [15,42,54,143,145-147] (Figure 4). Anti-angiogenic modulators secreted by adipose tissue include thrombospondin (TSP)-1 and -2, as well as a disintegrin and metalloproteinase (ADAM) and ADAM with thrombospondin motifs (ADAMTS) family members [42]. According to the 'angiogenic switch' model, angiogenesis in adipose tissue depends on the microenvironmental equilibrium of pro- and anti-angiogenic effectors, with angiogenesis being initiated by tipping the net balance in favor of the pro-angiogenic side [42,148].

In this context, local hypoxia is regarded a major physiological trigger for vascular growth and remodeling [15,48,149]. Via the master regulator hypoxia-inducible factor 1 alpha (HIF-1α), low pO_2 can elevate expression levels of adipose-related pro-angiogenic factors, especially leptin and VEGF [54,143,150] (Figure 4).

Members of the VEGF family are the cardinal pro-angiogenic factors, and VEGF-A (referred to as VEGF in this thesis) in particular, has been established as the most potent stimulator of endothelial cell proliferation and migration [21,42,52]. VEGF is involved in physiological as well as pathological angiogenesis and acts primarily via vascular endothelial growth factor receptor 2 (VEGFR-2) signaling [42]. As functional partners of VEGF, angiopoietins 1 and 2 (Ang-1/2) are involved in the early phase of angiogenesis [15].

Leptin directly exerts its pro-angiogenic effect via binding to leptin receptors expressed by endothelial cells [151], and additionally, it indirectly acts on endothelial cells by its involvement in FGF-2-triggered signaling [152-154]. It potentiates VEGF-mediated angiogenesis by increasing VEGF levels and mRNA expression [151,153,155], and synergistically stimulates angiogenesis with FGF-2 and VEGF [152,154]. Leptin further triggers matrix remodeling through activation of MMP-2 and MMP-9 [156] which, together with other matrix remodeling enzymes by local proteolysis of matrix proteins, control

sprouting activity at the tip region of nascent vessels and sequentially release matrix-sequestered growth factors such as VEGF [21,143].

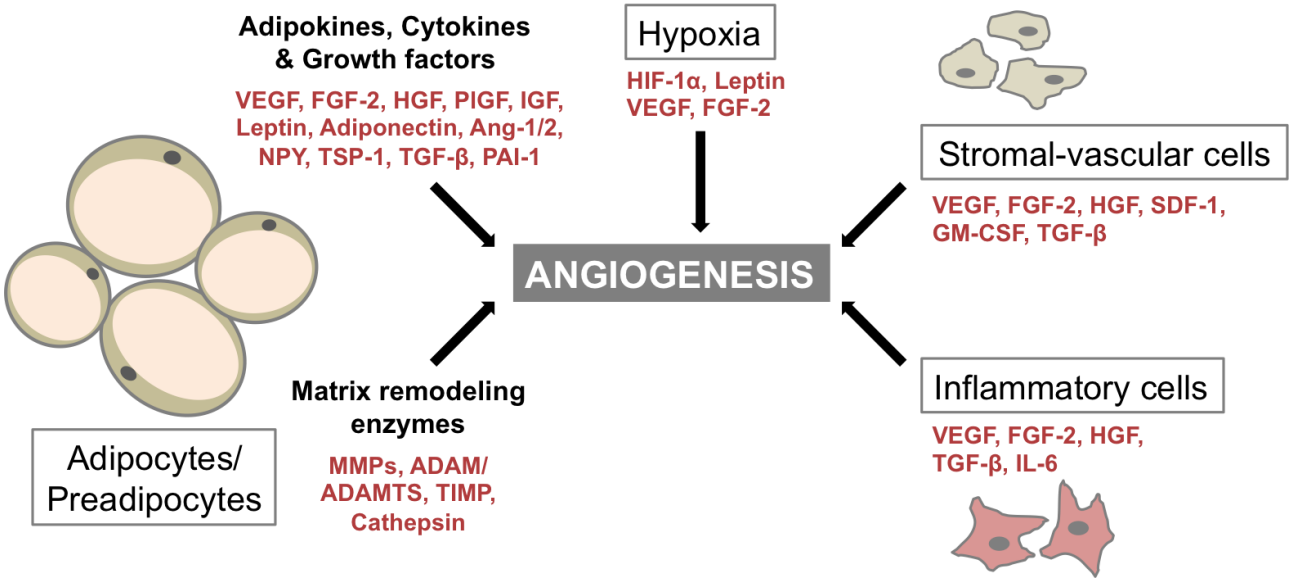

Figure 4. Regulation of adipose tissue angiogenesis. Multiple cellular components (adipocytes/preadipocytes, stromal-vascular cells, inflammatory cells) as well as environmental conditions such as hypoxia contribute to the production of pro- and anti-angiogenic mediators. (VEGF: vascular endothelial growth factor; FGF-2: fibroblast growth factor 2; HGF: hepatocyte growth factor; PlGF: placental growth factor; IGF: insulin-like growth factor; Ang-1/2: angiopoietin 1/2; NPY: neuropeptide Y; TSP-1: thrombospondin 1; TGF-β: transforming growth factor beta; PAI-1: plasminogen activator inhibitor 1; SDF-1: stem cell-derived factor 1; GM-CSF: granulocyte macrophage colony-stimulating factor; IL-6: interleukin 6; MMP: matrix metalloproteinase; ADAM: a disintegrin and metalloproteinase; ADAMTS: ADAM with TSP motifs; TIMP: tissue inhibitor of MMP; Adapted in part from [15,143].)

FGF-2 stimulates differentiation, migration and proliferation of endothelial cells [42,157] and is likewise acknowledged to promote adipogenesis *in vivo* [158,159]. Other adipose-secreted factors such as TGF-β, PAI-1 and adiponectin, function in a context-dependent manner, with the ability to both positively and negatively regulate angiogenesis [143]. Besides adipocytes and stromal cells, resident macrophages and inflammatory cells also produce factors with pro-angiogenic activity [160]. Here, TNF-α acts in a dual role, transmitting pro-angiogenic signals, but at the same time, acting as a potent inflammatory cytokine, linking inflammation, angiogenesis and adipogenesis [15,143].

In light of the above, it is not surprising that virtually all obesity-related disorders such as diabetes, cardiovascular disease and cancer, entail vascular dysfunction and pathological angiogenesis [15,21,143]. Accordingly, modulation of angiogenesis, for example by

administration of anti-VEGF antibodies or blockade of VEGFR-2 signaling [161,162], as pioneered in the cancer field, may constitute a strategy for the treatment of obesity [15,42]. Overall, the complexity of the described regulatory mechanisms and pathways profoundly highlights the central relevance of angiogenesis and the role of the adipose vasculature for the functioning and maintenance of fat tissue and related physiological systems. Apart from the need of a more detailed exploration of the tightly interwoven network of cells, growth factors and cytokines that underlies the interplay between adipogenesis and angiogenesis, the development of strategies that concomitantly integrate these two key processes into current approaches for adipose repair is strongly emphasized.

1.3 ADIPOSE TISSUE ENGINEERING

The loss of soft tissue mass due to trauma, chronic disease, oncological surgery or malformation, represents a tremendous functional and aesthetic impairment for the patient. In 2013, the American Society of Plastic Surgeons (ASPS) reported 5.7 million reconstructive procedures of which tumor removal was the primary cause for surgical intervention [163]. Despite the prevalent occurrence of fat tissue even in lean individuals, the adequate augmentation of soft tissue, notably large volumes, is an ongoing challenge in plastic and reconstructive surgery, explaining the major clinical demand for improved surgical solutions [36,108,164].

Current procedures for soft tissue reconstruction utilize composite tissue flaps, autologous adipose tissue, fat grafts and allogenic or synthetic implants [165]. Despite autologous grafts having routinely been applied for decades, there is considerable variability with respect to long-term outcomes [165,166]. Engrafted tissue is highly susceptible to volume loss by partial resorption and necrosis [167], undergoes fibrosis and is prone to cyst formation [111]. This is primarily rooted in insufficient vascularization of the transplant and a consequent shortage of oxygen and nutrient supply [13,24]. The use of pedicled implants ameliorates the lack of vascular integration to a certain extent, however the underlying surgical procedures are extensive and costly [24,168]. Less invasive methods such as cell-assisted lipotransfer (CAL) and stem cell-enriched tissue (SET) have more recently been developed and showed good efficiency [169-172]. Both procedures entail the co-engraftment of ASCs resulting in a better survival rate of the transplants [108]. Undoubtedly, the advancement of surgical procedures by integration of stem cell applications has introduced novel therapeutic options, yet it remains that central features required for an applicable tissue substitute are lacking, namely full tissue function, adequate vascularization, host integration and long-term maintenance of the initial volume and shape [24]. Further, the harvest of healthy tissue from a remote area of the body for autologous fat grafting is accompanied by substantial donor site morbidity and potential complications such as hypertrophic scar formation [111,173]. Alternatives to autologous tissue are limited and clinicians resort to implants or fillers manufactured from silicone or

collagen, which entail complications such as foreign body reactions, fibrotic encapsulation, material contraction and migration [174].

Accordingly, adipose tissue engineering aims at providing alternatives to the current standard of care by developing viable and functional substitutes that have the ability to adequately augment soft tissue defects. For the successful generation of adipose tissue equivalents that can reliably be used for reconstructive purposes, several critical aspects have to be considered including 1) maintenance of volume and shape of the construct at the implantation site, 2) rapid and sufficient construct vascularization, 3) the ability to replace the native tisse in cellular and extracellular composition, 3D organization and function, and 4) predictability of clinical outcome and long-term integration [24,36].

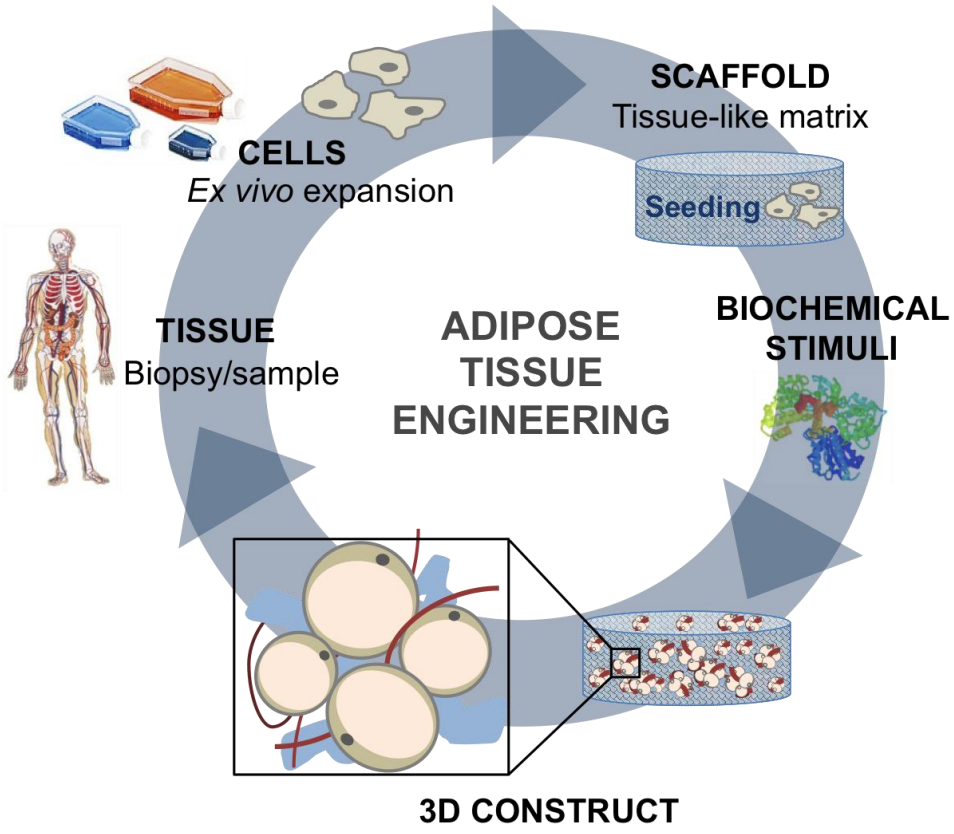

Figure 5. Concept of adipose tissue engineering. Autologous cells are isolated from tissue samples or biopsies obtained from the patient. The isolated cells are either expanded *ex vivo* or employed directly without *in vitro* manipulation. For construct preparation, the cells are placed onto a biomaterial scaffold and subjected to *in vitro* cultivation and bioinduction prior to reimplantation.

In order to address these requirements, adipose tissue engineering approaches combine cells with a strong regenerative capacity with a compliant biomaterial scaffold and a tissue-inductive microenvironment that transmits the appropriate cues and signals for growth and

differentiation [36,47,175]. Accordingly, as outlined in Figure 5, the starting point of any cell-based adipose tissue engineering approach is the harvest of tissue from the patient and the subsequent isolation and *ex vivo* expansion of the desired stem cell population, usually ASCs from fat tissue. In the following, isolated cells are reseeded onto an adequate scaffolding material and exposed to a specific bioinductive environment *in vitro* for differentiation. Finally, cell-seeded 3D tissue substitutes are grafted back into the defected site as replacement tissue [3,176,177].

Despite the apparent practicability of this strategic setup, the coordinated integration of adequate volume-stable scaffolds, the differentiation of cells and tissue-specific functionality, as well as immediate vascularization for long-term survival, remains profoundly challenging and has to date prevented the engineering of larger adipose tissue volumes. Since this is largely attributed to the physiological complexity of adipose tissue, reconsidering the fundamentals of adipose tissue structure and function, and simultaneously exploiting the vast potential offered by the available stem cell sources and tailored biomaterials, may aid the advancement of novel and innovative adipose tissue engineering approaches.

1.3.1 Cell sources for adipose tissue engineering

The selection of an appropriate cell source can strongly influence the experimental success and potential therapeutic value of engineered adipose tissue constructs. With tissue engineering approaches potentially aiming at clinical translation, the use of autologous human cells is desired, whereas murine preadipocyte cell lines such as 3T3-L1, 3T3-F442A or Ob17, which have been applied in earlier tissue engineering studies [47,178,179], are only suited for basic research [111,180]. Ideally, the applied cellular component should be autologous, non-immunogenic and easily accessible in sufficient quantities [24,181]. According to these minimal criteria, mature adipocytes, which self-evidently seem suitable for adipose repair, are impractical since they are postmitotic and have lost their proliferative potential [173,174]. Likewise, they are easily traumatized by mechanical force due to the buoyancy and fragility of the lipid vacuole [47,174].

Owing to their unique properties including self-renewal, vast proliferation and differentiation, stem cells are thus considered an attractive alternative for the regenerative treatment of tissue defects. There are two different types of stem cells: embryonic and non-

embryonic (adult) stem cells [101,182]. Theoretically, embryonic stem cells (ESCs) have the greatest regenerative potential since they are pluripotent and can differentiate into all three embryonic germ layers (mesoderm, endoderm and ectoderm) [165,183]. Safety concerns over potential tumorigenicity [184], immunocompatibility [185], and notably ethical and political barriers, yet abrogate the use of ESCs for basic research and clinical applications. Not stigmatized by ethical concerns, but still unsuitable for safe use in humans due to extensive *ex vivo* manipulation and possible chromosomal abberations, are induced pluripotent stem cells (iPSCs) [186,187].

Adult stem cells, such as MSCs, circumvent many of the ethical and technical issues associated with ESCs and iPSCs. As they are obtained from developed, adult tissues such as bone marrow [188,189], adipose [105], skeletal muscle [190] or skin [191], they can be isolated from the patient and reapplied autologously [108]. Although MSCs have lost pluripotency, their proliferative and multilineage differentiation capability together with promising data from translational clinical applications [108], has rendered them an attractive and relatively safe option for applications in tissue engineering and regenerative medicine.

Bone-marrow aspirates or biopsies are the primary sources of BM-MSCs [192]. The invasive and painful harvesting procedure as well as the comparatively low yield of cells featuring multilineage potential [193,194], limit the use of BM-MSCs for adipose regeneration, for which high seeding densities and large-volume constructs are desired. In contrast, adipose tissue is particularly rich in adult stem cells and is hence regarded an easily accessible, abundant, and reliable source for the isolation of ASCs [195,196]. Exhibiting a differentiation potential and gene expression pattern similar to BM-MSCs [106,197], ASCs may be harvested with lower morbidity [109,195], are yielded in larger quantities upon isolation [105,198] and exhibit higher proliferation in culture compared to BM-MSCs [12,105]. In many settings, ASCs can be obtained from waste products accrued in routine procedures such as abdominoplasties, liposuctions and other cosmetic surgeries [196]. By enzymatic digestion of the native tissue using collagenase or other lytic enzymes and discharge of the mature fat cells, the stromal-vascular fraction (SVF) can be isolated under standardized conditions [105,196,199]. Upon conventional 2D *in vitro* culture, the ASC population of adipose tissue is subsequently selected from the SVF cell pool by plastic adherence [24,47].

Despite considerable advances related to the use of ASCs for regenerative medicine [200], more recent work has also approached the direct application of uncultured SVF cells for tissue engineering approaches, promoting clinical translation by avoidance of *ex vivo* manipulation [196,201,202]. Providing a pool of tissue-inherent cells from one single source, the SVF contains ASCs, endothelial cells and pericytes among others, and represents a promising cell source option, particularly with respect to vascularization and *in vivo* engraftment of engineered constructs. For applications such as wound healing [201,203], cartilage repair [204] and especially, the generation of vasculogenic bone substitutes [205-207], SVF cells have already been successfully applied *in vitro* and *in vivo*, yet surprisingly, for adipose regeneration only limited *in vitro* experience with SVF cells [202], and apparently, no *in vivo* investigations exist to date. Thus, apart from exploiting the profound regenerative potential of ASCs for adipose construct generation, here, SVF cells were also explored as a potential new source of cells that might additionally aid the development of vascularized adipose substitutes.

1.3.2 Biomaterial scaffolds for adipose tissue engineering

The main strategy applied for adipose construct preparation involves the seeding of cells, most commonly ASCs or BM-MSCs, onto 3D biomaterial scaffolds [12,24]. The cell carrier thereby functions both as cell delivery vehicle, as well as matrix for cellular growth and tissue development [175,208]. In general, cell-based strategies are distinguished from acelluar approaches, which rely on matrix-guided *de novo* tissue development through the repopulation of the scaffold with host cells [7,35]. Biomaterial scaffolds are either designed solely as structural support providing passive stimuli or are equipped with biological cues to guide cell and tissue growth [208]. The latter especially applies to acellular approaches, where host cells are attracted by structure, topography, or growth factor release [159,209,210].

For adequate soft tissue repair, scaffolds should be biocompatible with low immunogenicity, biodegradable, mechanically stable and provide an *in vivo*-like environment for cells to attach, proliferate and/or differentiate [8,111,175,211-213]. Volume stability is particularly relevant for the clinical applicability of adipose tissue constructs, whereas at the cellular level, structural support is needed as adipocytes and maturing cells are highly susceptible to mechanical stress and require protection from mechanical

forces [174,175,214]. Further, to adequately sustain the developing tissue, the degradation kinetics of the biomaterial should match the dynamics of tissue formation and produce non-toxic and non-carcinogenic degradation products [12,24,111,213]. Non-degradable materials are rarely used due to unpredictable and possibly adverse effects of long-term *in vivo* persistence [213]. Delivery of cells to the cell carrier largely depends on the internal architecture of the scaffolds. Porous scaffolds or bulk materials are usually seeded directly using a cell suspension, whereas the use of hydrogels also allows the encapsulation of cells during gelation [24,208]. Accordingly, the seeding method greatly affects related parameters such as cell distribution and seeding density [215].

As far as the 3D scaffold microarchitecture is concerned, an adequate pore size is required to accommodate developing adipocytes [24,212,216], whereas pore interconnectivity should facilitate oxygen, nutrient and metabolite diffusion [8], and essentially, the establishment of coherent tissue and a functional capillary network *in vivo* [111,217]. Apart from porosity, the elasticity and hydrophilicity of the scaffolds may additionally be tailored to imitate the biological template of the ECM and create a 3D environment that promotes cellular development and matrix deposition [176]. Thereby, advances in biomaterial fabrication techniques such as 3D printing, sintering or electrospinning, enormously facilitate the adjustment of the carrier material to the requirements of the reconstructed tissue [218-221]. Since internal structure, physicochemical characteristics and mechanics of the cell carrier collectively transmit environmental cues, it is relevant to consider that biomaterial properties may both intentionally and unintentionally affect cellular differentiation and tissue formation *in vitro* and *in vivo* [111,222].

Scaffolds used for adipose tissue engineering can be subdivided by origin into synthetic and natural biomaterials (Table 1), which are employed to produce porous scaffolds as well as soft hydrogel carriers [2,8,47,223]. Natural materials inherently contain information that promotes cell attachment and differentiation, and usually feature low immunogenicity [214], however, batch-to-batch variations and scale-up difficulties need to be considered [2]. Using synthetic materials, parameters such as molecular weight, degradation time or hydrophilicity may be precisely tuned, yet in turn, insufficient cellular attachment and an overall reduced biocompatibility represent possible limitations [2,222,224].

Biomaterials for adipose tissue engineering	
SYNTHETIC	**NATURAL**
Poly(lactic adic) (PLA)	Collagen
Poly(glycolic acid) (PGA)	Hyaluronic acid
Poly(lactic-co-glycolic acid) (PLGA)	Fibrin
Poly(ethylene glycol) (PEG)	Alginate
Polyethylene terephthalate	Decellularized matrices
Polyurethanes	Matrigel (tumor product)

Table 1. Common synthetic and biologically derived scaffold materials for adipose tissue engineering applications.

Porous scaffolds

Porous carriers facilitate the definition of size and shape of engineered tissues, their application, however, usually requires surgery [24]. Advantages of scaffolds manufactured from synthetic materials include their availability in large quantities, control over chemical and physical properties and good reproducibility of cellular response [224].

Synthetic biodegradable polymers processed from poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PLGA) have been employed already in early adipose tissue engineering trials by Patrick *et al.* [225,226]. These polymers are considered safe for medical use by the FDA [223] and are widely applied either unmodified, derivatized or in combination with other materials for a wide range of tissue engineering applications [227,228]. Other materials suited for scaffold fabrication include polyethylene terephthalate and polycaprolactones, yet these are not always biodegradable [179,229]. Featuring large interconnected pores and good biodegradation, more recently designed scaffolds processed from polycaprolactone-based polyurethane (PU) appear promising, especially for the generation of mechanically stable constructs [230-232]. Combining soft texture with intrinsic bulk stability, such biodegradable polycaprolactone-based PU scaffolds [233] were utilized in this work as mechanical support structures for the generation of volume-stable adipose tissue constructs.

ECM components such as collagen and hyaluronic acid have been employed for the manufacture of biocompatible sponges and fiber meshes [111,234,235], and were shown

suitable for adipose development [236]. Collagen modifications such as cross-linked collagen-chitosan similarly supported adipogenic differentiation of rat preadipocytes [237]. Fast degradation and limited volume retention meanwhile restrict the use of collagen scaffolds for the development of volume-stable adipose tissue constructs [24,214]. Silk fibroin in contrast, gives rise to highly resilient and biocompatible scaffolds [238], which appear more promising candidates for improved volume maintenance of engineered adipose tissue [239,240]. Alternatively, hyaluronic acid-based spongy scaffolds embedded with ASCs have been described as stable carriers for engineering of volume stable tissues [213]. More recently, decellularized matrices from different tissues including placenta, skin, jejunum or adipose have been applied as scaffolding materials, providing a preexisiting tissue structure together with the underlying vascular network as well as low immunological response [12,224,241-244].

Hydrogels and injectables

Cell-encapsulating hydrogels have great scaffolding potential for tissue engineering applications since they overcome some of the constraints associated with solid polymeric scaffolds. They facilitate the repair of irregularly shaped defects and can directly, in a minimally invasive manner, be administered to the site of injury for *in situ* gelation, reducing the incidence of scars and infections [214]. Alternatively, hydrogels may be prefabricated prior to *in vivo* implantation and, depending on the application, precultivated *in vitro* for a certain time. Due to their high water content and good biocompatibility, the ability to uniformly encapsulate cells and retain matrix components, as well as their mechanical properties that can parallel the texture of soft tissues, hydrogels may closely recapitulate the *in vivo* microenvironment [175,208].

Like porous scaffolds, hydrogels can be processed from synthetic materials such as poly(ethylene glycol) (PEG), PEG-diacrylate (PEGDA) or Pluronic F-127 [214,222]. Since PEG can be modified extensively, it is a frequently employed hydrogel component for tissue engineering approaches [208]. PEGDA for example, can be applied to generate stable gels with excellent volume retention *in vivo* [245,246]. By surface modification with peptide sequences derived from fibronectin (RGD), laminin (IKVAV, YIGSR) or other ECM components, the non-adhesive nature of PEG can be improved and specific guidance of cellular behavior facilitated [208,247]. Similarly, the strong cell-inductive effect of

Matrigel, which is a tumor product of Engelbreth-Holm-Swarm mouse sarcoma [248], is largely ascribed to its high laminin, collagen IV and FGF-2 content [249-251]. However, although Matrigel is profoundly supportive of adipose formation and vascularization [209,252], it is inapplicable for clinical use due to its neoplastic origin. Here, more recently developed adipose-derived ECM gels prepared from decellularized tissue represent an attractive alternative as injectable, cell-instructive carrier materials [253-256].

Natural polymer hydrogels made from collagen, hyaluronic acid, alginate and fibrin have successfully been used for adipose tissue engineering *in vitro* and *in vivo*, advocating their use for soft tissue repair [208,217,251,257-260]. Fibrin, in particular, is a natural polymer with good biocompatibility [261] that has been investigated for the bioengineering of bone, cartilage, adipose, skin and ligament, among others [175,262,263]. Fibrin is conducive of angiogenesis and was shown to promote endothelial development [264]. The 3D fibrin network is generated by the enzymatic cleavage of fibrinogen by thrombin [263,265], resulting in clots of variable stiffness depending on the fibrinogen concentration [266]. As a blood component critical for hemostasis, fibrin may be produced from autologous plasma, reducing the risk of foreign body reactions or infection [175,267].

Despite its versatility, fibrin features low mechanical stiffness and disadvantageous degradation kinetics resulting in rapid dissolution [267,268], limiting its applicability as a volume-stable scaffold [24]. However, to exploit the advantageous properties of this natural material, different strategies emerged to enhance fibrin stability, such as the combination with PEG [269,270], a reduction of the seeding density to prevent intrinsic proteolytic activity [271] or the use of protease inhibitors such as aprotinin or tranexamic acid [267,272,273]. By variation of fibrinogen concentration, ionic strength as well as pH, the development of a stable fibrin formulation with prolonged stability for tissue engineering applications was effectively approached in our group [215]. Additionally, the suitability of the stable fibrin to support the development of bovine chrondrocytes was proven [215]. Consequently, the comprehensive investigation of this promising biomaterial for adipose tissue engineering *in vitro* and *in vivo* represented a central part of the present work.

Composite scaffolds

Both porous scaffolds and hydrogels feature beneficial intrinsic properties for the engineering of adipose tissue, yet individually applied, each scaffolding option entails considerable disadvantages [231]. Soft hydrogels for example, often lack the intrinsic load bearing capacity and mechanical integrity of porous scaffolds required for *in vivo* maintenance [222,223], whereas seeding efficiency and ECM retention are inadequate in porous scaffolds [208,274]. Owing to the absolute necessity to mechanically protect developing adipose tissue within engineered constructs and to prevent volume loss upon implantation, the concept of joining solid scaffolds and soft hydrogels has emerged [13,24]. Advocating the strategy of providing additional protection, pionieering studies utilized hollow support structures fabricated from silicone, polycarbonate or reinforced PGA to protect hydrogel implants within a chamber space [168,259,275,276]. Although especially non-degradable scaffolds are difficult to reason for clinical application, proof-of-principle was impressively provided. To better imitate soft tissue texture, a different group utilized sponge-like porous scaffolds fabricated from gelatin, PGA and polypropylene, however, again only the non-degradable polypropylene mesh maintained the initial construct volume [277]. Integrating these approaches, the application of macroporous biodegradable scaffolds that feature sufficient elasticity together with a pore-filling hydrogel component as cell carrier may provide constructs featuring appropriate texture and intrinsic rigidity. The effectiveness of this concept was demonstrated before employing porous biodegradable PU scaffolds together with fibrin hydrogels for cartilage repair [231], however, for adipose tissue engineering, such biomaterial combinations remain to be explored. Thus, in this work, composite constructs were investigated as promising scaffolding option for the engineering of functional adipose tissue constructs with sufficient volume retention *in vivo*.

1.3.3 Vascularization strategies for adipose tissue engineering

Providing immediate blood supply is regarded a central requirement for growing complex and large-volume substitute tissues [4,278,279]. On account of its naturally high vascular density, adipose tissue is particularly susceptible to deficient blood supply [53,147] and thus, the establishment of a functional vasculature remains one of the major challenges in adipose tissue engineering. Similar to grafted autologous fat, oxygen and nutrient supply to

tissue-engineered constructs is diffusion-limited to a distance of approximately 200 μm upon implantation, which is insufficient to sustain a larger tissue volume [280,281].
Accordingly, in addition to achieving adipogenic development within engineered constructs, it is crucial to ensure adequate vascular integration upon implantation to prevent construct dysfunction and volume loss. To generate viable implants, different vascularization strategies *in vitro* and *in vivo* have been applied to improve current adipose tissue engineering approaches.

Integration of a vascular predicle

As an *in vivo* vascularization strategy, the microsurgical integration of vessel loops or pedicles exploits the natural angiogenic potential of the host blood vessels for immediate implant vascularization [279]. In this context, a variety of modalities may be utilized, including arteriovenous (AV) loops, flow-through and ligated AV bundles, from which new vessels invade the construct by angiogenic sprouting [1,24]. In previous adipose tissue engineering studies, this technique has often been applied in combination with protecting growth chambers prepared from silicone or polycarbonate, in which vascularized adipose tissue was successfully generated and maintained for up to 1 year [168,282]. Similarly, a flow-through vessel loop was shown to facilitate capillary network formation in engineered adipose tissue constructs [283]. In this work, aiming at the generation of volume-stable adipose tissue and additionally, adequate vascularization thereof, the microsurgical insertion of a flow-through AV bundle into composite adipose tissue constructs was approached as possible *in vivo* vascularization strategy.

Application of pro-angiogenic growth factors

The incorporation of factors that centrally regulate angiogenesis, most prominently FGF-2 and VEGF, into biomaterial scaffolds is another potent strategy to generate vascularized adipose tissue *in vivo* [4,159,284-288]. Growth factor release from the implanted scaffolds into the local environment thereby triggers the ingrowth of host blood vessels [8]. The pro-angiogenic effect can be prolonged using sustained release systems, such as PLGA particles or gelatin microspheres, which are more effective compared to administration of the free protein [285,287,289-291]. Difficulties are associated with the restriction of protein delivery to the site of interest and general control over release kinetics [4,292,293]. Further, since

pro-angiogenic factors solely accelerate vessel ingrowth from the surrounding tissue, the time course of vascularization to reach central regions of the construct may still be delayed, especially in larger tissue volumes [24,279].

Implantation of growth factor-producing cells

As an alternative cell-based approach, the paracrine action of factors secreted by the implanted cells may be exploited to promote vascularization of engineered constructs in a biomimetic manner [278,279,294]. ASCs are strong modulators of adipose tissue angiogenesis and release multiple pro-angiogenic growth factors including VEGF, HGF, FGF-2 and leptin [42], and their strong regenerative potential has been postulated to be at least partly attributable to paracrine angiogenesis-supportive mechanisms [294]. To enhance the release of endogenous growth factors by the implanted cells, they may be transfected to overexpress pro-angiogenic factors that modulate vascular cell migration, proliferation and maturation [8,279,295], functioning as an *in vivo* controlled release system upon implantation [278]. Alternatively, hypoxia may be employed as a physiological stimulus that enhances the release of endogenous pro-angiogenic factors by ASCs [296-298]. In this setup, ASC-seeded constructs may be preconditioned under hypoxic conditions *in vitro* prior to implantation to increase the production of angiogenic modulators, which then trigger the ingrowth of blood vessels from the surrounding tissue upon *in vivo* implantation. Prior to conducting extensive *in vivo* studies, however, it is necessary to evaluate the effects of hypoxic preculture on adipogenic and angiogenic capabilites of ASCs *in vitro* in basic investigations. Eminently, the hypoxia-triggered upregulation of pro-angiogenic growth factor production by ASCs was considered worth exploring, especially since this would enable the dual use of ASCs as both building blocks for adipose formation, but also as a source of bioactive components for the paracrine stimulation of angiogenesis.

Endothelial cell cocultures

Endothelial cell cocultures represent an *in vitro* vascularization strategy by which prevascular structures are formed as a starting point for integration *in vivo* [279,299,300]. Theoretically, via this approach, a continuous 3D pre-capillary network may be established inside the engineered constructs and, upon anastomosis with the host vessel system, full vascularization may be achieved more rapidly [1,278,300-302]. Coculture setups employing

ASCs and endothelial cells showed that in principle, prevascular structures can be generated *in vitro* to serve as a basis for the development of vascularized tissue [240,264,302,303]. The establishment of a network-like capillary system may be supported by nanofabrication of tubular structures and microchannels within the constructs, which can be perfused and lined with endothelial cells upon seeding *in vitro* or implantation *in vivo* [245,279,304-306]. The complex processes underlying vascular assembly, demanding the coordinated release of signaling molecules and the contribution of other cell types such as smooth muscle cells and pericytes [278], diverging microenvironmental requirements of the co-implanted cell types, as well as the necessity of functional anastomosis [279], however currently restrict coculture systems to *in vitro* models for basic research and proof-of-principle studies [24]. Circumventing some of the obstacles associated with endothelial cell cocultures, more recently, the SVF of adipose tissue was shown to feature a tremendous vascularization potential [201,206] and may thus be applied as a more physiological 'coculture'-system, providing the required cell types for vascular assembly, including endothelial cells, perivascular co-cells and smooth muscle cells, in a physiological ratio [201]. Despite numerous studies employing the SVF for bone and cartilage regeneration [204-207], these cells have only sporadically been investigated with regard to adipogenesis [202,203]. Hence, SVF cells, with their potential to give rise to vascularized tissues and additionally, as a source of ASCs, represent an ideal cellular component for the generation of vascularized adipose tissue constructs.

Collectively, to successfully realize the sufficient vascularization of adipose tissue substitutes, further research is needed to improve the currently applied approaches *in vitro* and *in vivo*. Many of the above-described vascularization strategies appear promising as such and proof-of-principle studies have been conducted, yet their practicability is highly dependent on the reconstructive purpose. It is thus necessary to identify methods that can effectively be employed for adipose construct vascularization, which is crucial for the generation and maintenance of larger volumes, and subsequently, to refine and integrate these strategies for the development of fully functional, volume-stable adipose tissue substitutes.

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Chapter 2

Goals of the Thesis

Advances in tissue engineering research and improved surgical treatments have contributed to novel therapeutic concepts for soft tissue regeneration. For large-volume soft tissue losses and more complex injuries, however, the treatment options are still limited and entail substantial and compromising disadvantages [1,2]. To meet this strong clinical demand, adipose tissue engineering seeks to provide transplantable soft tissue substitutes, yet current approaches are still confronted with a considerable lack of volume-stable scaffolding options and applicable strategies for adequate vascularization of engineered adipose tissue constructs [3-6].

Therefore, this thesis aimed at the development of volume-stable 3D adipose tissue constructs and the investigation of effective vascularization strategies *in vitro* and *in vivo* for the generation of functional soft tissue substitutes for reconstructive surgery.

To achieve this goal, the specific aims included:

1. The development of 3D volume-stable composite constructs based on the combination of fibrin hydrogels with porous scaffolds, and the comprehensive investigation of adipogenesis in the generated constructs *in vitro* and *in vivo* upon seeding with adipose-derived stem cells (ASCs).

2. The evaluation of hypoxic culture as an *in vitro* prevascularization strategy for adipose tissue constructs, and the investigation of the impact of hypoxia on adipogenic development of ASCs.

3. The application of the stromal-vascular fraction (SVF) as a novel cell source for adipose tissue engineering approaches *in vitro* and *in vivo*, putting a special focus on the vascularization potential of the cells and the detailed visualization of tissue structure *in vivo* via 3D whole mount staining.

1. Development and characterization of volume-stable 3D adipose tissue constructs

Volume loss and resorption of engineered adipose tissue constructs have been observed in numerous studies before and were predominantly attributed to insufficient mechanical strength and fast degradation of the applied scaffold materials [4]. Thus, to enhance long-term maintenance and structural integrity of engineered adipose tissue, the

development of composite constructs, consisting of a hydrogel component as cell carrier and a porous scaffold for structural support, was pursued. Composite constructs were generated on the basis of stable fibrin gels with optimized degradation behavior established in our group [7], and porous biodegradable poly(ε-caprolactone)-based polyurethane (PU) scaffolds. Both scaffolding materials were newly introduced to the field of adipose tissue engineering. Aiming at the development of clinically applicable constructs, human ASCs were utilized as cell source. These cells are multipotent, can be harvested in large quantities directly from the patient, and give rise to mature adipocytes upon hormonal induction [8-10]. Accordingly, stable fibrin gels and porous scaffolds were individually assessed for construct development with respect to material properties and cellular response of ASCs. Commercially available fibrin hydrogels (TissuCol, Baxter), which have been demonstrated to facilitate adipogenesis before [11], were included in the study for comparison (**Chapter 3**).

In continuative work, the preparation of volume-stable adipose tissue constructs via combination of ASCs-seeded stable fibrin gels with porous PU scaffolds was approached. Apart from the evaluation of construct stability and cell viability, adipogenic differentiation of ASCs in composite constructs *in vitro* was comprehensively analyzed at the cellular and molecular levels. Subsequently, a pilot study in nude mice aimed at assessing the applicability of volume-stable composite constructs *in vivo*. Here, to ensure immediate oxygen and nutrient supply upon implantation, a first approach towards construct vascularization was integrated into the experimental design and involved the microsurgical insertion of a vascular pedicle (**Chapter 4**).

2. Hypoxic culture as prevascularization strategy for adipose tissue constructs

To accelerate the establishment of a capillary network that can sufficiently maintain engineered implants, hypoxic pretreatment of adipose tissue constructs was investigated as a possible *in vitro* prevascularization strategy. Hypoxia as a strong physiological regulator of angiogenesis was previously demonstrated to upregulate the secretion of adipokines and pro-angiogenic factors by mature adipocytes and ASCs [12,13]. Precultivation of adipose tissue constructs under hypoxic conditions was thus intended to enhance the pro-angiogenic properties inherent to adipose-derived stem cells and to

accustom ASCs to the hypoxic conditions they encounter upon implantation *in vivo*. In addition, it was of central relevance to concomitantly investigate the effect of hypoxia on the adipogenic differentiation potential of ASCs. This was accomplished by evaluating the response of ASCs towards different culture setups of hypoxia in 2D monolayer culture in initial experiments. Employing the established ASC-seeded stable fibrin gels for 3D culture experiments, the suitability of hypoxic pretreatment for *in vitro* prevascularization, in comparison to normoxia as control condition, was thoroughly assessed in terms of adipogenic development, adipokine and pro-angiogenic factor secretion, as well as endothelial development on the cellular and molecular level (**Chapter 5**).

3. **Evaluation of the stromal-vascular fraction for the generation of vascularized adipose tissue**

   SVF cells isolated from adipose tissue have recently been employed as an alternative cell source to ASCs for tissue engineering applications such as bone, cartilage and wound healing [14-17]. Since SVF cells have, as of yet, only sporadically been evaluated for adipose repair [18], a broader application of these cells *in vitro* and, for the first time, their investigation *in vivo* were approached. Containing a variety of cell types that are involved in adipose regeneration, most prominently ASCs and endothelial cells, the use of SVF cells was intended to support functional assembly and vascularization of engineered adipose tissue *in vitro* and *in vivo*.

   To gather an impression of the behavior of SVF cells in 3D constructs, initial experimental work explored the capacity of SVF cells for adipogenic and endothelial development *in vitro* on the basis of previously applied stable fibrin gels as cell carriers. Different culture conditions were tested to provide the different cell types with adequate stimuli for differentiation. To facilitate interpretation of results, SVF-seeded constructs were compared to the already established 3D constructs seeded with ASCs alone. In preparation of *in vivo* studies, the establishment of a whole mount staining (WMS) technique purposed the detailed 3D visualization of tissue structure and particularly, the analysis of adipogenesis and vascular development within the constructs (**Chapter 6**).

   In successive experimental work further investigating SVF cells in fibrin gels for adipose construct generation, parameters that potentially exert a decisive impact on

tissue formation *in vivo* were assessed in an animal study in nude mice. Specifically, the influence of the applied cell carrier on adipose tissue development was addressed by comparison of SVF-seeded stable fibrin and TissuCol gels. It was further evaluated whether adipogenic precultivation of the constructs *in vitro* prior to implantation has an effect on tissue formation *in vivo*. To correlate *in vitro* and *in vivo* results, SVF-seeded adipose tissue constructs were extensively analysed focusing on adipogenic and endothelial development. With respect to the visualization of tissue formation *in vivo*, the pre-established WMS technique was utilized as an effective tool for adipose construct analysis, focusing on the capacity of SVF cells to contribute to the vascularization of adipose tissue constructs (**Chapter 7**).

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Chapter 3

Development and Characterization of Adipose Tissue Constructs *In Vitro*

3.1 INTRODUCTION

The necessity of tissue-engineered adipose substitutes is rooted in the still constricted efficiency of surgical procedures and the unresolved need for functional and full-volume replacement of injured and diseased soft tissue [1-3]. The first issue with regard to a successful adipose tissue engineering approach is the selection of a suitable cell carrier [4,5]. Thereby, the choice of scaffold depends on the tissue of interest and the specific application [6]. For adipose tissue, shape stability and mechanical integrity of engineered implants are required to reliably restore contour deformities and in particular, critically sized soft tissue defects [1,7,8]. Therefore in this study, biomaterials exhibiting adequate stability and tissue-like soft texture that can be combined with cells committed to the adipocyte lineage were investigated in preliminary experimental work to prepare the generation of applicable volume-stable soft tissue constructs for reconstructive purposes.

Fibrin is a versatile biopolymer featuring outstanding compatibility with cells as well as adhesive properties, and has repeatedly been employed as scaffolding material for various tissue engineering applications [9-12]. Fibrin is formed by thrombin-mediated cleavage of fibrinopeptide A and B from fibrinogen monomers, by which polymerization sites are exposed, leading to self-association and formation of insoluble fibrin [10,13]. In the form of a soft and extracellular matrix (ECM)-like hydrogel, fibrin is inherently cell-instructive and promotes regeneration as reflected by its profound role in hemostasis and wound healing [5,14-16].

However, despite excellent cytocompatible properties [17,18], the applicability of fibrin hydrogels for soft tissue reconstruction is hampered due to instability and fast degradation *in vitro* and *in vivo* by fibrinolysis within a time span of merely few weeks [11,12,19-22]. This downside has been eliminated by the development of a stable fibrin formulation in our group [19]. By manipulation of pH, ionic strength, thrombin and fibrinogen concentration, the polymerization process underlying fibrin formation and thereby, its material properties, may be altered [19,23-25]. Apart from changing a series of parameters, degradation of fibrin can also be prevented by addition of fibrinolysis inhibitors such as aprotinin or tranexamic acid to the culture medium, or directly to the fibrin gels [26-28]. Integrating these approaches, the developed long-term stable fibrin gels featured an excellent durability in

cell culture medium for at least 12 months [19]. Having to this point not been investigated for adipose tissue engineering, the developed stable fibrin gels, with respect to their optimized degradation properties and the overall suitability of fibrin as cell carrier, were considered an attractive option for the development of more resilient adipose tissue constructs.

To fill larger-volume defects, an appealing strategy to additionally enhance the mechanical integrity of fibrin-based adipose tissue constructs, and hydrogels in general, is offered by the integration of a stabilizing porous support structure. Combination of preadipocyte-seeded fibrin gels with a dome- or tube-shaped mechanical support [12,29], and the use of solid chamber configurations made from silicone or polycarbonate [30,31], were shown particularly supportive of tissue development *in vivo*, where unpredictable high mechanical loads potentially compromise adipose formation [12]. Pursuing a modified and more translational setup in this study, biodegradable porous poly(ε-polycaprolactone)-based polyurethane (PU) scaffolds were evaluated as support structures instead of the solid or non-degradable materials employed in previous studies. By combining these polymeric scaffolds with the stable fibrin hydrogels, the generation of biodegradable composite constructs with an inherent mechanical strength and texture related to that of adipose tissue was intended. Here, in preparative experiments prior to composite construct generation, the porous PU scaffolds were first evaluated as biomaterial alone in terms of general handling, biomaterial properties and interaction with cells.

As cellular component, adipose-derived stem cells (ASCs) were employed, since they are highly proliferative and exhibit a robust differentiation potential upon adipogenic induction *in vitro* [11,32]. Due to these properties, ASCs are regarded a suitable functional replacement for mature adipocytes, which are prone to mechanical damage and ischemia [8,33,34]. For research applications, ASCs may be obtained in high quantities from liposuctioned or excised adipose tissue, whereas in a clinical setting, ASCs can potentially be isolated from autologous adipose tissue sampled from the patient [1,35,36].

Consequently, with the aim to develop composite adipose tissue constructs for soft tissue reconstruction, the basic evaluation of the suitability of stable fibrin hydrogels and porous PU scaffolds for adipose tissue engineering was approached in preparative experiments. Specifically, in a first step, the recently developed stable fibrin gels were investigated as cell

carriers for the encapsulation of human ASCs, including their potential to facilitate adipogenesis *in vitro*. As an alternative fibrin formulation, commercially available TissuCol fibrin gels (Baxter) were included in the experimental setup for comparison, since they had previously been shown highly supportive of adipose development [11,37]. The second stage of construct development included the evaluation of biodegradable porous PU scaffolds in terms of biomaterial properties and interaction with cells, to potentially serve as support structures for the generation of volume-stable composite fibrin/PU constructs in upcoming studies.

3.2 MATERIALS AND METHODS

3.2.1 Cell isolation and expansion

Human ASCs were isolated from subcutaneous adipose tissue of healthy female donors obtained in lipoaspiration procedures. The study was approved by the ethics committee of the University of Wuerzburg, Germany. Written informed consent was obtained from all patients. Patients were between 20 and 40 years of age and had a body mass index (BMI) ranging from 28 to 33.

For ASC isolation, fat tissue was digested with 0.1% collagenase NB4 from Clostridium histolyticum (Serva Electrophoresis, Heidelberg, Germany) in freshly prepared collagenase buffer for 2 h at 37 °C on an orbital shaker. The resulting suspension was filtered through a 100 μm nylon mesh and centrifuged at 300 g for 10 min. The fat layer on top was aspirated and pelleted cells were washed with phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria). The obtained stromal-vascular fraction (SVF) was resuspended in preadipocyte growth medium 2 (PGM-2) consisting of preadipocyte basal medium 2 (PBM-2, Lonza, Walkersville, USA) supplemented with 1% penicillin-streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 10% fetal bovine serum (FBS), both obtained from Invitrogen (Karlsruhe, Germany). For cryopreservation, PGM-2 further contained 5% DMSO (Sigma-Aldrich, Steinheim, Germany). SVF cells were cryopreserved at a cell density of approximately 1.5×10^6 cells/mL.

For expansion of ASCs, cryopreserved SVF cells were thawed and plated on tissue culture-treated plastic flasks (175 cm^2). The ASC subpopulation was subsequently selected via plastic adherence. The cells were cultured to a subconfluent level and expanded for 2 passages in PGM-2 until seeding of the scaffolds. For passaging, trypsin-EDTA at 0.25% from Invitrogen (Karlsruhe, Germany) was used.

3.2.2 2D cell culture

For experiments in 2D culture, ASCs were seeded in 12-well plates at a density of 3×10^4 cells/cm^2 and cultured in PGM-2. Two days after seeding, adipogenesis was induced by addition of differentiation medium [PGM-2 with 1.7 μM insulin, 1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine (IBMX) and 200 μM indomethacin]. For medium preparation, bovine insulin was kindly provided by Sanofi-Aventis (Frankfurt, Germany),

IBMX was obtained from Serva Electrophoresis (Heidelberg, Germany) and indomethacin, as well as dexamethasone were purchased from Sigma-Aldrich (Steinheim, Germany). Cells subjected to non-induced control conditions were cultured in PGM-2 devoid of additional supplements. Cells in 2D culture were analyzed after 10 days *in vitro*.

3.2.3 PU scaffolds

Poly(ε-caprolactone)-based polyurethane scaffolds were manufactured as previously described [38]. Briefly, polycaprolactone diol (28.3 wt%), polycaprolactone triol (17.0 wt%), poly(ethylene glycol) (19.5 wt%), poly(tetramethylene glycol) (5.7 wt%) and a slight excess of isophorone diisocyanate (28.6 wt%) were mixed with small amounts of distilled water (0.2 wt%) and Pluronic P-123 (0.1 wt%) and heated to 40 °C. Diazabicycloundecene (0.6 wt%) was dissolved in methylal and added to the previous mixture at 35 °C, leading to a gas foaming process and pore formation. All reagents were either obtained from Acros Organics (Geel, Belgium) or Sigma-Aldrich (Steinheim, Germany). The scaffolds were annealed for 3 h at 50 °C. Residual solvents were removed by treating the scaffolds with boiling water, followed by drying to constant weight at 10^{-2} mbar. Discs measuring 5 mm in diameter and 2 mm in height were prepared using sterile biopsy punches (Stiefel Laboratories, Sligo, Ireland). For sterilization, the PU scaffolds were autoclaved. For seeding with cells, autoclaved polymeric discs were placed in sterile glass rings (inner diameter 5 mm) in 12-well plates.

3.2.4 Preparation of TissuCol hydrogels

TissuCol® Kit 1.0 Immuno was obtained from Baxter (Unterschleissheim, Germany). 20 µL of the thrombin-L solution were premixed with an equal volume of the TissuCol fibrinogen component and quickly transferred to sterile glass rings that had been placed in 12-well culture dishes. The gels were subjected to 45 min of gelation at 37 °C and 5% CO_2 in an incubator.

3.2.5 Preparation of stable fibrin hydrogels

Stable fibrin hydrogels (final fibrinogen concentration 50 mg/mL or 25 mg/mL, 20 mM $CaCl_2$ and 2.5 U/mL thrombin) were prepared as described previously [19]. Bovine fibrinogen and aprotinin from bovine lung were obtained from Sigma-Aldrich (Steinheim, Germany). In brief, 20 µL of either 100 mg/mL (final concentration 50 mg/mL) or

50 mg/mL (final concentration 25 mg/mL) fibrinogen dissolved in an aprotinin solution [10 000 kallikrein inhibitory units (KIU)/mL] were mixed with an equal volume of a 5 U/mL thrombin solution, and the resulting gels were subjected to the same gelation conditions as TissuCol. For preparation of the thrombin component, a 500 U/mL thrombin solution was prepared from thrombin-S according to the instructions in the TissuCol® Kit and the resulting solution was further diluted 1:100 with thrombin dilution buffer containing 40 mM $CaCl_2$.

3.2.6 3D cell culture

Fibrin gels (40 µL; height 2 mm, diameter 5 mm) were seeded with 1×10^6 ASCs. Cells were suspended in the thrombin component before mixing with the fibrinogen component. Subsequently, the mixture was transferred to sterile glass rings placed in 12-well plates. After gelation, constructs were removed from the glass rings and cultured dynamically on an orbital shaker at 50 rpm (37 °C, 5% CO_2). For two days, constructs were cultured in PGM-2. Two days after seeding (referred to as day 0), adipogenesis was induced by the addition of differentiation medium. Medium exchange was performed every other day. Cell-seeded constructs in the non-induced control groups were cultured in PGM-2 for the entire culture period without adipogenic induction. Constructs were harvested at the in the results section indicated time points.

3.2.7 Mechanical testing

Stable fibrin gels (50 mg/mL and 25 mg/mL fibrinogen) and TissuCol gels were prepared as described above. Gel cylinders measuring 5 mm in height were prepared using sterile glass rings. Mechanical testing was performed using a Z010 AllroundLine Materials Testing Machine and the testXpert® 2 software (version 3.1; Zwick GmbH & Co. KG, Ulm, Germany). A load cell with a capacity of 100 N was used. The fibrin gels were subjected to unconfined compression at a rate of 0.5 mm/min. The force required for the deformation of fibrin gels by 20% (1 mm) of the initial height was measured.

3.2.8 Scanning electron microscopy (SEM)

Porosity and interconnectivity of polyurethane scaffolds were investigated by SEM. Scaffolds were mounted on aluminium stubs, sputtered with silver at 1.5 kV and analyzed at

5.0 kV. Pore structure and interconnectivity were assessed employing a Philips SEM 525 M instrument.

3.2.9 PKH staining

Distribution of ASCs within the PU scaffolds was assessed by labeling the cells with a fluorescent membrane dye (PKH67 green fluorescent cell linker; Sigma-Aldrich, Steinheim, Germany). The expanded cells were trypsinized, washed with PBS and resuspended in Diluent C provided in the staining kit. The staining solution was added to the resuspended cells and incubated whilst shaking gently for 3 min at room temperature. 1 mL of FBS was added to terminate the staining process and after 1 min of incubation, 1 mL PGM-2 was added. The cells were subjected to a centrifugation step (300 g, 5 min) and subsequently washed with PBS thrice. The cells were resuspended in PGM-2 and counted with a hemacytometer. PKH-stained cells seeded on the PU scaffolds were imaged with a fluorescence microscope and images were processed with cellSens™ Dimension Microscope Imaging Software (Olympus, Hamburg, Germany).

3.2.10 BODIPY staining

To visualize the porous structure of the PU scaffolds and to confirm their lipophilic nature, BODIPY staining was performed. 10 µm cross-sections were cut from the PU scaffolds which had been embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, Zoeterwonde, Netherlands). The slides were shortly rinsed with water to remove excess TissuTek and subsequently stained employing a 1:100 dilution of BODIPY® 493/503 (stock 1 mg/mL; Invitrogen, Karlsruhe, Germany) in PBS. After thorough washing with PBS, the slides were coverslipped and microscopically analyzed. Images were processed with Olympus cellSens™ Dimension Microscope Imaging Software.

3.2.11 Histological investigation of adipogenesis

Adipogenesis was histologically investigated by staining cells seeded in 3D constructs as well as in 2D monolayer culture for lipid inclusions with Oil Red O. Prior to staining, the cells were subjected to a fixation step using buffered formalin (3.7% in PBS). The Oil Red O staining solution was prepared by dissolution of Oil Red O (Sigma-Aldrich, Steinheim, Germany) at a concentration of 3 mg/mL in 60% isopropanol. Cells in 2D monolayer culture were incubated with the staining solution for 5 min and rinsed shortly

with distilled water to subsequently counterstain nuclei with hematoxylin (Bio Optica, Milan, Italy).

Whole 3D constructs were incubated in Oil Red O staining solution overnight on an orbital shaker. In the following, constructs were washed thrice with PBS and dehydrated by applying increasing sucrose concentrations (10-60%) over a period of 4 days. The samples were then embedded in Tissue-Tek® O.C.T. compound and 10 µm sections were cut from the embedded, Oil Red O-stained constructs. The slides were rinsed with water, counterstained with hematoxylin for visualization of nuclei and coverslipped with Glycergel Mounting Medium (Dako, Carpinteria, USA). Microscopic images of 2D and 3D cultures were taken and images were processed with Olympus cellSens™ Dimension Microscope Imaging Software.

3.2.12 Leptin quantification by ELISA

Leptin concentration in cell culture supernatants was measured using a sandwich Quantikine® Human Leptin Immunoassay from R&D Systems (Minneapolis, USA). Samples from cell culture medium were collected between day 19 and 21 of culture and stored below -20 °C until analysis. Leptin levels were normalized to the total DNA content of the respective samples.

3.2.13 Quantification of DNA

For DNA content measurements, Hoechst 33258 intercalating dye was purchased from Polysciences (Warrington, USA). Samples were sonified in phosphate-saline buffer. Quantification of DNA content was carried out with a Tecan GENios pro spectrofluorometer (Tecan Deutschland GmbH, Crailsheim, Germany) at an excitation wavelength of 340 nm and an emission wavelength of 465 nm.

3.2.14 Statistics

Results are presented as mean values ± standard deviation (SD). Statistical significance was assessed by either one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni post-test at the level of $p < 0.05$. Statistical analysis was performed using GraphPad Prism, Version 5.0 (GraphPad Software, La Jolla, USA). For the experiments, if not stated otherwise, the number of samples per group was n=3.

3.3 RESULTS

3.3.1 Mechanical resilience of fibrin hydrogels

Stable fibrin gels were investigated as cell carriers for the development of adipose tissue constructs and compared to commercially available TissuCol gels. Already by general handling and in the course of protocol establishment, distinct differences between the fibrin formulations with respect to softness and texture were observed. Thus, prior to performing cell culture experiments, the mechanical stabilities of stable fibrin gels and TissuCol were compared (Figure 1).

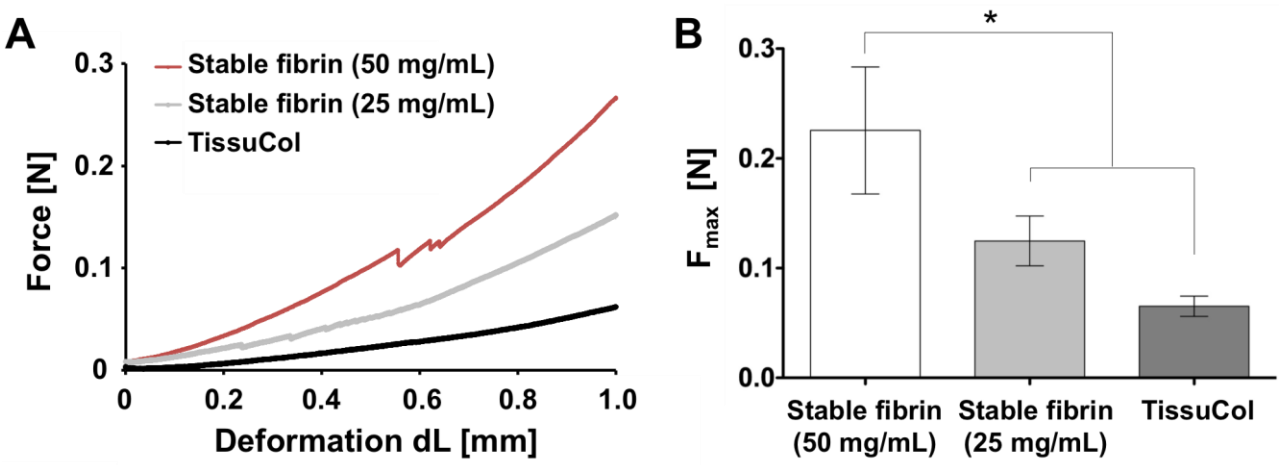

Figure 1. Mechanical resilience of fibrin gels. Stable fibrin gels, prepared at two different fibrinogen concentrations (50 mg/mL and 25 mg/mL), and TissuCol gels were subjected to compression from 0 to 20% strain at a rate of 0.5 mm/min; representative results are shown (A). Maximum force (F_{max}) required for 20% (1 mm) deformation of stable fibrin gels (50 mg/mL and 25 mg/mL) and TissuCol gels (n=5 per group) (B). * Statistically significant differences between the respective fibrin formulations ($p < 0.05$).

Stable fibrin gels were prepared at two different fibrinogen concentrations, namely 50 mg/mL and 25 mg/mL, and TissuCol gels according to the instructions provided by the manufacturer. By measuring the force needed for the compression of the hydrogels at a rate of 0.5 mm/min until reaching a deformation of 20% (1 mm) of the initial height, distinct differences in the mechanical properties of the fibrin gels were observed (Figure 1 A). The respective maximum forces required for a 20% deformation are included in Figure 1 B. TissuCol gels displayed the softest texture and were easily deformed, whereas stable fibrin gels were more resilient. Specifically, for stable fibrin gels at 50 mg/mL, the required

compressive force to reach a 20% deformation of the gel cylinder was significantly higher than that needed for stable fibrin gels at 25 mg/mL and TissuCol gels, respectively (Figure 1 B). Due to the observed high mechanical integrity of stable fibrin gels at a fibrinogen concentration of 50 mg/mL, these gels were in the following employed for the *in vitro* experiments and compared to TissuCol.

3.3.2 Fibrin construct preparation and establishment of in vitro culture

As a first step towards the generation of adipose tissue constructs, stable fibrin and TissuCol gels were seeded with ASCs and subjected to adipogenic culture *in vitro* (Figure 2).

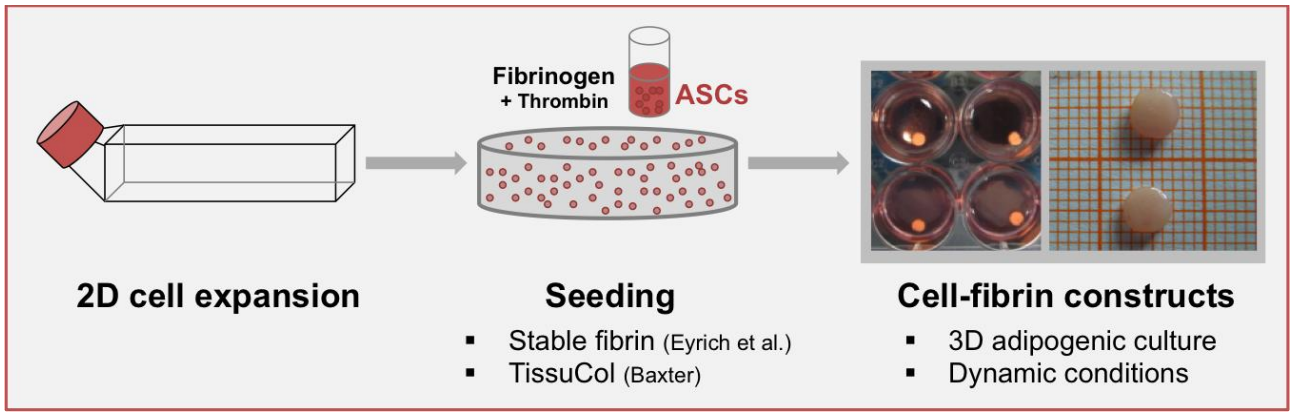

Figure 2. Outline of construct preparation and *in vitro* culture conditions. ASCs were expanded in 2D culture prior to seeding of the fibrin gels. Stable fibrin gels were compared to TissuCol gels. Cell-seeded constructs were subjected to 3D adipogenic culture *in vitro* under dynamic conditions at 50 rpm on an orbital shaker.

ASCs were expanded in 2D culture prior to encapsulation within the gels in order to obtain sufficient cell numbers. For seeding, the cells were suspended in the thrombin component of the respective hydrogel formulation and by adding an equal volume of fibrinogen, fibrin polymerization occurred by enzymatic cleavage of fibrinogen monomers. To achieve a high seeding density, 1×10^6 ASCs were used for construct preparation in 40 µL gels. The positive effect of a high seeding density had previously been shown [39]. After gelation, ASC-seeded fibrin gels were subjected to dynamic culture conditions and a culture setup according to Figure 3.

Following seeding (day -2), constructs were cultured in PGM-2 for 2 days prior to adipogenic induction of ASCs (day 0). Adipogenesis was induced by addition of a commonly used hormonal cocktail containing insulin, IBMX, dexamethasone and

indomethacin. Cell-seeded constructs were also cultured under non-induced conditions in PGM-2 for comparison.

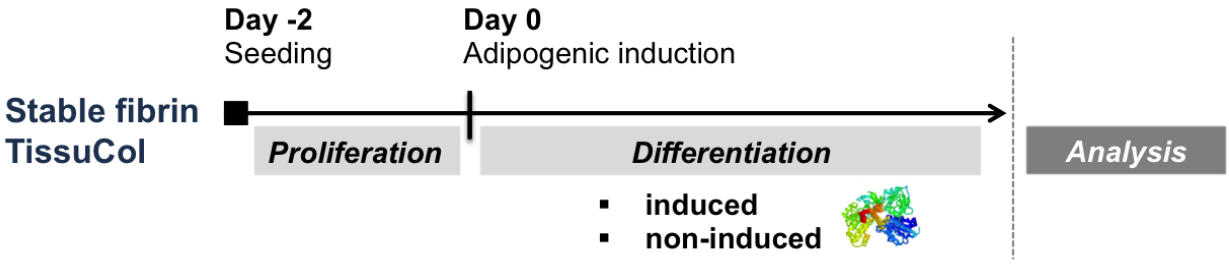

Figure 3. Experimental setup. Stable fibrin and TissuCol gels were seeded with ASCs (day -2). Adipogenic differentiation was induced by addition of a hormonal cocktail on day 0. For comparison, non-induced constructs were cultured under control conditions. Constructs were subsequently analyzed on the cellular and molecular level.

To compare the 3D differentiation capacity of ASCs to conventional 2D culture, ASCs were also seeded in 12-well plates at a seeding density of 3 x 10^4 cells/cm^2 and treated according to the 3D constructs. General biocompatibility of the stable fibrin formulation had previously been demonstrated by Eyrich *et al.* [19], hence the parameters evaluated in this chapter only relate to the specific application for adipose tissue engineering.

In the course of adipogenic culture, the macroscopic appearance of stable fibrin gels pointed towards an adequate volume stability with no visible contraction or shrinkage. In contrast, TissuCol gels were susceptible to degradation and presumably contraction, resulting in a significant size and volume reduction over time. (For the detailed macroscopic assessment of the fibrin gels and their comparison with composite fibrin/PU scaffolds, the reader is referred to Chapter 4.)

3.3.3 Adipogenic differentiation in fibrin gels in vitro

Cellular differentiation of ASCs in 3D fibrin constructs and conventional 2D culture was assessed by staining cytoplasmatic triglycerides with Oil Red O (Figure 4). In both fibrin formulations, the cells acquired a rounded morphology, displayed distinct Oil Red O-positive lipid vacuoles and appeared to differentiate to a similar extent (Figure 4 A). There was no visible difference in the degree of differentiation between outer and inner areas of the constructs. ASCs in the induced groups were homogeneously distributed in the fibrin gels. Enhanced cell density and less homogenously distributed cells were observed in TissuCol gels without adipogenic induction. This effect was less pronounced in non-

induced stable fibrin gels. Overall, in groups that did not receive adipogenic induction, no substantial lipid accumulation was detectable. Cells in 2D culture displayed a spreaded cell shape, even under induced conditions (Figure 4 B), whereas ASCs in 3D hydrogels had a more rounded, 'adipocyte'-like morphology (Figure 4 A). Adipogenic induction in 2D culture similarly resulted in the accumulation of intracellular lipids as reflected by Oil Red O-stained vacuoles.

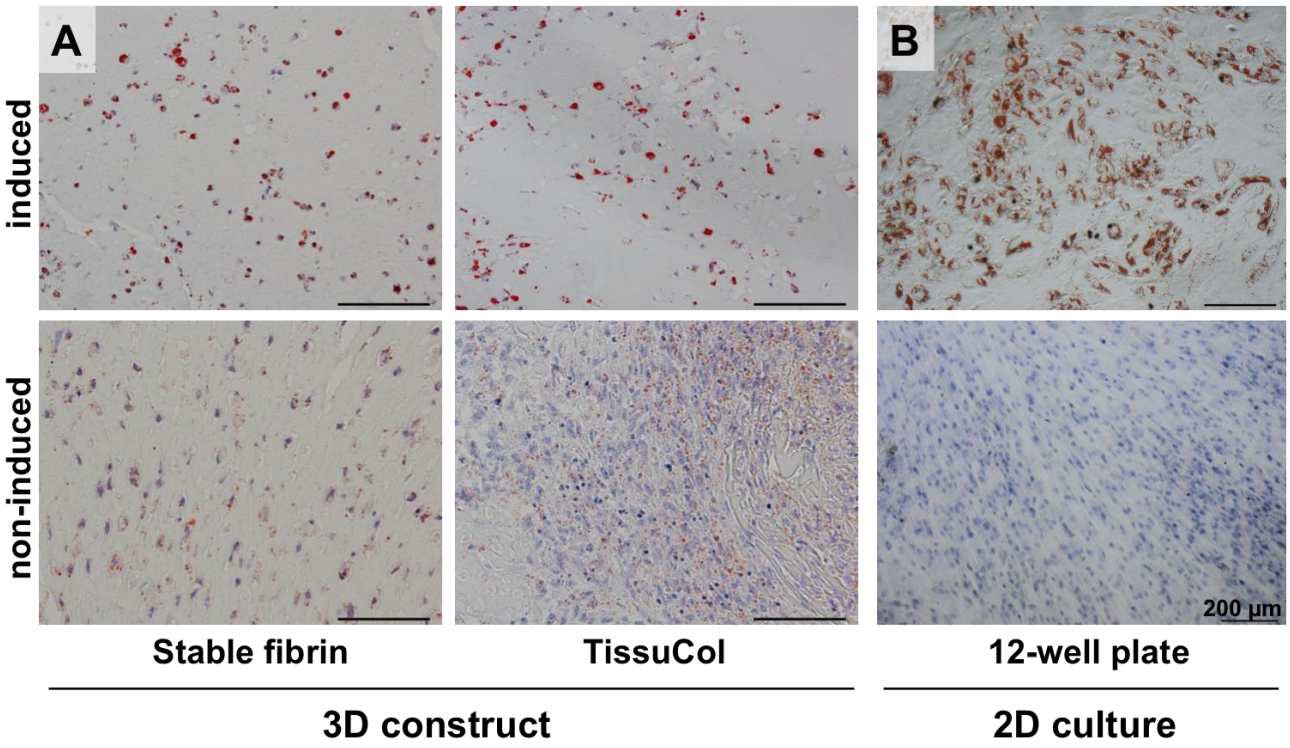

Figure 4. Differentiation of ASCs in stable fibrin and TissuCol gels (A), as well as in 2D culture (B). Oil Red O staining was performed at day 10 after induction; nuclei were counterstained with hematoxylin. Scale bars represent 200 µm.

On the molecular level, leptin served as a marker for late adipogenesis (Figure 5). Leptin secretion by ASCs seeded in stable fibrin and TissuCol gels was functionally assessed after 21 days of adipogenic culture using a sandwich ELISA. Upon advanced differentiation, induced ASCs in both stable fibrin and TissuCol gels produced significantly higher amounts of leptin than in non-induced constructs. In stable fibrin gels, leptin secretion was further significantly elevated compared to TissuCol hydrogels.

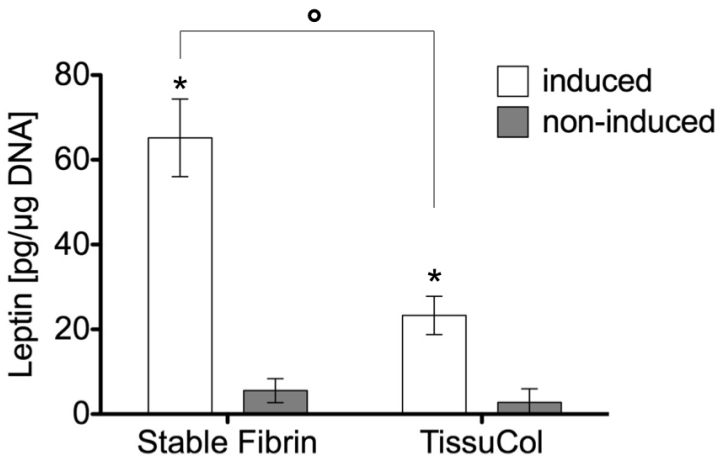

Figure 5. Quantification of leptin secretion. Leptin levels were analyzed in cell culture supernatants of ASC-seeded stable fibrin and TissuCol gels collected between day 19 and 21 of culture; the obtained values were normalized to total DNA content. * Statistically significant differences between induced and non-induced constructs in the respective groups ($p < 0.05$). ° Statistically significant differences between stable fibrin and TissuCol gels ($p < 0.05$).

3.3.4 Investigation of poly(ε-caprolactone)-based polyurethane scaffolds as scaffolding material

Porous PU scaffolds were investigated as potential support structures for the generation of composite constructs via combination with stable fibrin gels. To first demonstrate the general suitability of porous PU scaffolds as biomaterial component for adipose tissue engineering, the scaffolds alone were characterized in terms of handling and material properties, as well as interaction with ASCs.

In first experiments, wettability, hydrophobicity and porosity of the scaffolds were tested (Figure 6). Upon addition of culture medium to the scaffolds using a micropipette, the aqueous liquid was rejected and remained as a drop on top of the porous structures (Figure 6 A). Repeatedly exerting a weak manual pressure using the tip of a micropipette, or optionally a thin cannula, resulted in the absorption of the culture medium into the scaffolds. In a second test, the scaffolds were cryosectioned, mounted on cover slips and stained with BODIPY, a lipophilic fluorescent dye that is usually applied to visualize lipid vacuoles (Figure 6 B). The PU scaffolds strongly took up the lipophilic dye and revealed a highly porous structure upon microscopic analysis. The internal 3D microarchitecture of the PU scaffolds was further investigated by scanning electron microscopy (SEM) (Figure 6 C). The scaffolds were shown to feature uniformly sized, interconnected pores. Overall, the

internal structure appeared homogeneous, and interconnective pores were evenly distributed, displaying approximately 4-6 interconnections per larger pore. (For a more detailed evaluation of pore dimensions, the reader is referred to Chapter 4.)

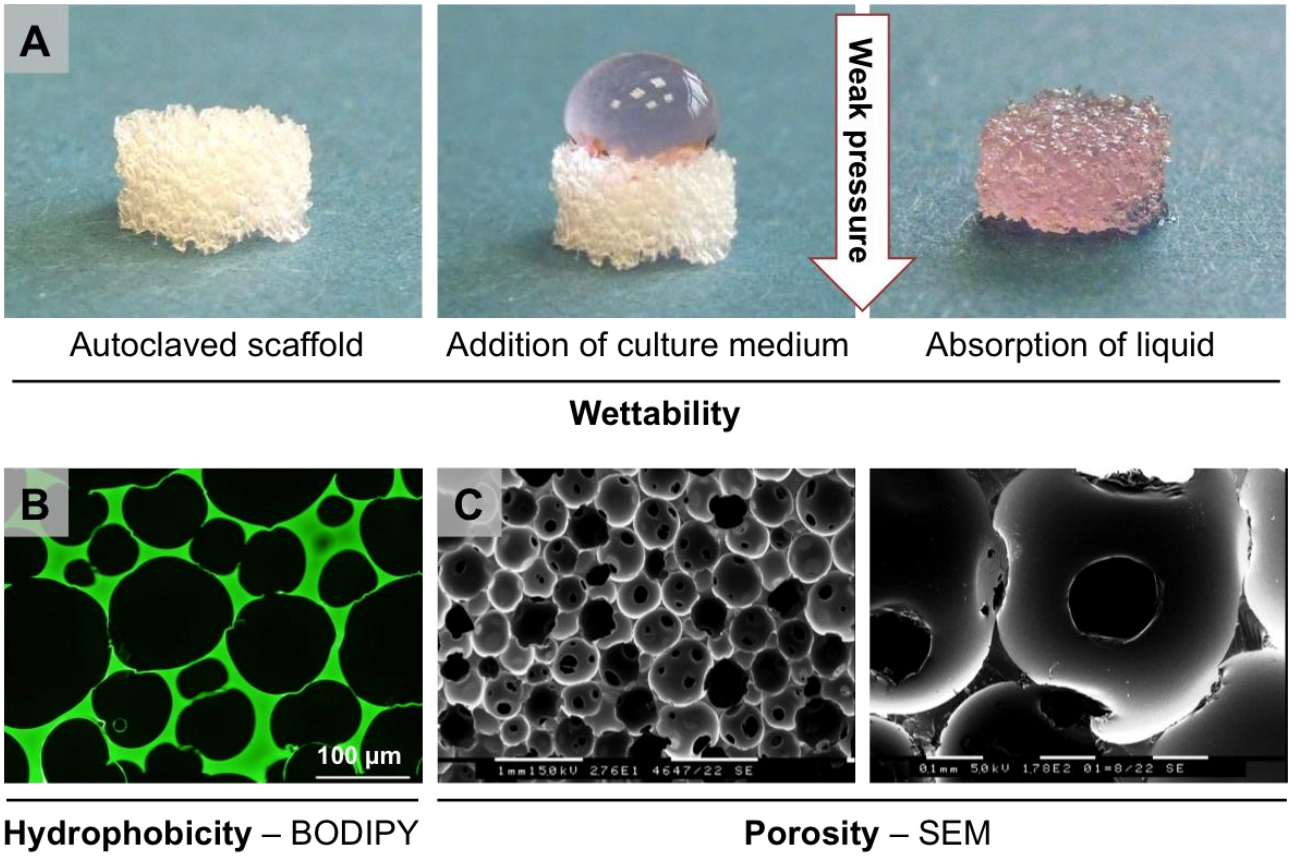

Figure 6. Characteristics of porous PU scaffolds. Wettability was tested by incorporation of cell culture medium (A). Staining with lipophilic BODIPY revealed scaffold hydrophobicity (B). SEM measurements were conducted to visualize pore size and pore interconnectivity (C).

To investigate seedability and the response of cells towards the material, the PU scaffolds were directly seeded with ASCs and subjected to adipogenic and non-induced culture under dynamic conditions (Figure 7). To facilitate tracking, cells were labeled with a fluorescent membrane dye (PKH staining) prior to seeding. The efficiency of the PKH dye for labeling of ASCs *in vitro* over extended culture periods had been confirmed in previous experiments (data not shown). As reflected by the reduction of the fluorescence signal over time, the cell load inside the scaffolds diminished during the course of adipogenic culture between day 0 and day 21 (Figure 7 A). The cells apparently formed round, spheroid-like structures inside the pores as indicated by intensely stained cell clusters.

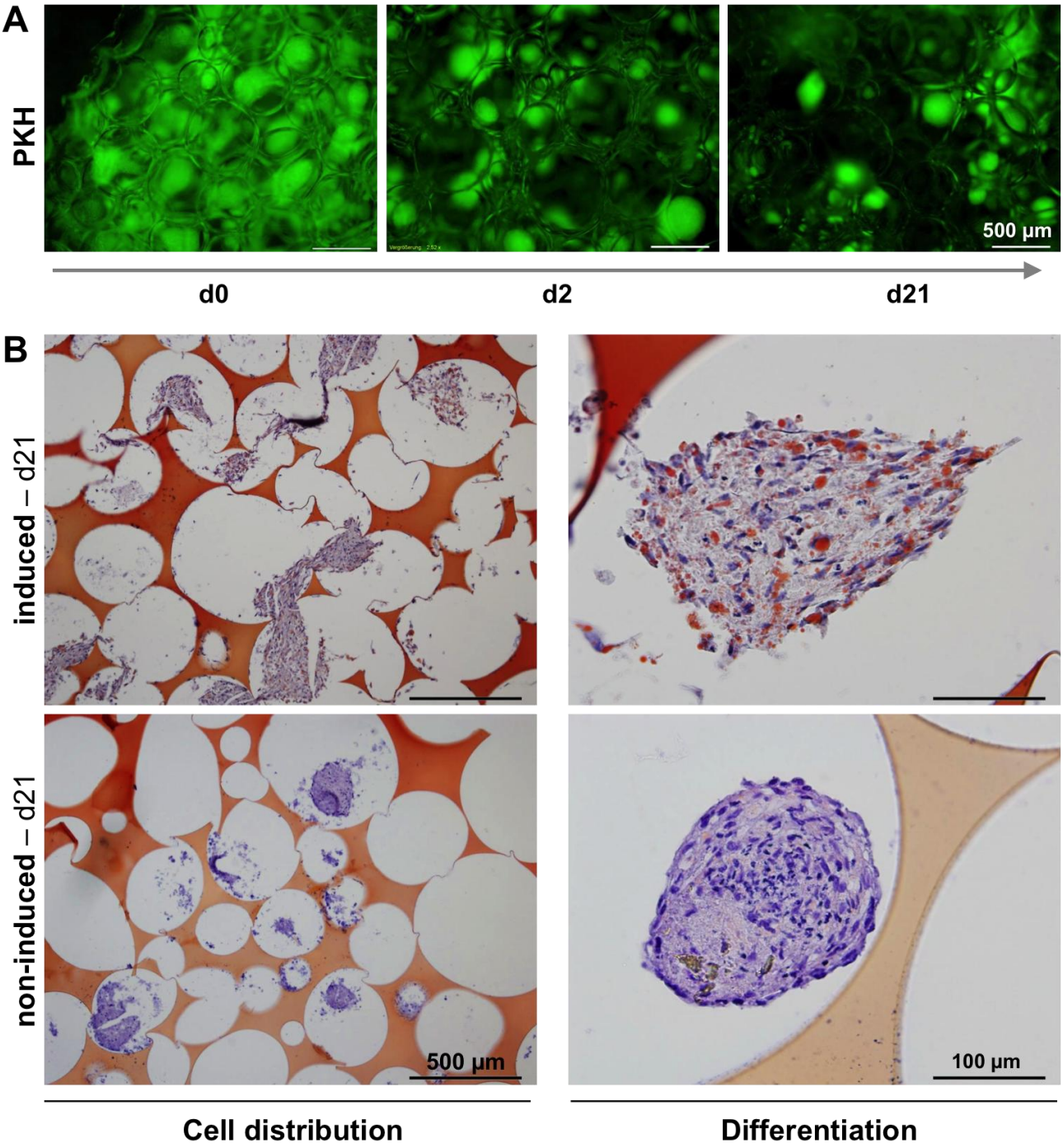

Figure 7. Seedability and cell compatibility of PU scaffolds. PHK-labeled ASCs were directly seeded onto the porous scaffolds (A). Diminishing amounts of ASCs were observed during culture between day 0 and day 21; scale bar represents 500 µm. Cell distribution and differentiation of ASCs in PU scaffolds after 21 days of culture under induced and non-induced conditions (B). Lipid formation was assessed by Oil Red O staining; nuclei were counterstained with hematoxylin; scale bars represent 500 and 100 µm, respectively.

In accordance with the results from the PKH-experiment, histological cross-sections of the porous scaffolds after 21 days of adipogenic culture revealed that many pores were devoid of cells and that the overall cell density was low (Figure 7 B). The formation of rounded cell

clusters inside the porous structures was confirmed. As reflected by Oil Red O staining of accumulated lipid droplets, ASCs encapsulated in the cell clusters differentiated upon adipogenic induction. Smaller and more densely packed spheroid structures were formed under non-induced conditions, where no cellular differentiation was observed. Overall, it appeared that no direct interaction between the cells and the porous scaffolds occurred.

3.4 DISCUSSION

On the basis of biocompatible carrier materials and tissue-specific cells, promising advances may be made towards the generation of functional adipose tissue substitutes with inherent long-term stability for soft tissue replacement. Therefore, with the overall goal to bioengineer volume-stable composite adipose tissue constructs, the basic evaluation of stable fibrin hydrogels and porous PU scaffolds as potential biomaterial components for this purpose was successfully approached in the present work.

In a first step, the suitability of a recently developed stable fibrin formulation for construct generation was demonstrated. As far as stability was concerned, stable fibrin as hydrogel carrier was shown to display an enhanced mechanical resilience and prolonged longevity in comparison to a conventional fibrin formulation (TissuCol). Specifically, at two different fibrinogen concentrations, namely 50 mg/mL and 25 mg/mL, stable fibrin gels endured higher compressive forces and were less susceptible to deformation than commercially available TissuCol gels with a comparable fibrinogen content (manufacturer: 35-55 mg/mL). This observation was well in agreement with the thorough rheological characterization of stable fibrin gels by Eyrich *et al.*, by which the mechanical superiority of gels prepared at fibrinogen concentrations ranging from 25 to 50 mg/mL was demonstrated [19]. Apart from a softer texture, TissuCol gels were increasingly prone to degradation, in accordance with reports by Torio-Padon *et al.* [11] and our own observations (Chapter 4), rendering the stable fibrin formulation an appealing alternative to commercially available fibrin as scaffolding material.

The ease of handling and simple preparation procedure, as well as the physiological polymerization and crosslinking conditions [6], facilitated the seeding of both stable fibrin gels and TissuCol, and recommended their use for cell delivery *in vivo* upon implantation but also as injectable tissue fillers. Direct cell encapsulation further promoted an adequate cell distribution and favorable cell-matrix interactions, which are considered prerequisites for homogeneous tissue formation *in vitro* and *in vivo* [5,40]. With the intention of engineering cartilage constructs, the general biocompatibility of stable fibrin gels had been demonstrated in cell culture experiments before using bovine chrondrocytes [19], and was now similarly noted for ASCs.

Stable fibrin gels were shown permissive of adipogenic development of ASCs as mirrored by lipid accumulation comparable to TissuCol gels, which evidently promote adipogenesis [11,37]. The presence of uniformly differentiated cells in outer and inner areas of the constructs was partially attributed to the applied dynamic culture conditions, which had previously been found advantageous compared to static conditions by Fischbach *et al.* [41]. The homogeneous cellular response to adipogenic stimuli pointed towards beneficial mass transport properties of the fibrin hydrogels, providing sufficient nutrient and oxygen supply to the cells. Further, the cell-instructive and tissue supportive nature of fibrin was reflected by spreading, proliferation and migration of cells encapsulated in non-induced constructs.

Adipokine secretion by adipogenically induced ASCs served as an additional positive indicator for the ability of stable fibrin gels to promote adipogenesis. Despite histological results showing no evident differences in lipid accumulation between the two fibrin formulations, leptin secretion of ASCs in stable fibrin gels was elevated relative to TissuCol. It is thinkable that this finding is related to a direct effect of the biomaterial properties on cellular function, for example via alteration of gene expression and adhesion in response to differences in matrix stiffness [42,43].

The observations on the cellular and molecular level, such as lipid accumulation and leptin secretion, rendered stable fibrin gels eligible for sustained adipose tissue growth *in vitro* as well as *in vivo*. Coherent tissue formation may additionally be improved by enhanced retention of matrix components and the adequate presentation of growth factors by the stable fibrin network [5,13]. The profound stability of stable fibrin gels also potentially allows culture setups that include the adipogenic preconditioning of encapsulated ASCs *in vitro* and subsequent *in vivo* implantation of the constructs at the recipient site once cells have reached an adequate differentiation status [44]. The fact that TissuCol and other FDA-approved fibrin sealants such as Evicel or Crosseal all have in common that they are rapidly degraded and thus rather unfavorable as scaffolding materials [10], encourages the use of the developed stable fibrin gels for adipose construct generation. Since the two key components fibrinogen and thrombin can be extracted from autologous plasma, the application of stable fibrin, although not FDA approved, together with human primary ASCs permits the generation of autologous implants [5,15,27].

In a second step towards the design of volume-stable constructs for adipose repair, porous biodegradable PU scaffolds were evaluated as mechanically stable support structures. Various approaches before have rendered the principle of mechanical protection effective for long-term adipose tissue development. This primarily included the implantation of solid structures to shield the developing tissue *in vivo* [12,30,31]. However, in the context of soft tissue reconstruction, the implantation of solid and non-degradable support structures was considered inapplicable for clinical translation. Here, biodegradable PU scaffolds, exhibiting a soft and sponge-like texture, represented an attractive alternative. Important scaffold characteristics such as high porosity and interconnectivity were regarded to promote cell delivery, adequate supply of nutrients and oxygen, as well as upon implantation *in vivo*, the establishment of coherent tissue and a functional vascular network [1,5,7]. Despite low wettability and deficient cell adhesion, and as a result, insufficient seeding and inhomogeneous cell distribution, PU scaffolds displayed adequate cytocompatibility as reflected by adipogenic differentiation of ASC-clusters within the pores.

Consequently, the generation of composite constructs, using a combination of the porous PU scaffolds with the pre-evaluated stable fibrin gels as cell carriers, is considered advantageous to on the one hand overcome the poor cell-biomaterial interaction in PU scaffolds, and on the other, to add mechanical strength to fibrin as hydrogel component. Specifically, the open-pored structure of the PU scaffolds and the efficiency of stable fibrin gels for cell delivery are expected to facilitate seeding of composite fibrin/PU constructs. Supported by the interconnectivity of the porous scaffolds, the advantageous mass transport properties of stable fibrin gels may further enhance the differentiation potential of incorporated cells in composite constructs. Possibly, the restricted interaction of ASCs with the inert PU scaffolds might even prove advantageous in this context, preventing a differential response of the incorporated cells towards the two structurally distinct biomaterials.

Joining the beneficial properties of fibrin gels and porous scaffolds is further intended to promote the generation of inherently stable constructs without having to increase hydrogel stiffness by enhanced crosslinking or fiber density. Especially, by means of their homogeneous interconnected 3D architecture, the porous PU scaffolds are attributed great

potential to reinforce the hydrogel substrate without compromising texture or coherence. Accordingly, on the basis of this material combination, long-term stable cartilage constructs have previously been successfully prepared [40]. As far as persistence *in vivo* is concerned, the hydrophobic nature of the PU likely prevents excessive biodegradation through hydrolysis, with the scaffolds featuring an estimated period of degradation in the range of 1 to 1.5 years (information provided by the manufacturer).

Ultimately, in this study, well-suited biomaterials for the generation of adipose tissue constructs with improved volume stability were identified. Meeting the physiological requirements for adipose regeneration, stable fibrin gels were shown to provide a tissue-inductive environment that supports 3D organization and adipogenic differentiation of ASCs. Biodegradable PU scaffolds with large, interconnected pores and adequate compatibility with cells were regarded highly applicable as structual support. Overall, the suitability of the two individual biomaterials for the generation of composite constructs that integrate the matrix-like properties of fibrin gels with the structural integrity provided by the porous PU scaffolds was demonstrated, advocating the investigation of composite fibrin/PU constructs in continuative work.

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Chapter 4

Development of Volume-Stable Adipose Tissue Constructs Using Polycaprolactone-Based Polyurethane Scaffolds and Fibrin Hydrogels

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Development of Volume-Stable Adipose Tissue Constructs Using Polycaprolactone-Based Polyurethane Scaffolds and Fibrin Hydrogels

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4.1 ABSTRACT

Adipose tissue engineering aims at the restoration of soft tissue defects and the correction of contour deformities. It is thereby crucial to provide functional adipose tissue implants with appropriate volume stability. Here, we investigate two different fibrin formulations alone or in combination with biodegradable polyurethane (PU) scaffolds as additional support structures, with regard to their suitability to generate volume-stable adipose tissue constructs. Human adipose-derived stem cells (ASCs) were incorporated in a commercially available fibrin sealant as well as a stable fibrin hydrogel previously developed by our group. The composite constructs made from the commercially available fibrin and porous poly(ε-caprolactone)-based PU scaffolds exhibited increased volume stability as compared to fibrin gels alone; however, only constructs using the stable fibrin gels completely maintained their size and weight for 21 days. Adipogenesis of ASCs was not impaired by the additional PU scaffold. After induction with a common hormonal cocktail, for constructs with either fibrin formulation, strong adipogenic differentiation of ASCs was observed after 21 days *in vitro*. Furthermore, upregulation of adipogenic marker genes was demonstrated at mRNA (PPARγ, C/EBPα, GLUT4, aP2; qRT-PCR) and protein levels (leptin; ELISA). Stable fibrin/PU constructs were further evaluated in a pilot *in vivo* study, resulting in areas of well-vascularized adipose tissue within the implants after only 5 weeks.

4.2 INTRODUCTION

For the reconstruction of soft tissue defects resulting from trauma, congenital disease or tumor resection, the development of viable and volume-stable adipose tissue is of major clinical interest [1,2]. To date, soft tissue augmentation is frequently carried out by transplantation of either free or pedicled autologous fat flaps [3,4] or, alternatively, synthetic materials such as silicone are implanted. However, autologous grafts are prone to shrinkage or fibrosis leading to unpredictable outcomes with regard to flap shape and retention [2,5], whereas synthetic implants are associated with encapsulation or foreign body reactions [6]. Especially for the restoration of large tissue volumes, i.e. after mastectomy or trauma, the therapeutic options are limited.

Adipose tissue engineering holds the promise to provide answers to this still growing clinical need. However, maintenance of volume and shape, the integration into the host tissue and a functional vasculature for long-term survival of engineered adipose tissue still represent crucial challenges [5]. Volume stability is of particular importance for clinical application as well as, on the cellular level, for the protection of maturing adipocytes, which are sensitive to mechanical stress due to their high cytoplasmatic lipid content [7]. The principle of shielding the implant from surrounding destabilizing forces using hollow support structures has been repeatedly shown to be beneficial for volume retention and favorable development of adipose tissue constructs [5]. For example, Walton *et al.* developed constructs with a built-in vascular supply protected by silicone domes in rats and demonstrated *de novo* adipogenesis in Matrigel [8]. In several studies, Morrison and co-workers have employed tube-shaped chamber configurations made from silicone and, integrating a vascular pedicle, the generation of adipose tissue was shown [9-11]. While the protective effect of the support structures was demonstrated, the persistence of non-degradable silicone shields or other solid structures after implantation is considered disadvantageous for clinical application and the transfer to biodegradable materials is desirable.

Therefore, in this study, biodegradable poly(ε-caprolactone)-based PU scaffolds with large interconnected pores were investigated with regard to their use as support structures and employed in combination with hydrogels as cell carriers. The PU scaffolds provide not only

volume stability but also mechanical resilience to the constructs, which is required to withstand compressive forces *in vivo* after implantation [12,13]. In turn, hydrogels can feature ECM-like properties that support cell viability, attachment and differentiation, and the limitations of solid scaffolds, such as inhomogeneous cell distribution and impaired matrix retention, are eliminated [14-16]. As the hydrogel component, fibrin was used, which is a physiological and thus biocompatible material that can be prepared as an autologous implant from the patient's blood components [16]. Here, a fibrin formulation previously developed by our group [17] was employed, as its long-term stability (provided by specific fibrinogen concentration, ion concentration and pH) renders it a suitable component for the generation of volume-stable implants. For comparison, TissuCol Immuno, a commonly applied commercially available fibrin sealant [14] was investigated.

Human ASCs were employed for seeding, as they can easily be isolated from the patient's fat depots in a clinical setting, thus abolishing the risk of immune reactions and implant rejection [1,18]. Additionally, ASCs are preferred over mature adipocytes due to their higher resistance to ischemia and mechanical stress, and they can be readily differentiated into the adipogenic lineage [5,14].

Specifically, two fibrin gels with different stabilities were seeded with ASCs and were investigated either alone or in combination with the porous PU scaffolds *in vitro*. The generated constructs were evaluated with regard to cell viability, construct stability and adipogenic differentiation on the cellular and molecular level. The *in vivo* performance of the volume-stable constructs was assessed in a pilot study in nude mice, integrating a femoral arteriovenous (AV) bundle for effective vascularization.

4.3 MATERIALS AND METHODS

4.3.1 Cell isolation and expansion

Human ASCs were isolated from subcutaneous adipose tissue of healthy female donors obtained in lipoaspiration procedures. The study was approved by the ethics committee of the University of Wuerzburg, Germany, and written informed consent was obtained from all patients. Patients were between 20 and 40 years of age and had a body mass index (BMI) in the range of 28 to 33.

For ASC isolation, fat tissue was digested with 0.1% collagenase NB4 from Clostridium histolyticum (Serva Electrophoresis, Heidelberg, Germany) in freshly prepared collagenase buffer for 2 h at 37 °C on an orbital shaker. The resulting suspension was filtered through a 100 μm nylon mesh and centrifuged at 300 g for 10 min. The fat layer on top was aspirated and pelleted cells were washed with phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria). The obtained stromal-vascular fraction (SVF) was resuspended in basal medium containing Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12; Invitrogen, Karlsruhe, Germany) supplemented with 1% penicillin-streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 10% fetal bovine serum (FBS), both obtained from Invitrogen (Karlsruhe, Germany). 5% DMSO (Sigma-Aldrich, Steinheim, Germany) were added to the medium for cryopreservation of the SVF at a cell density of approximately 1.5×10^6 cells/mL.

For ASC expansion, cryopreserved SVF cells were thawed and plated on tissue culture-treated plastic flasks (175 cm^2). ASCs were selected via plastic adherence. The cells were cultured to a subconfluent level and expanded for two passages in basal medium until seeding of the scaffolds. For passaging, trypsin-EDTA at 0.25% from Invitrogen (Karlruhe, Germany) was used.

4.3.2 PU scaffolds

Poly(ε-caprolactone)-based polyurethane scaffolds were manufactured as previously described [19]. Briefly, polycaprolactone diol (28.3 wt%), polycaprolactone triol (17.0 wt%), poly(ethylene glycol) (19.5 wt%), poly(tetramethylene glycol) (5.7 wt%), and a slight excess of isophorone diisocyanate (28.6 wt%) were mixed with small amounts of distilled water (0.2 wt%) and Pluronic P-123 (0.1 wt%) and heated to 40 °C.

Diazabicycloundecene (0.6 wt%) was dissolved in methylal and added to the previous mixture at 35 °C leading to a gas foaming process and pore formation. All reagents were either obtained from Acros Organics (Geel, Belgium) or Sigma-Aldrich (Steinheim, Germany). The scaffolds were annealed for 3 h at 50 °C. Residual solvents were removed by treating the scaffolds with boiling water, followed by drying to constant weight at 10^{-2} mbar. Discs measuring 5 mm in diameter and 2 mm in height were prepared using sterile biopsy punches (Stiefel Laboratories, Sligo, Ireland). For sterilization, the PU scaffolds were autoclaved.

Polymeric discs were placed within sterile glass rings (inner diameter 5 mm) in 12-well plates. Combined fibrin/PU constructs were made by pipetting 40 µL of cell-hydrogel suspension onto the polymeric discs, which was subsequently taken up into the scaffold.

4.3.3 Preparation of TissuCol hydrogels

TissuCol® Kit 1.0 Immuno was obtained from Baxter (Unterschleissheim, Germany). 20 µL of the thrombin-L solution were premixed with an equal volume of the TissuCol fibrinogen component and quickly transferred into sterile glass rings that had been placed in 12-well culture dishes. The hydrogels were subjected to 45 min of gelation at 37 °C and 5% CO_2 in an incubator.

4.3.4 Preparation of stable fibrin hydrogels

Stable fibrin hydrogels (final fibrinogen concentration 50 mg/mL, 20 mM $CaCl_2$ and 2.5 U/mL thrombin) were prepared as described previously [17]. Bovine fibrinogen and aprotinin from bovine lung were obtained from Sigma-Aldrich (Steinheim, Germany). In brief, 20 µL of 100 mg/mL fibrinogen dissolved in an aprotinin solution [10 000 kallikrein inhibitory units (KIU)/mL] were mixed with an equal volume of a 5 U/mL thrombin solution and were subjected to the same gelation conditions as TissuCol. Before, for preparation of the thrombin component, a 500 U/mL thrombin solution was prepared from thrombin-S according to the instructions in the TissuCol® Kit and the resulting solution was further diluted 1:100 with thrombin dilution buffer containing 40 mM $CaCl_2$.

4.3.5 3D cell culture

Fibrin gels (40 µL) as well as fibrin/PU composites measuring 5 mm in diameter and 2 mm in height were seeded with 1 x 10^6 ASCs. Cells were suspended in the thrombin component

before mixing with the fibrinogen component. Subsequently, the mixture was either transferred into sterile glass rings or pipetted onto the PU scaffolds that were placed within the glass rings. The constructs were cultured dynamically on an orbital shaker at 50 rpm (37 °C, 5% CO_2). For two days, constructs were cultured in preadipocyte growth medium 2 (PGM-2), consisting of preadipocyte basal medium 2 (PBM-2; Lonza, Walkersville, USA) supplemented with 10% FBS and 1% penicillin-streptomycin. Two days after seeding (referred to as day 0), adipogenesis was induced by the addition of differentiation medium [PGM-2 with 1.7 µM insulin, 1 µM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine (IBMX) and 200 µM indomethacin]. For medium preparation, bovine insulin was kindly provided by Sanofi-Aventis (Frankfurt, Germany), IBMX was obtained from Serva Electrophoresis (Heidelberg, Germany), and indomethacin and dexamethasone were purchased from Sigma-Aldrich (Steinheim, Germany). Constructs were cultured for 21 days and media exchange was performed every other day. Cell-seeded constructs in the non-induced control groups were cultured in PGM-2 for the entire culture period without adipogenic induction.

4.3.6 Scanning electron microscopy (SEM)

Porosity and interconnectivity of polyurethane scaffolds were investigated by SEM. Scaffolds were mounted on aluminum stubs, sputtered with silver at 1.5 kV and analyzed at 5.0 kV. Pore structure and interconnectivity were assessed employing a Philips SEM 525 M instrument. The average pore size of the PU scaffolds was calculated by measuring 30 pores each in 8 overview images from different regions of the scaffolds; for the interconnective pores, 20 pores each were measured.

4.3.7 Live/dead staining

Cell viability was evaluated using a live/dead cell staining kit from PromoKine (Heidelberg, Germany). Whole constructs were washed three times with PBS and stained by applying 0.5 mL of staining solution containing 4 µM ethidium bromide homodimer III (EthD-III) and 2 µM calcein acetoxymethyl ester (calcein-AM) in PBS to the constructs within each well. After 1 hour, the dye was removed and the constructs were washed with PBS and cut transversely in half. The middle sections were subsequently viewed under a fluorescence microscope (ex/em 460-490 nm/520 nm and ex/em 510-550 nm/590 nm, respectively) and

the resulting images overlaid (Olympus cellSens™ Dimension Microscope Imaging Software; Olympus, Hamburg, Germany).

4.3.8 Histological investigation of adipogenesis

Adipogenesis was histologically investigated by staining of the constructs for lipid inclusions with Oil Red O after fixation using buffered formalin (3.7% in PBS). The Oil Red O staining solution was prepared by dissolution of Oil Red O (Sigma-Aldrich, Steinheim, Germany) in 60% isopropanol at a concentration of 3 mg/mL. After staining, constructs were dehydrated by applying increasing sucrose concentrations (10-60%) over a period of 4 days and finally embedded in Tissue-Tek® O.C.T. compound manufactured by Sakura Finetek (Zoeterwonde, Netherlands). Sections (10 µm) were cut from the embedded, Oil Red O-stained constructs. Slides were rinsed with water and counterstained with hematoxylin (Bio Optica, Milan, Italy) for visualization of nuclei. Microscopic images were taken and processed with the Olympus cellSens™ software.

4.3.9 Molecular investigation of adipogenesis by real-time qRT-PCR

For real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), constructs were lysed prior to RNA isolation employing a TissueLyser from Qiagen (Hilden, Germany). TRIzol® Reagent and Ambion RNaseZAP were obtained from Invitrogen (Karlsruhe, Germany). First-strand cDNA was synthesized from total RNA using the ImProm-II™ Reverse Transcription System (Promega, Madison, USA). Real-time quantitative PCR analysis was carried out using specific QuantiTect® Primer Assays (Qiagen) for the adipogenic transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα), and the adipogenic marker genes glucose transporter 4 (GLUT-4) and adipocyte protein 2 (aP2). For detection, MESA GREEN qPCR MasterMix Plus with Meteor*Taq* polymerase (Eurogentec, Seraing, Belgium) was used at the following cycling conditions: 95 °C for 15 min followed by 40 cycles at 95 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec. mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each construct group and time point. Fold increase in expression levels for each gene was determined using the $2^{-\Delta\Delta CT}$ method. For easier comparison, the obtained values were further normalized to the values of the stable fibrin/PU constructs at day 0.

4.3.10 Adipokine quantification by ELISA

Concentration levels of the adipokine leptin in cell culture media were measured using a sandwich Quantikine® Human Leptin Immunoassay from R&D Systems (Minneapolis, USA). Cell culture supernatants were collected between day 19 and 21 of culture and stored below -20 °C until analysis. Leptin levels were normalized to the total DNA content of the respective samples.

4.3.11 Quantification of DNA

For DNA content measurements, Hoechst 33258 intercalating dye was purchased from Polysciences (Warrington, USA). Samples were sonified in phosphate-saline buffer. Quantification of DNA content was carried out with a Tecan GENios pro spectrofluorometer (Tecan Deutschland GmbH, Crailsheim, Germany) at an excitation wavelength of 340 nm and an emission wavelength of 465 nm.

4.3.12 In vivo implantation

In a pilot study, stable fibrin/PU constructs were implanted with an integrated AV bundle (flow-through type) in a nude mouse model. Female NMRI nu/nu mice (Charles River, Wilmington, USA) of 6 to 7 weeks were used. The study was conducted with permission of the Bavarian Ethics Committee. The animals were sedated with a mixture of meditomidine (Pfizer GmbH, Berlin, Germany), midazolam (Ratiopharm, Ulm, Germany) and fentanyl (Janssen-Cilag, Baar, Switzerland) (MMF) before making a small incision and dissecting the femoral vessels. A small cut was made transversely through the fibrin/PU constructs and the AV bundle was inserted. Two sutures were used to close the cut through the construct and the incision was closed with interrupted sutures. ASC-seeded stable fibrin/PU constructs were prepared and cultured *in vitro* under adipogenic induction in differentiation medium for 7 days (see section 4.3.5). After this precultivation period, the constructs were washed with PBS and subsequently implanted into the animals. One construct each was inserted on either side of the animal. Additionally, two constructs per animal were implanted without vascular pedicle; they were placed subcutaneously between the scapulae by making a small incision in the neck region and preparing two small pockets to the left and right of the incision. Each construct was placed in one pocket and marked with a prolene suture. Again, the incision was closed with interrupted sutures. Four animals were

employed, i.e., eight constructs/group were implanted. After surgery, the MMF was antagonized with atipamezol (Pfizer GmbH, Berlin, Germany), flumazenil (Hameln Pharma Plus GmbH, Hameln, Germany) and naloxone (Inresa Arzneimittel GmbH, Freiburg, Germany) (AFN). For explantation after 5 weeks *in vivo*, the mice were again sedated with MMF, all implants were removed and the mice were sacrificed.

4.3.13 Evaluation of tissue formation in vivo

The explanted constructs were washed twice with PBS and fixed in 3.7% buffered formalin over 4 days. After dehydration in increasing concentrations of ethanol, constructs were embedded in paraffin, sectioned and afterwards deparaffinized and stained with hematoxylin and eosin (H&E; Bio Optica, Milan, Italy). Microscopic images of the middle sections were taken to evaluate tissue formation.

Anti-human vimentin staining was employed to investigate the origin of the cells within the constructs. Deparaffinized cross-sections were treated with pepsin solution (Invitrogen, Karlsruhe, Germany) for antigen retrieval and subsequently incubated with the primary antibody against human vimentin (Clone SP20) overnight. The cross-sections were washed thrice with wash buffer and anti-rabbit AP-Polymer (ZUC031-006) was added for 30 min. Again, the slides were washed and incubated with Permanent AP Red. The colour reaction was terminated by addition of distilled water and finally, counterstaining with hematoxylin was carried out. All antibodies and reagents were obtained from Zytomed-Systems (Berlin, Germany).

4.3.14 Statistics

Results are presented as mean values ± standard deviation (SD). Statistical significance was assessed by two-way analysis of variance (ANOVA) in conjunction with a Bonferroni post-test at the level of $p < 0.05$. For each *in vitro* experiment, the number of samples per group was n=3. Each assay was conducted at least three times with cells from three different donors; in the figures, representative results from one experiment are shown.

4.4 RESULTS

4.4.1 Volume stability and macroscopic appearance

To confirm the structure of the polyurethane scaffolds, they were imaged using SEM. The pore size was shown to measure 438 ± 73 µm on average, pores had a round geometry and were evenly distributed within the scaffolds. Additionally, interconnections of 152 ± 28 µm between the pores were observed (Figure 1 A).

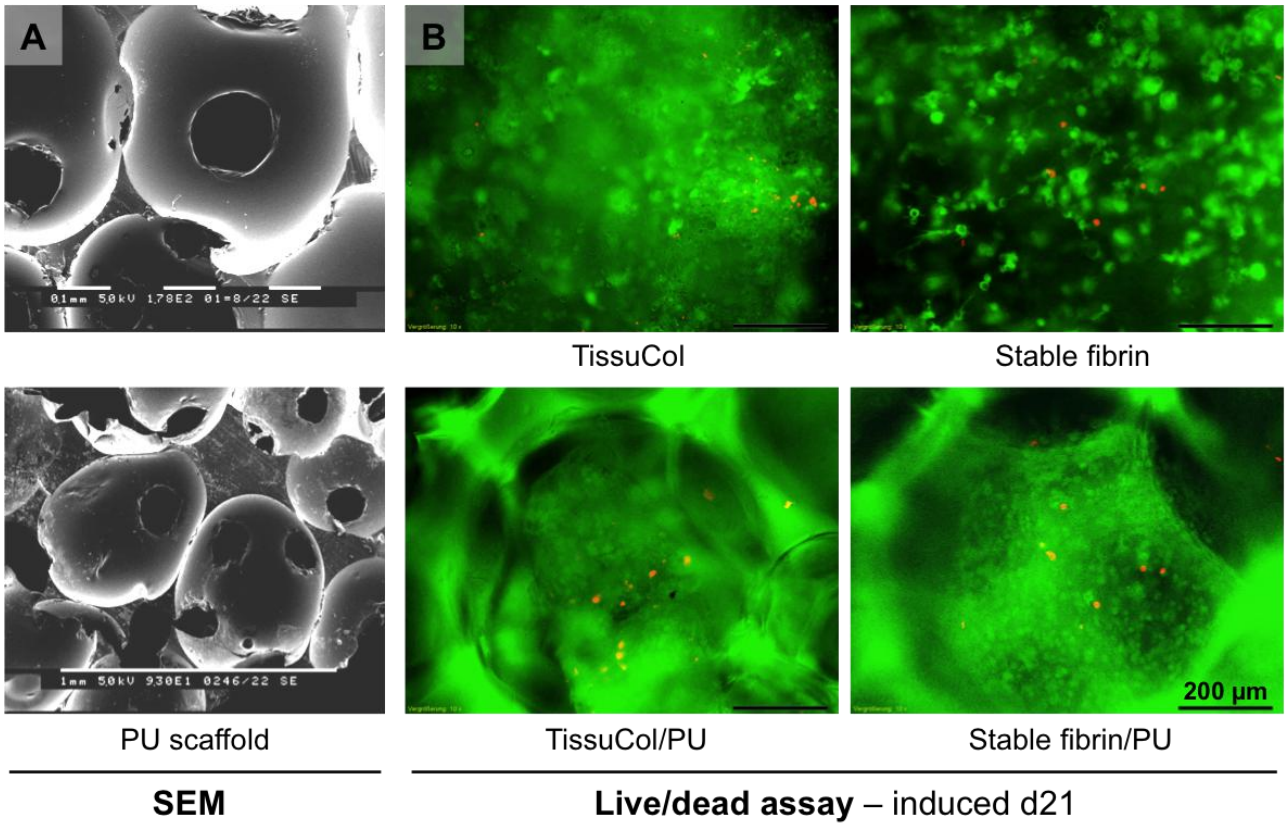

Figure 1. SEM images of biodegradable PU scaffolds with an average pore size of 438 ± 73 µm and interconnecting openings measuring 152 ± 28 µm (A). Live/dead assay from middle sections of fibrin gels and combined fibrin/PU constructs seeded with 1 x 10^6 ASC after 21 days of adipogenic culture. Viable cells appear green, dead cells red; scale bars represent 200 µm (B).

Human ASCs in all constructs, i.e. in fibrin gels alone and in combined constructs, displayed a homogeneous cell distribution throughout and good cell viability even in the centers of the constructs, where only a negligible number of dead cells were detected (Figure 1 B). The macroscopic assessment of the cell-seeded fibrin gels alone after 21 days of dynamic cultivation showed that the stable fibrin gels had a superior volume retention compared to TissuCol gels, which were reduced to approximately 40% of the initial

construct diameter (5 mm) in the adipogenically induced group (Figure 2 A). Degradation of TissuCol was also reflected by a significant weight loss of the constructs to about one fifth of the original weight, which was in accordance with the size measurements (Figure 2 B, upper diagram). In combined TissuCol/PU constructs, the initial size was maintained (Figure 2 A), however, a weight loss similar to TissuCol alone was still apparent (Figure 2 B, lower diagram). In contrast, adipogenically induced constructs generated with stable fibrin gels alone or stable fibrin/PU displayed very good volume retention and no significant weight loss (Figure 2 A and B).

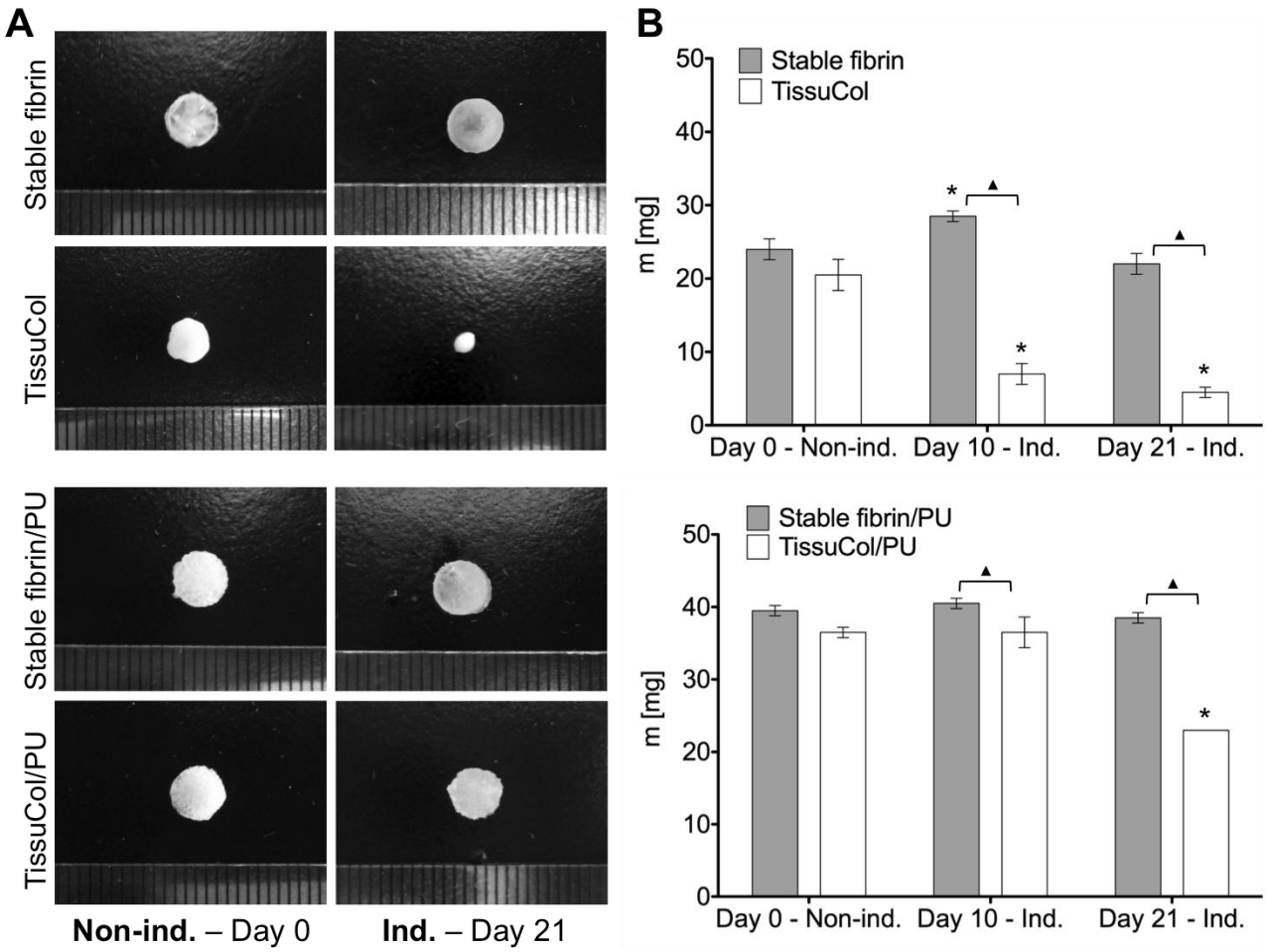

Figure 2. Stability of cell-seeded fibrin hydrogels and fibrin/PU constructs. Size of induced (Ind.) constructs after 21 days of culture compared to day 0 (Non-ind.) (A). Weight of induced constructs after 10 and 21 days compared to day 0 (B). * Statistically significantly differences between induced constructs at the time point indicated and constructs at day 0 ($p < 0.05$). ▲ Statistically significantly differences between constructs generated with TissuCol and stable fibrin ($p < 0.05$).

4.4.2 Adipogenesis

Adipogenesis was analysed in cell-seeded fibrin gels and composite fibrin/PU constructs by staining of lipid inclusions with Oil Red O. Histological sectioning showed that the fibrin gels could penetrate all pores of the scaffolds and hence, a homogeneous cell distribution was achieved.

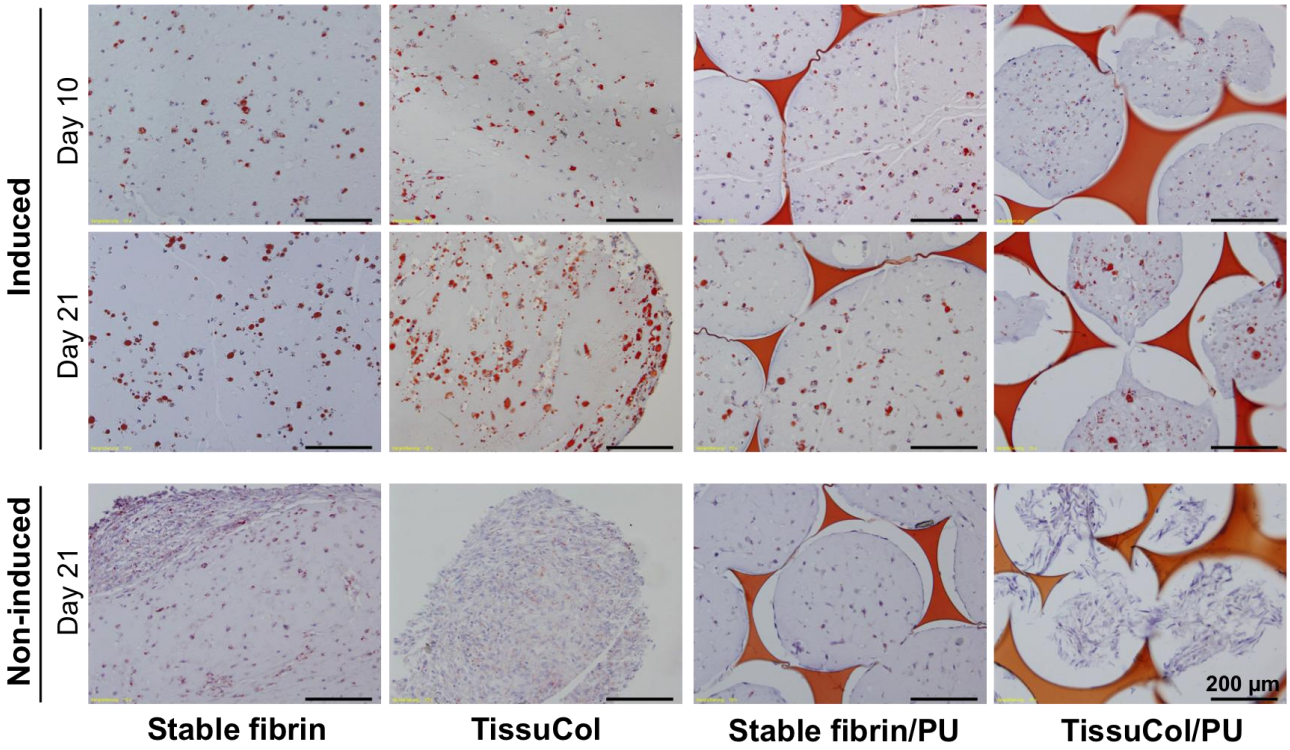

Figure 3. Adipogenesis of ASCs in fibrin gels and combined fibrin/PU constructs. Constructs were cultured for 21 days under adipogenic induction (Induced) and non-induced control conditions (Non-induced). Cryosections were stained for intracellular lipids with Oil Red O; lipid droplets appear red and cell nuclei blue (hematoxylin staining); scale bars represent 200 µm.

In all constructs, already after 10 days of adipogenic induction (Figure 3, first row), lipid vacuoles were observed after Oil Red O staining. Triglyceride storage further increased from day 10 to day 21 as indicated by the enlargement of the vacuoles in all groups (Figure 3, second row). In ASC-seeded constructs that had not received adipogenic stimulation, no differentiation occurred as indicated by the lack of Oil Red O-stained vacuoles (Figure 3, third row).

Histology confirmed the enhanced stability of the stable fibrin gels compared to TissuCol, as no signs of degradation were observed in stable fibrin alone as well as in combined stable fibrin/PU constructs. Fibrin gels based on TissuCol shrank considerably and displayed a

high cell density, especially in the non-induced group (Figure 3, third row). In combined TissuCol/PU constructs, the fibrin gel contracted within the pores of the polyurethane scaffold and was strongly degraded in non-induced constructs.

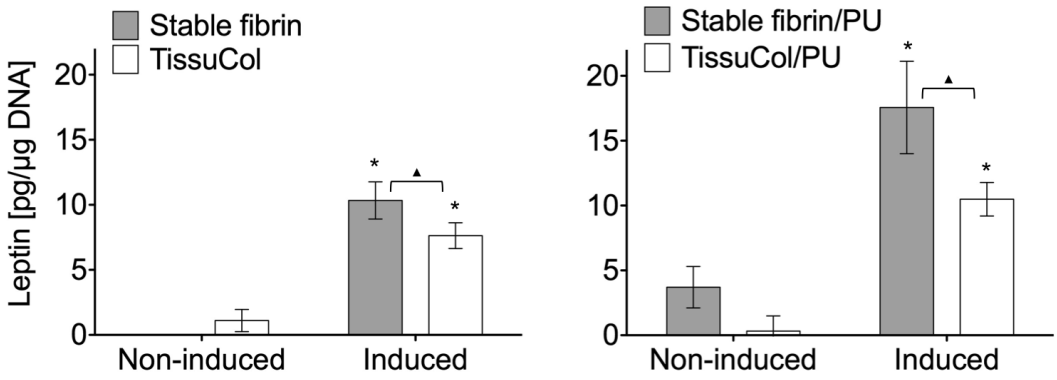

Figure 4. Leptin secretion of ASCs. Cell culture media (conditioned between day 19 and 21) were evaluated by ELISA for fibrin gels and combined fibrin/PU constructs. * Statistically significant differences between adipogenic induction (Induced) and non-induced control conditions (Non-induced) ($p < 0.05$). ▲ Statistically significant differences between constructs generated with TissuCol and stable fibrin ($p < 0.05$).

To further characterize the adipogenic differentiation of ASCs within the constructs, an ELISA for human leptin, a prominent adipokine that is recognized as a marker for terminal differentiation of adipocytes, was performed (Figure 4). In fibrin gels as well as in combined constructs, a strong increase of leptin secretion under adipogenic induction was detected. When stable fibrin gels were used for construct generation, greater amounts of leptin were produced under induction relative to constructs made with TissuCol (Figure 4).

Combined fibrin/PU constructs were then selected for additional characterization of adipogenic marker gene expression by real-time qRT-PCR. To determine early differentiation, the two most relevant transcription factors involved in adipogenesis, namely peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα) were chosen, whereas adipocyte protein 2 (aP2) and glucose transporter 4 (GLUT-4) served as late adipogenic markers (Figure 5). Gene expression in the non-induced constructs was low at all time points. In contrast, for all four genes investigated, expression levels were significantly elevated upon adipogenic induction and increased from day 0 to day 21 (Figure 5). Apart from C/EBPα expression at day 10 (higher levels in TissuCol/PU constructs) and GLUT-4 expression at days 2 and 10 (higher levels in

stable fibrin/PU constructs), no statistically significant differences were detected between the two types of fibrin/PU constructs.

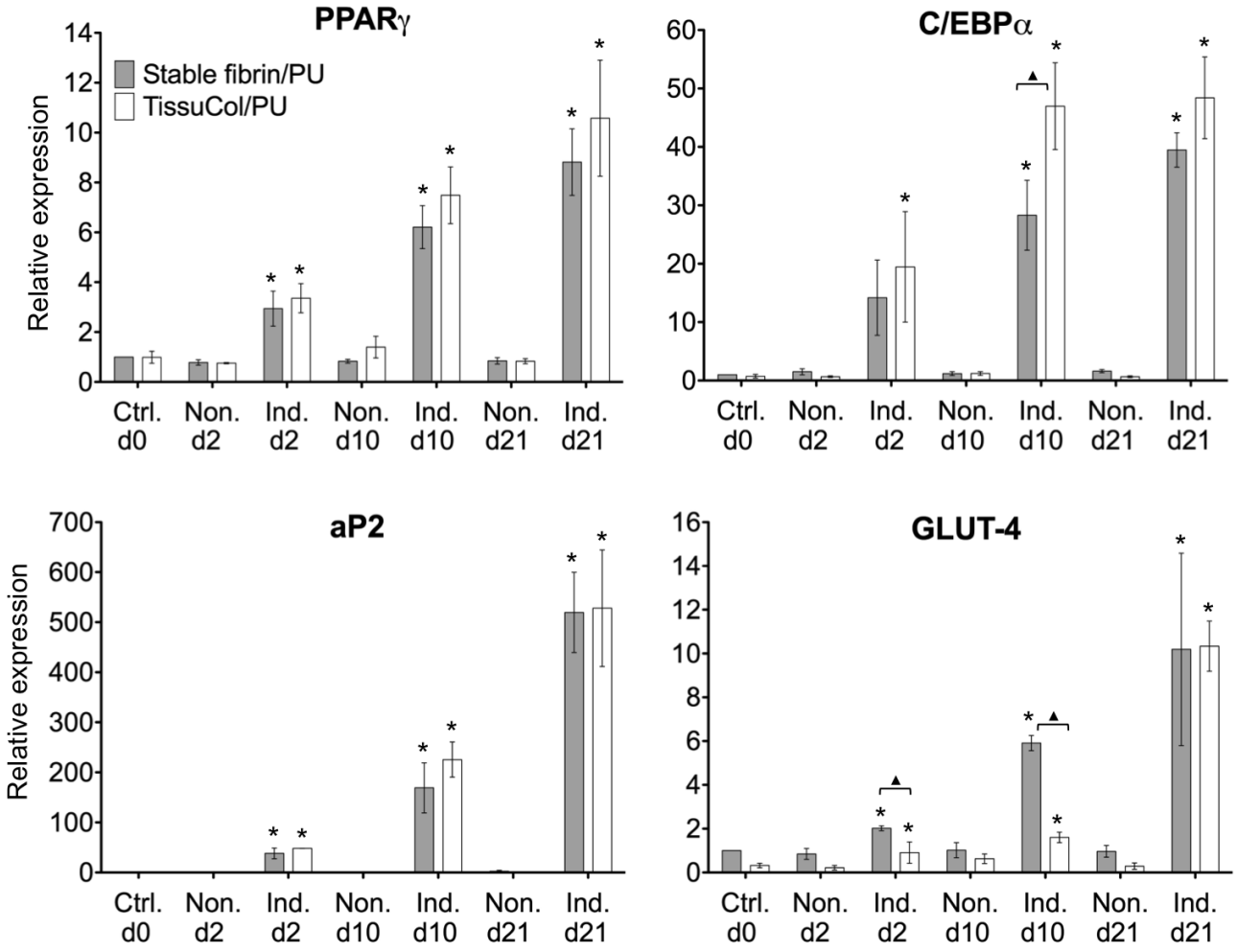

Figure 5. Adipogenic marker gene expression of ASCs in combined fibrin/PU constructs as determined by qRT-PCR. Gene expression was normalized to GAPDH; the obtained values were normalized to the values of stable fibrin/PU constructs at day 0. * Statistically significant differences between adipogenic induction (Ind.) and non-induced conditions (Non.) ($p < 0.05$). ▲ Statistically significant differences between TissuCol/PU and stable fibrin/PU constructs ($p < 0.05$).

4.4.3 Pilot in vivo study

In a pilot *in vivo* study, the suitability of the combined constructs for the generation of adipose tissue was evaluated. The stable fibrin/PU constructs were selected for implantation as they had shown favorable volume and weight stability and were considered to best withstand compressive forces and mechanical stress *in vivo*. Moreover, analysis at the cellular as well as molecular levels had demonstrated good adipogenic development *in vitro*. In order to foster vascularization of the tissue constructs, the technique of running an

AV bundle through the constructs was applied. For comparison, control constructs were implanted without an AV bundle in the neck region (Figure 6 A and B).

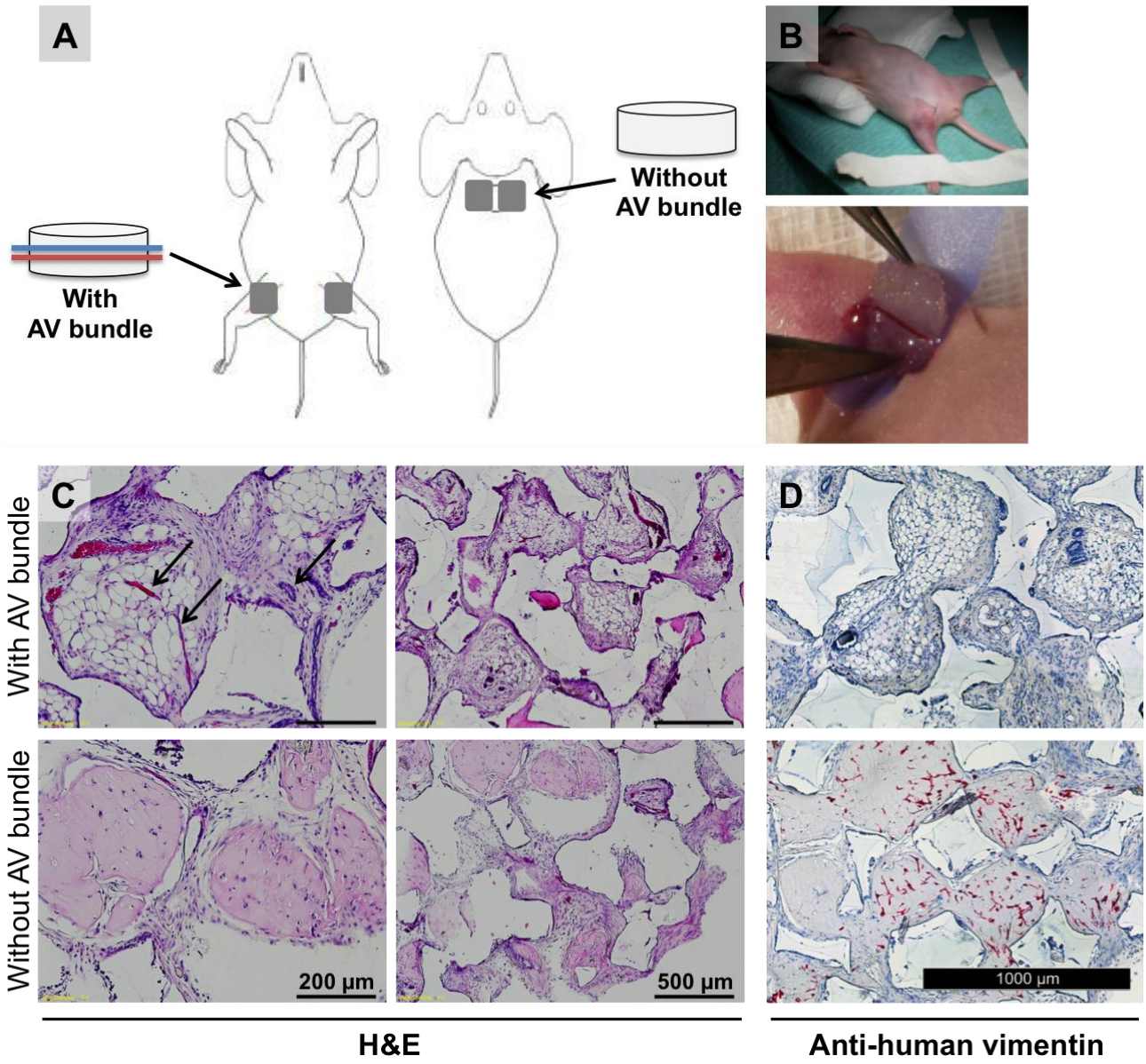

Figure 6. Evaluation of combined stable fibrin/PU constructs *in vivo*. Constructs were implanted in nude mice with an integrated AV bundle; the femoral vessels were placed within the scaffolds by a sandwich technique. For comparison, a second set of constructs was placed between the scapulae without an AV bundle. Constructs were implanted after 7 days of adipogenic precultivation and remained in the animals for 5 weeks (A,B). Paraffin cross-sections of explanted fibrin/PU constructs stained with hematoxylin and eosin (H&E); arrows indicate examples for capillaries; scale bars represent 200 µm (left column) and 500 µm (right column), respectively (C). Paraffin cross-sections stained against human vimentin (red) and counterstained with hematoxylin (blue); scale bar represents 1000 µm (D).

The macroscopic appearance of the constructs, at the time point of explantation after 5 weeks, indicated that the constructs had approximately maintained their initial size. Histological cross-sections of the outer areas of the constructs displayed no evidence for the enhanced formation of fibrous tissue around the constructs. Within the pores of the PU scaffold, histological analysis revealed many large patches of mature adipose tissue in constructs that had received a vascular pedicle. Thereby, the fibrin hydrogel appeared to have been completely degraded and replaced by coherent tissue perfused by a network of capillaries (Figure 6 C, upper row). No cells positive for human vimentin were detected in the constructs, i.e. apparently the adipose tissue had been generated by invading host cells (Figure 6 D, upper image). At a less vascularized implantation site between the scapulae, where constructs had been inserted without an AV bundle, only few mature adipocytes were found. A majority of the pores in the constructs were still filled with fibrin and the initially implanted ASCs, positive for human vimentin, which had hardly changed their morphology (Figure 6 C and D, lower rows).

4.5 DISCUSSION

In plastic and reconstructive surgery, the restoration of large soft tissue defects still represents a major challenge. Autologous tissue transplantation is associated with significant drawbacks, including necrosis or resorption of the implant. Promising proof-of-principle studies generating bioengineered soft tissues describe adipose tissue formation *in vivo*, however, constructs did not reach the desired sizes and lacked volume retention [5,20,21].

In this study, we successfully applied biodegradable poly(ε-caprolactone)-based PU scaffolds in combination with stable fibrin gels for the generation of volume-stable adipose tissue constructs. The suitability of a support structure to enhance the volume stability of soft tissue constructs has been demonstrated in various studies before [5]. Hollow silicone or polycarbonate chambers have been employed to protect developing adipose tissue against mechanical forces *in vivo* [8,9], however, the unresorbable nature of these materials limits their clinical applicability. Our approach uses biodegradable poly(ε-caprolactone)-based PU scaffolds as support structure to add volume stability to fibrin hydrogels which serve as cell carriers. With an estimated degradation time frame of 1 to 1.5 years, the PU scaffolds sustain the initial implant volume until functional new tissue has been formed. Stable fibrin gels developed by our group were incorporated within the pores of the PU scaffolds and this combination resulted in the maintenance of the size and weight of the constructs (Figure 2). In contrast, TissuCol, a different fibrin formulation that had been employed in other adipose tissue engineering studies so far [14], rapidly degraded within the pores of the PU scaffold (Figure 3), although the macroscopic deformation of the combined TissuCol/PU constructs was prevented by the extra structural support (Figure 2). In another study, also a biodegradable support structure, which was made by reinforcing poly(glycolic acid) (PGA) fiber-based matrices with poly(lactic acid) (PLA), was employed and combined with a commercially available fibrin gel. Adipose tissue development was reported in an *in vivo* experiment, however, fibrin behavior within the support structure and adipogenesis *in vitro* was not investigated [13].

In the present study, the fibrin gels facilitated cell delivery into the porous scaffold, promoting adequate pore penetration, cell adhesion as well as a homogeneous cell

distribution (Figure 3). By employing the hydrogels for seeding, cell losses frequently observed when seeding cells directly on porous scaffolds could be avoided. As demonstrated by SEM analysis and live/dead staining, the homogeneous internal structure of the PU scaffold did not impair cell viability and displayed advantageous characteristics for soft tissue engineering, such as evenly distributed interconnecting pores and an average pore size of 438 ± 73 µm to host and protect maturing adipocytes (Figure 1).

Upon adipogenic induction, cells took up a rounded-up morphology and cytoplasmatic lipid accumulation increased over time. Already after 10 days, a large proportion of induced cells had started to differentiate, rendering both fibrin gels a suitable environment for adipogenesis of ASCs. In non-induced fibrin gels, the cell density was distinctly elevated compared to the induced specimens, as cells continued to proliferate and spread along the fibrin fibers (Figure 3).

In consistency with the histological data, elevated gene expression levels of PPARγ, C/EBPα, GLUT-4 and aP2 in induced constructs confirmed the suitability of combined fibrin/PU constructs to support adipogenesis *in vitro* at the molecular level (Figure 5). PPARγ and C/EBPα have an indispensable role in transcriptional activation and the distinct increase over time is in accordance with their acknowledged cross- and autoactivation that is necessary to maintain long-term differentiation [22].

Leptin production, as a late marker of adipogenesis, emphasized that ASCs in the induced constructs had reached an advanced physiological state [23] (Figure 4). More recently, adipokines such as leptin or adiponectin have been found to support vascularization, e.g. by activation of vascular endothelial growth factor (VEGF) expression and recruitment of endothelial cells [23-25] and are considered to foster neovascularization of adipose tissue implants.

With the long-term objective to design clinically applicable adipose tissue implants, the enhanced stability of stable fibrin/PU constructs and their distinct adipogenic development was regarded as advantageous for *in vivo* investigation. Potentially, these constructs allow an early implantation time point, as the structure is capable of sustaining itself *in vivo* and encapsulated cells within the scaffold are sufficiently protected to proliferate and differentiate appropriately. Stable fibrin/PU constructs precultivated for 1 week were hence chosen for *in vivo* testing. This short *in vitro* precultivation was assumed to be sufficient to

induce tissue formation. An earlier study of our group, utilizing 3T3-L1 cells and PGA scaffolds, had proven a short *in vitro* preconditioning of either 2 or 9 days as advantageous for *in vivo* adipogenesis, whereas long-term cultured constructs containing fully matured adipocytes possessed the same limitations as free fat grafts [26].

Previously, it was demonstrated that a vascular pedicle together with a protecting chamber is advantageous for the engineering of larger volumes of adipose tissue [9]. Similarly, in a further study, a flow-through vessel loop was shown to promote the neovascularization of adipose tissue constructs [27]. In our pilot study, the subcutaneous implantation of the combined stable fibrin/PU constructs with insertion of a flow-through AV bundle led to the formation of mature adipose tissue within 5 weeks and confirmed the suitability of the constructs for the development of adipose tissue implants *in vivo*. Adipose tissue development in pedicled constructs was accompanied by the formation of a capillary network perfusing the tissue (Figure 6). By placing control constructs between the scapulae, it was shown that a different environment with a lack of vascularization significantly delays tissue formation. The high cellularity and formation of adipose tissue in pedicled constructs was apparently evoked by a strong migration of host cells into the scaffolds, whereas the implanted ASCs could not be detected anymore after 5 weeks *in vivo* by anti-human vimentin staining. It remains to be determined in future studies, e.g. by also implanting unseeded constructs for comparison, whether or not, and how, the implanted cells affect the host response. These questions have been addressed in few studies before, however, the results varied significantly, indicating that the scaffold and the preconditioning of the ASCs can influence the *in vivo* development of engineered adipose tissue [14,28,29].

Altogether, this study demonstrates the potential of stable fibrin hydrogels combined with porous biodegradable poly(ε-caprolactone)-based PU scaffolds for the engineering of volume-stable adipose tissue constructs. Supporting adipose tissue development *in vitro* and *in vivo* and being completely biodegradable, these combined scaffolds exhibit favorable characteristics for soft tissue implants and were shown to meet the requirements for both adipogenesis and angiogenesis within the constructs.

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Chapter 5

Hypoxic Culture as Prevascularization Strategy for Adipose Tissue Engineering

5.1 INTRODUCTION

Providing oxygen supply to implanted cells currently represents one of the greatest challenges in adipose tissue engineering [1-3]. Being restricted to merely 150 to 200 μm, the diffusion distance of oxygen is insufficient to sustain cells on the tissue level, implying that adipocytes further away from an oxygen source, i.e. a capillary, are exposed to hypoxia and inevitably compromised in survival and function [4-6]. Thus, physiologically, an extensive microvascular network nurtures adipose tissue [7-9] and effectively upholds its functional role as an energy depot and endocrine organ [10]. Owing to the lack of pre-existing blood vessels, encapsulated cells in engineered constructs are exposed to temporary oxygen deprivation upon *in vivo* implantation [11,12]. This entails functional impairment and ultimately cell death, severely limiting the regenerative potential of the implants [13,14]. Accordingly, for adipose tissue engineering approaches to be successful and clinically applicable, innovative strategies for rapid vascularization upon implantation are required [3,15-17].

Providing an intrinsic blood supply via integration of a femoral pedicle, the vascularization of engineered volume-stable constructs *in vivo* was previously approached (Chapter 4, [18]). Apart from a direct intervention *in vivo*, which is frequently associated with a complex surgical procedure [19], *in vitro* vascularization strategies aiming at the preformation of capillary networks, for example by coimplantation of endothelial cells [1,2,20], application of pro-angiogenic factors [21-23] or prefabrication of microchannels [24], have been introduced.

Alternatively, hypoxia is a potent trigger of angiogenesis and plays a pivotal role in neovascularization via the upregulation of pro-angiogenic factors [25-27]. In contrast to the detrimental effects of hypoxia and ischemia on mature adipocytes [13,28,29], low oxygen is a basic microenvironmental condition for the maintenance of adipose-derived stem cells (ASCs) within their niche [30-33]. Similarly, regenerative processes such as wound healing rely on hypoxic stimuli for mesenchymal stem cell (MSC) recruitment to the site of injury [34-37]. Hypoxia further promotes the adoption of specific cellular functions required for angiogenesis by adult stem and progenitor cells [38,39]. Since ASCs themselves are attributed a wide array of pro-angiogenic properties [40-42] and even have the capacity to

acquire characteristics of endothelial cells [43-47], exposure to hypoxic conditions may further enhance their profound reparative traits [36,48,49]. Consequently, preconditioning of ASCs under hypoxia has emerged as an *in vitro* prevascularization strategy for tissue engineering and other regenerative applications [41,42,48,49]. By this, the generation of a pro-angiogenic milieu inside bioengineered constructs is expected to promote vessel ingrowth and host-induced neovascularization, resulting in a higher degree of functional engraftment. Besides, hypoxic pretreatment may acclimatize implanted cells to reduced oxygen levels and increase cellular tolerance towards hypoxic conditions prevalent prior to full vascular integration of the constructs *in vivo*.

Supporting this concept, previous studies investigating the impact of hypoxia on bone marrow-derived mesenchymal stem cells (BM-MSCs) and ASCs *in vitro* have demonstrated a stimulatory effect of low oxygen on the expression of pro-angiogenic factors [12,41,48,50]. Stubbs *et al.* reported hypoxic preconditioning of ASCs to effectively enhance cell viability upon simulated ischemia and a possible protective role of vascular endothelial growth factor (VEGF) in this context [51]. Differential reports however exist concerning the effect of hypoxia on adipogenic development of MSCs. The general consensus on the impact of hypoxia is that it maintains stemness but inhibits differentiation [30,33,52-55]. In line with the latter, various groups observed enhanced survival and proliferation rates under hypoxia [30,56], whereas adipogenesis was impaired [53,57-59]. In contrast, Valorani *et al.* reported a positive effect of hypoxic treatment on adipogenic differentiation of murine MSCs [60] and Fink *et al.* identified an adipocyte-like phenotype in human MSCs cultured under hypoxia [61].

As outlined above, the effects of low oxygen on basic functional properties of MSCs and ASCs have been thoroughly addressed in previous work, yet, to date, no detailed reports on the role of hypoxia, and hypoxic preconditioning in particular, in engineered 3D adipose tissue constructs are available. In this study, it was thus intended to apply hypoxic culture as a possible *in vitro* prevascularization strategy for adipose tissue engineering. Thereby, hypoxic pretreatment of 3D adipose tissue constructs was intended to induce a pro-angiogenic environment in ASC-seeded 3D constructs to facilitate vascular ingrowth upon implantation *in vivo*, as well as to enhance the cells' tolerance towards ischemia. To investigate the effectiveness of this method *in vitro*, the previously established constructs

based on ASCs and stable fibrin gels (Chapter 3 and 4) were exposed to hypoxic culture and evaluated in terms of pro-angiogenic factor secretion and endothelial marker expression. Apart from focusing on angiogenesis and ischemic protection of ASCs, the impact of hypoxia on adipogenic differentiation of ASCs on the cellular and molecular level was studied in 2D monolayer culture as well as in ASC-seeded 3D constructs.

5.2 MATERIALS AND METHODS

5.2.1 Cell isolation and expansion

Human ASCs were isolated from subcutaneous adipose tissue obtained from abdominal depots of healthy female donors. The study was approved by the ethics committee of the University of Wuerzburg, Germany. Written informed consent was obtained from all patients. Patients were between 22 and 56 years of age and had a body mass index (BMI) ranging from 26 to 33.

For ASC isolation, fat tissue was digested with 0.1% collagenase NB4 from Clostridium histolyticum (Serva Electrophoresis, Heidelberg, Germany) in freshly prepared collagenase buffer for 2 h at 37 °C on an orbital shaker. The resulting suspension was filtered through a 100 µm nylon mesh and centrifuged at 300 g for 10 min. The fat layer on top was aspirated and pelleted cells were washed with phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria). The obtained stromal-vascular fraction (SVF) was resuspended in basal medium containing Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12) supplemented with 1% penicillin-streptomycin (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 10% fetal bovine serum (FBS), all obtained from Invitrogen (Karlsruhe, Germany). For cryopreservation of the SVF in liquid nitrogen, 5% DMSO (Sigma-Aldrich, Steinheim, Germany) was added to the medium.

For expansion of ASCs, cryopreserved SVF cells were thawed and plated on tissue culture-treated plastic flasks (175 cm^2). The ASC subpopulation was selected via plastic adherence. The cells were cultured to a subconfluent level and expanded for 2 to 3 passages in basal medium. For passaging, trypsin-EDTA at 0.25% from Invitrogen (Karlsruhe, Germany) was used. Cell expansion was carried out under normoxic conditions at 21% O_2/5% CO_2 as well as under hypoxia at either 2 or 4% O_2/5% CO_2 as indicated.

5.2.2 Oxygen monitoring

Oxygen tension was measured non-invasively using an SDR SensorDish Reader system kindly provided by PreSens (Regensburg, Germany). Oxygen levels were detected via a luminescent dye patch at the bottom of a 24-well plate (OxoDish®, OD-24). The applied multi-well plates were sterilized and calibrated by the manufacturer. Oxygen measurements were performed in a controlled environment within an incubator at 37 °C. Partial oxygen

pressure was recorded every 60 sec. Low oxygen concentrations were obtained by continuously flushing the incubator with a balanced gas mixture of either 2 or 4% O_2, 5% CO_2 and N_2.

5.2.3 2D cell culture

For experiments in 2D culture, ASCs (normoxia/hypoxia; passage 2 or 3) were seeded in 12- or 24-well plates at a density of 3 x 10^4 cells/cm^2. Cells were cultured in preadipocyte growth medium 2 (PGM-2) consisting of preadipocyte basal medium 2 (PBM-2, Lonza, Walkersville, USA) supplemented with 1% penicillin-streptomycin and 10% FBS. Two days after seeding, adipogenesis was induced by addition of differentiation medium [PGM-2 with 1.7 µM insulin, 1 µM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine (IBMX) and 200 µM indomethacin]. For medium preparation, bovine insulin was kindly provided by Sanofi-Aventis (Frankfurt, Germany), IBMX was obtained from Serva Electrophoresis (Heidelberg, Germany) and indomethacin, as well as dexamethasone were purchased from Sigma-Aldrich (Steinheim, Germany). Cells subjected to non-induced control conditions were cultured in PGM-2 devoid of additional supplements. 2D cell culture was performed under normoxic conditions at 21% O_2/5% CO_2 or under hypoxia at 4% O_2/5% CO_2. Cells were harvested after 10 days *in vitro*.

5.2.4 Preparation of stable fibrin gels

Stable fibrin gels were prepared as described previously [18,62] with a fibrinogen concentration of 25 mg/mL, 20 mM $CaCl_2$, and 2.5 U/mL thrombin. Bovine fibrinogen and aprotinin from bovine lung were obtained from Sigma-Aldrich (Steinheim, Germany). 20 µL of 50 mg/mL fibrinogen dissolved in an aprotinin solution [10,000 kallikrein inhibitory units (KIU)/mL] were mixed with an equal volume of a 5 U/mL thrombin solution and subjected to 45 min of gelation at 37 °C and 5% CO_2. The thrombin component was prepared using a 1:100 dilution of thrombin-S (500 U/mL, TissuCol® Kit 1.0 Immuno; Baxter, Unterschleissheim, Germany) in thrombin dilution buffer.

5.2.5 3D cell culture

Stable fibrin gels (40 µL; height 2 mm, diameter 5 mm) were seeded with 1.0 x 10^6 ASCs (normoxia/hypoxia; passage 2 or 3). Cells were suspended in the thrombin component. 20 µL of the thrombin-cell suspension were mixed with 20 µL fibrinogen (50 mg/mL) and

transferred to sterile glass rings with an inner diameter of 5 mm. Constructs were removed from the glass rings after gelation and cultured dynamically on an orbital shaker at 50 rpm (37 °C, 5% CO_2). PGM-2 was employed for *in vitro* culture of the constructs. Two days after seeding (referred to as day 0), adipogenesis was induced by the addition of differentiation medium as described for 2D culture experiments. Cell-seeded constructs in the control groups were cultured in PGM-2 without adipogenic inducers. Medium exchange was performed every other day. 3D cell culture was performed under normoxic conditions at 21% O_2/5% CO_2 or under hypoxia at 2% O_2/5% CO_2. Constructs were maintained *in vitro* for 10, 14 or 21 days as indicated for the respective experiments in the results section.

5.2.6 Oil Red O staining

Adipogenesis was histologically investigated by staining cells in 2D monolayer culture and 3D constructs for lipid inclusions with Oil Red O. Cells were subjected to a fixation step using buffered formalin (3.7% in PBS). The Oil Red O staining solution was prepared by dissolution of Oil Red O (Sigma-Aldrich, Steinheim, Germany) in 60% isopropanol at a concentration of 3 mg/mL. Cells in 2D monolayer culture were incubated with the staining solution for 5 min and rinsed shortly with distilled water.

3D constructs were dehydrated in increasing sucrose series (10-60%) prior to embedding in Tissue-Tek® O.C.T. compound (Sakura Finetek, Zoeterwonde, Netherlands). 8 µm sections were cut and in the following stained with Oil Red O solution. Counterstaining was performed with hematoxylin (Bio Optica, Milan, Italy) for visualization of nuclei and afterwards, slides were coverslipped with Glycergel Mounting Medium (Dako, Carpinteria, USA). Microscopic images were taken and processed with Olympus cellSens™ Dimension Microscope Imaging Software (Olympus, Hamburg, Germany).

5.2.7 Perilipin staining

Lipid vacuole formation of ASCs in stable fibrin gels was investigated by immunostaining against perilipin. 8 µm cryosections were prepared as described above. After rehydration in PBS, unspecific binding was blocked with blocking solution (1.5% BSA in PBS) prior to overnight incubation with a mouse anti-human perilipin antibody (Creative Diagnostics, Shirley, USA). Slides were thoroughly washed with PBS and an Alexa Fluor® 488-conjugated AffiniPure goat anti-mouse secondary antibody (Jackson

Immuno Research, West Grove, USA) was added for 30 min in the dark. Nuclei were counterstained with IS Mounting Medium DAPI (Dako, Carpinteria, USA). Slides were coverslipped and viewed under a fluorescence microscope. The resulting images were overlaid using Olympus cellSens™ software.

5.2.8 CD31 staining

Endothelial development of ASCs seeded in stable fibrin gels was analyzed by staining with an antibody directed against CD31. 8 µm cryosections were prepared as described above. After rehydration in PBS, unspecific binding was blocked with blocking solution prior to overnight incubation with a monoclonal mouse anti-human CD31 antibody (clone JC70A; Dako, Carpinteria, USA). Slides were thoroughly washed with PBS and a Cy™3-conjugated AffiniPure donkey anti-mouse secondary antibody (Jackson Immuno Research, West Grove, USA) was added for 30 min in the dark. Nuclei were counterstained with IS Mounting Medium DAPI (Dako, Carpinteria, USA). Slides were coverslipped and viewed under a fluorescence microscope. Resulting images were processed and overlaid using Olympus cellSens™ software.

5.2.9 Quantification of soluble factor secretion by ELISA

Leptin and VEGF levels in cell culture supernatants were quantified using sandwich Quantikine® Human Immunoassays from R&D Systems (Minneapolis, USA). Medium samples from 2D and 3D cultures were collected over a period of two days at the time points indicated in the results section and stored below -20°C until analysis. Leptin and VEGF secretion was normalized to total protein (BCA assay) or DNA content as described for the respective experiments in the results section.

5.2.10 Quantification of protein content

Total protein content was quantified using a BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). The assay was conducted according to the instructions provided by the manufacturer. Spectroscopic quantification was performed at 562 nm using a MRX microplate reader (Dynatech Laboratories, Chantilly, Virginia). Total protein contained in the samples was calculated by correlating the measured absorbances with standard dilutions of albumin.

5.2.11 Quantification of DNA

For DNA content measurements, Hoechst 33258 intercalating dye was purchased from Polysciences (Warrington, USA). Samples were sonified in phosphate-saline buffer. Quantification of DNA content was carried out with a Tecan GENios pro spectrofluorometer (Tecan Deutschland GmbH, Crailsheim, Germany) at an excitation wavelength of 340 nm and an emission wavelength of 465 nm.

5.2.12 Statistics

Results are presented as mean values ± standard deviation (SD). Statistical significance was assessed by either one-way or two-way analysis of variance (ANOVA) in conjunction with a Bonferroni post-test at the level of $p < 0.05$. Statistical analysis was performed using GraphPad Prism, Version 5.0 (GraphPad Software, La Jolla, USA). For the experiments, if not stated otherwise, the number of samples per group was n=3.

5.3 RESULTS

5.3.1 Establishment of culture conditions

Cellular response of ASCs towards hypoxic culture conditions was investigated in preliminary experiments in 2D culture format and afterwards in stable fibrin gels. Oxygen monitoring was implemented using a SDR SensorDish Reader system in 24-well format provided by PreSens. Via a sensor spot equipped with a luminescent dye at the bottom of each well, the non-invasive readout of the partial oxygen pressure (pO_2) was enabled. With the luminescence lifetime of the dye being oxygen-dependent, pO_2 was calculated by the software from the measured signal.

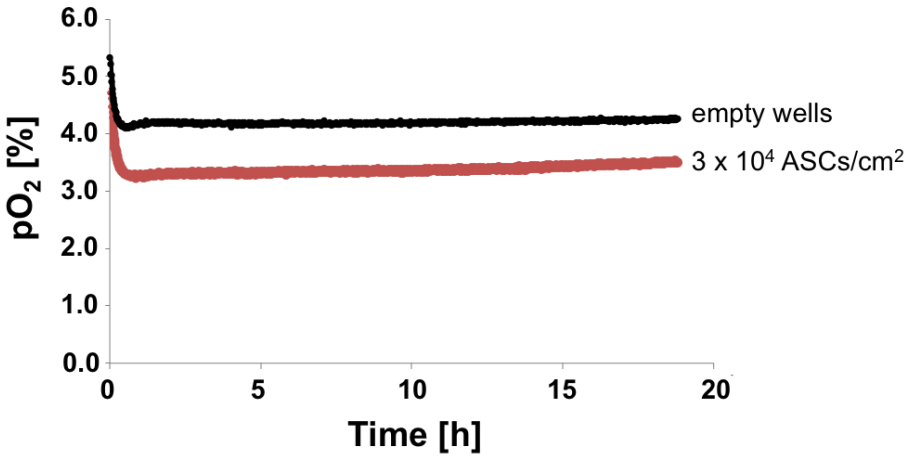

Figure 1. Oxygen monitoring in 2D cultures. Oxygen levels were set to 4% pO_2 and recorded in empty 24-well plates and wells seeded at a density of 3 x 10^4 cells/cm^2 ; representative results are shown.

Oxygen consumption of ASCs seeded in the sensor dishes was compared to empty wells containing solely culture medium (Figure 1). The initial pO_2 was set to 4% and cells were cultured in the presence of adipogenic inducers (insulin, IBMX, dexamethasone, indomethacin) for 2 days prior to monitoring. Oxygen levels were recorded overnight, revealing that in the cell-seeded wells, pO_2 was reduced by approximately 1% relative to unseeded, empty wells.

To subsequently evaluate the adipogenic differentiation capacity of ASCs under hypoxia in 2D, different culture setups were implemented by which hypoxic conditions were applied during the expansion and/or differentiation phase. Prior to adipogenic culture at either 21% pO_2 (normoxia) or 4% pO_2 (hypoxia), ASCs were expanded under normoxia (21% pO_2) or

hypoxia (4% pO_2) in conventional T175 tissue culture flasks. The conditions were recombined according to the Table in Figure 2 A. For the experiments, the cells were seeded in 12-well plates at a density of 3×10^4 cells/cm^2 (day -2) and subjected to a short proliferation phase of two days before they were adipogenically induced (day 0). After 10 days of adipogenic culture in 2D, Oil Red O staining was performed (Figure 2 B).

A

2D expansion	2D differentiation	
Normoxia	Normoxia	NN
	Hypoxia	NH
Hypoxia	Normoxia	HN
	Hypoxia	HH

B

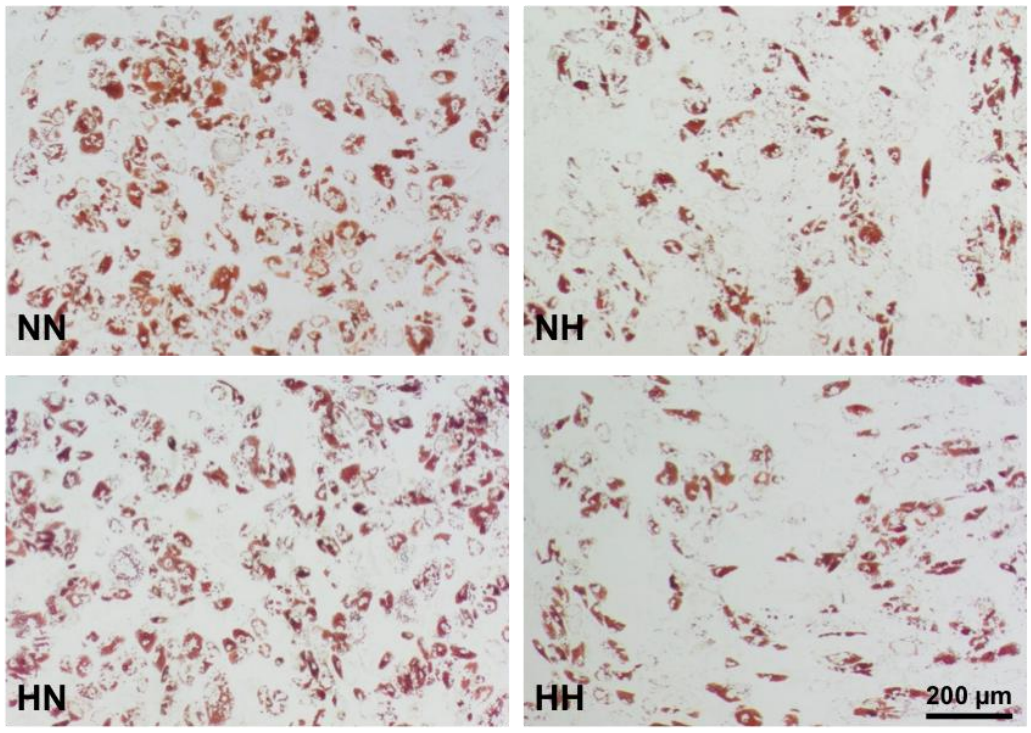

Figure 2. Culture setup for normoxic and hypoxic culture of ASCs in 2D format. ASCs were expanded in tissue culture flasks (normoxia: 21% pO_2/hypoxia: 4% pO_2) and afterwards adipogenically differentiated in 12-well plates (3×10^4 cells/cm^2) under 21% or 4% pO_2 resulting in the 4 recombinations: normoxia/normoxia (NN), normoxia/hypoxia (NH), hypoxia/normoxia (HN) and hypoxia/hypoxia (HH) (A). Adipogenic differentiation at day 10 was investigated by Oil Red O staining. Scale bar represents 200 µm.

Histology showed that in general, ASCs were able to form lipid droplets under the respective normoxic and hypoxic conditions in 2D culture, however distinct differences in

the extent of lipid accumulation were observed. Cells that had been expanded and differentiated at 21% pO_2 (NN) displayed elevated numbers of Oil Red O-positive cells relative to cells in the NH and HH groups. ASCs expanded at 4% pO_2 and differentiated at 21% pO_2 (HN) were observed to accumulate comparable amounts of intracellular trigylcerides to the NN group. Cell morphologies differed slightly as reflected by the more rounded cell shape of NN and HN cells, whereas ASCs cultured under NH and especially, HH conditions, appeared more stretched and elongated.

In addition to histological assessment, proliferation and adipokine secretion by ASCs were examined by quantification of the total protein content using a BCA assay and by an ELISA for human leptin (Figure 3).

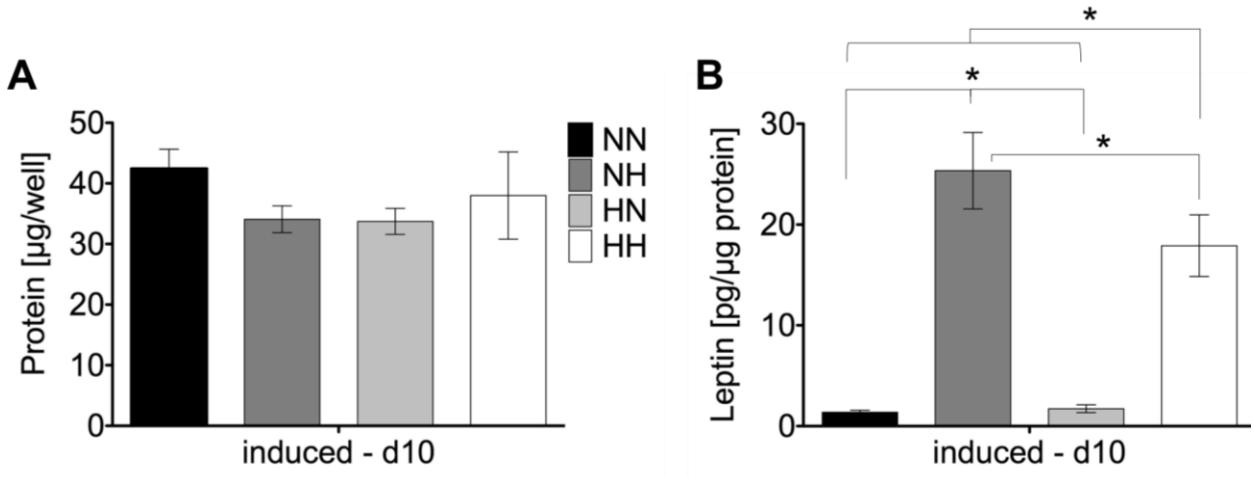

Figure 3. Proliferation and leptin secretion of ASCs in 2D culture. Hypoxic and normoxic conditions were applied during the expansion phase as well as during adipogenic differentiation according to the culture setup displayed in Figure 2 A. Total protein content was measured in induced cultures at day 10 using a BCA assay (A). Secreted leptin was measured in cell culture supernatants collected between day 8 and 10 of culture and evaluated by ELISA; leptin levels were normalized to total protein content (B). * Significant differences between the indicated groups ($p < 0.05$).

Total protein quantification confirmed that there were no significant variations in cell number between the respective groups at day 10 of adipogenic induction (Figure 3 A). This indicated further that the alterations in differentiation observed in Figure 2 B were not due to deviating cell numbers.

Leptin secretion varied greatly among the differentially treated cells (Figure 3 B). Leptin production of ASCs in the NH and HH group significantly exceeded the two other groups in which normoxic conditions had been applied during the differentiation phase. In addition,

adipokine levels under NH conditions were significantly higher than in the HH group. ASCs in the HN and NN group displayed both low but comparable leptin production.

5.3.2 Hypoxic culture of ASC-seeded constructs

To investigate the effect of hypoxia in engineered adipose tissue constructs, stable fibrin gels were seeded with ASCs. The gels were prepared at a lower fibrinogen concentration (25 mg/mL) than previously applied, resulting in reduced matrix stiffness (Chapter 3). With regard to the vast cell numbers needed for construct preparation, the following experiments were carried out solely comparing normoxic (NN) with hypoxic (HH) conditions, neglecting further recombinations. For culture of 3D constructs, oxygen levels were set to 2% pO_2 to more clearly distinguish hypoxic conditions from physiological 'tissue normoxia', which is reported to lie in the range of 3 to 6% [63] or even below 3% [32,64,65]. The culture setup was adopted from previous studies (Chapter 3) and extended with regard to the applied pO_2 (Figure 4).

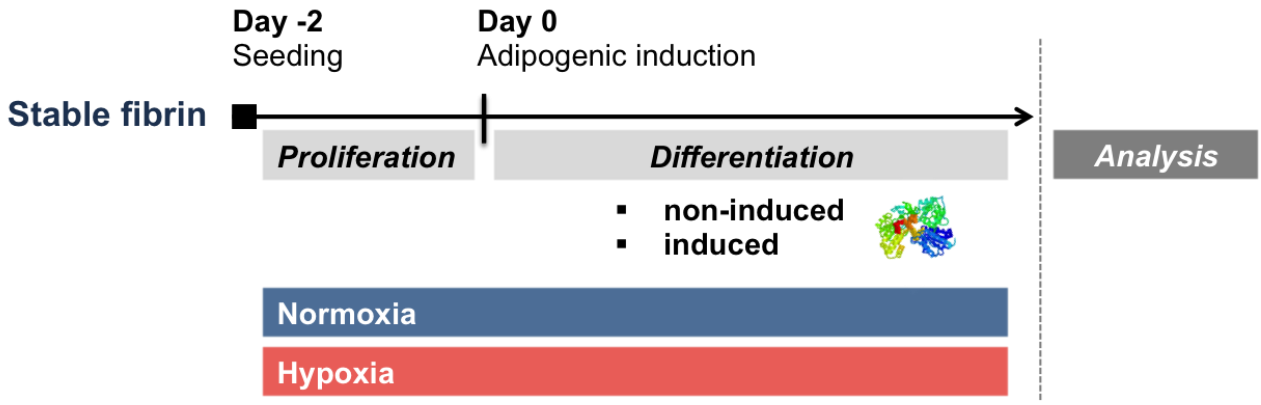

Figure 4. Culture setup for adipose tissue constructs. Stable fibrin gels were seeded with ASCs (day -2) and subjected to normoxic (21% pO_2) or hypoxic (2% pO_2) culture. Prior to seeding, ASCs were expanded under normoxia (21% pO_2) or hypoxia (2% pO_2), respectively. Adipogenic differentiation was induced by addition of a hormonal cocktail on day 0. Non-induced constructs were cultured under control conditions devoid of adipogenic inducers. Constructs were analyzed on the cellular and molecular level.

Stable fibrin gels were seeded with 1.0 x 10^6 ASCs (day -2) and subjected to culture in maintenance medium for 2 days (Figure 4). On day 0, adipogenesis was induced by addition of a hormonal cocktail, whereas non-induced specimens were cultured under control conditions. Constructs were exposed to the respective conditions for 14 days and then analyzed for adipogenic differentiation by Oil Red O staining (Figure 5).

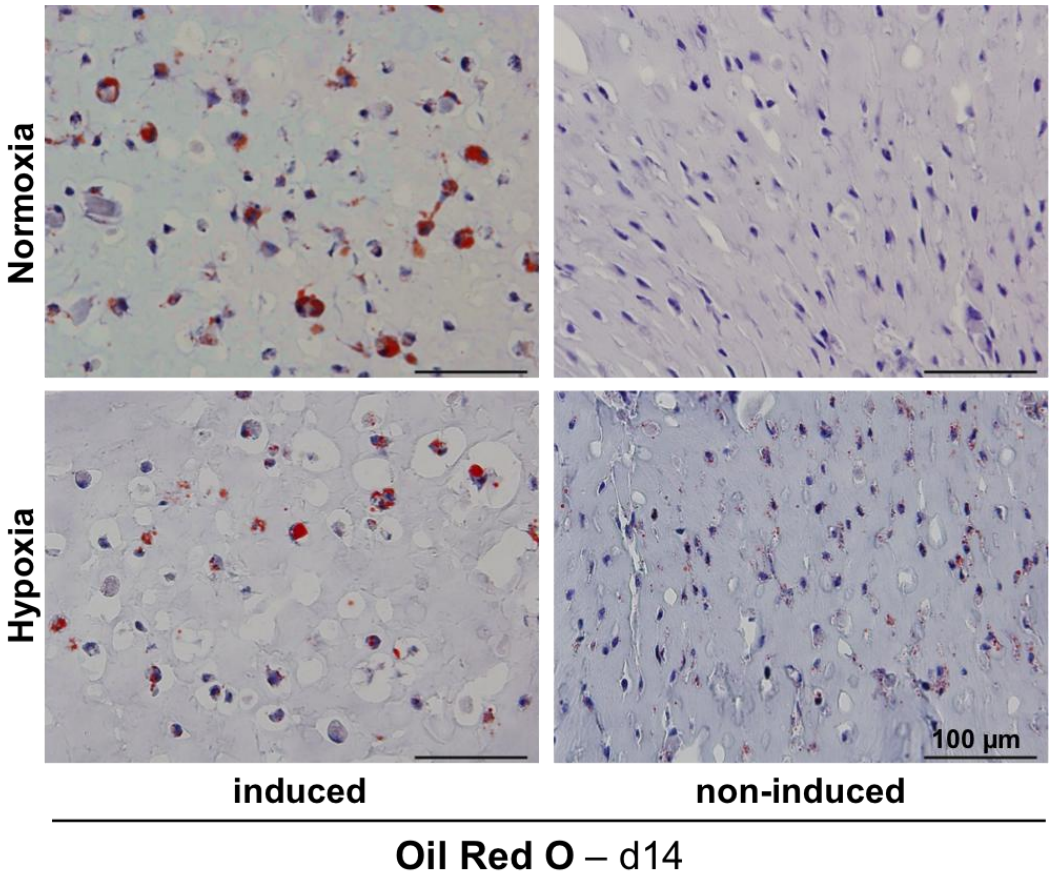

Figure 5. Adipogenic differentiation of ASCs seeded in stable fibrin gels under normoxic and hypoxic culture. Adipogenically induced and non-induced constructs were analyzed after 14 days. Oil Red O staining was used to determine lipid accumulation and nuclei were counterstained with hematoxylin. Scale bars represent 100 µm.

As noticed before in 2D culture, the amount of Oil Red O-stained lipid droplets in ASC-seeded 3D constructs under hypoxic conditions was reduced compared to normoxic culture. It further appeared that under hypoxia, the intracellular vacuoles were smaller than in the normoxic group. Non-induced constructs featured higher cell numbers relative to their induced counterparts, and a marginal tendency towards increased proliferation was observed for hypoxic relative to normoxic conditions. Overall, cell distribution in the constructs as well as cell morphology in the respective induced and non-induced groups was homogeneous.

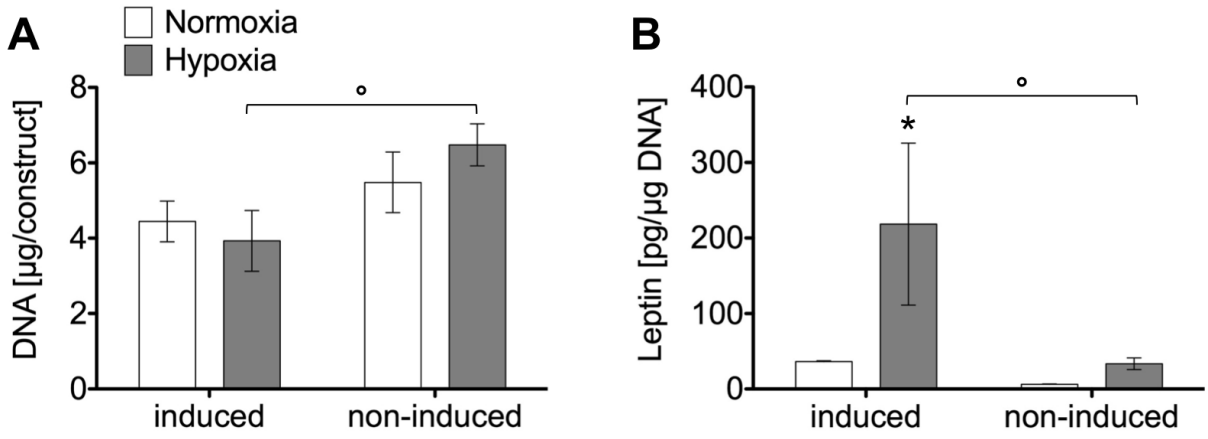

Figure 6. Proliferation and leptin secretion of ASCs seeded in stable fibrin gels under hypoxic and normoxic culture. Proliferation was investigated in induced and non-induced constructs at day 14 by quantification of DNA content (A). Secreted leptin was quantified in cell culture supernatants collected between day 12 and 14 of culture by ELISA; leptin levels were normalized to DNA content (B). * Statistically significant differences between normoxic and hypoxic culture ($p < 0.05$). ° Statistically significant differences between the indicated induced and non-induced groups ($p < 0.05$).

Proliferation and adipokine production were examined after 14 days of culture (Figure 6). With regard to cell numbers, no difference in DNA content was found between the induced groups independent of oxygen tension (Figure 6 A). Under non-induced culture, cells apparently displayed enhanced proliferation compared to induced conditions. On the molecular level, leptin secretion upon hormonal induction of ASCs resulted in higher leptin levels relative to non-induced cultures (Figure 6 B). This was particularly pronounced under hypoxic culture, resulting in significantly enhanced leptin secretion upon induction. Overall, leptin production under hypoxic conditions was higher than under normoxia irrespective of adipogenic treatment.

With regard to the pro-angiogenic properties of ASCs, upregulation of the endothelial marker CD31 in response to hypoxic treatment was subject to further investigations. Constructs were again seeded with 1.0×10^6 cells and subjected to induced and non-induced culture for 21 days. Cryosections were immunolabeled against CD31 and endothelial differentiation was additionally correlated with adipogenesis by staining adipocytes with an antibody directed against perilipin, a protein associated with the protection of the lipid vacuole against lipolysis [66,67] (Figure 7).

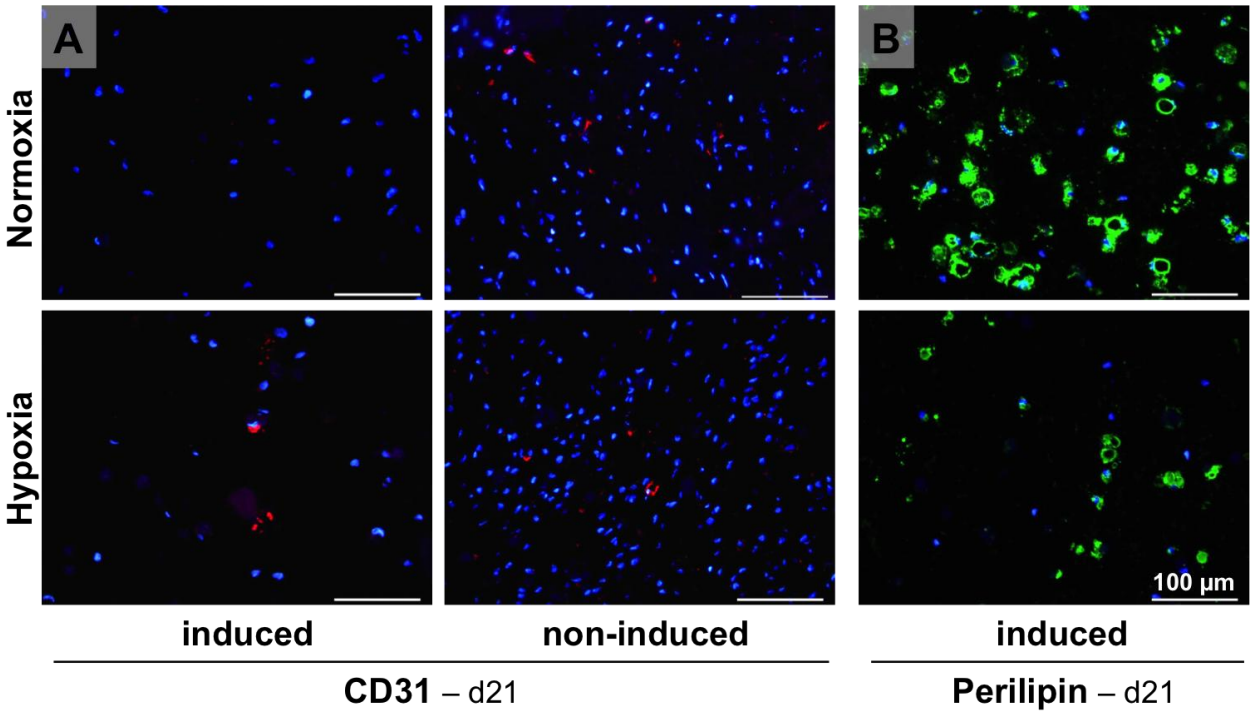

Figure 7. Endothelial and adipogenic differentiation of ASCs seeded in stable fibrin gels under normoxic and hypoxic culture conditions. Adipogenically induced and non-induced constructs were analyzed at day 21. Staining against CD31 (red) was used to determine endothelial development (A). Staining against perilipin (green) was performed to highlight lipid droplets (B). Nuclei (blue) were counterstained with DAPI; scale bars represent 100 µm.

Adipogenic induction in conjunction with hypoxia resulted in several CD31-expressing ASCs within the constructs (Figure 7 A). In contrast, the induced counterparts under normoxia did not show any signs of endothelial differentiation. Again, the cell densities in the non-induced groups were increased relative to induced specimen. Concerning endothelial differentiation in non-induced constructs, cells under both normoxic and hypoxic conditions sporadically expressed CD31. The possibility that $CD31^+$ cells were contaminating cells from the primary cell population was excluded by additional investigation of constructs on day -2, which exhibited no $CD31^+$ cells (data not shown). Staining against perilipin in induced constructs inversely correlated with CD31 expression of ASCs (Figure 7 B). As indicated by elevated numbers of rounded $perilipin^+$ vacuoles, ASCs under normoxic culture featured enhanced lipid accumulation relative to cells exposed to hypoxia, confirming results from earlier experiments (Figure 5). Non-induced cultures did not contain $perilipin^+$ structures (data not shown).

Apart from CD31, VEGF was assessed as soluble factor related to a possible pro-angiogenic profile of ASCs provoked by hypoxic treatment. Leptin, which usually serves as marker for adipogenesis, was additionally quantified owing to its dual role as adipokine and pro-angiogenic modulator (Figure 8).

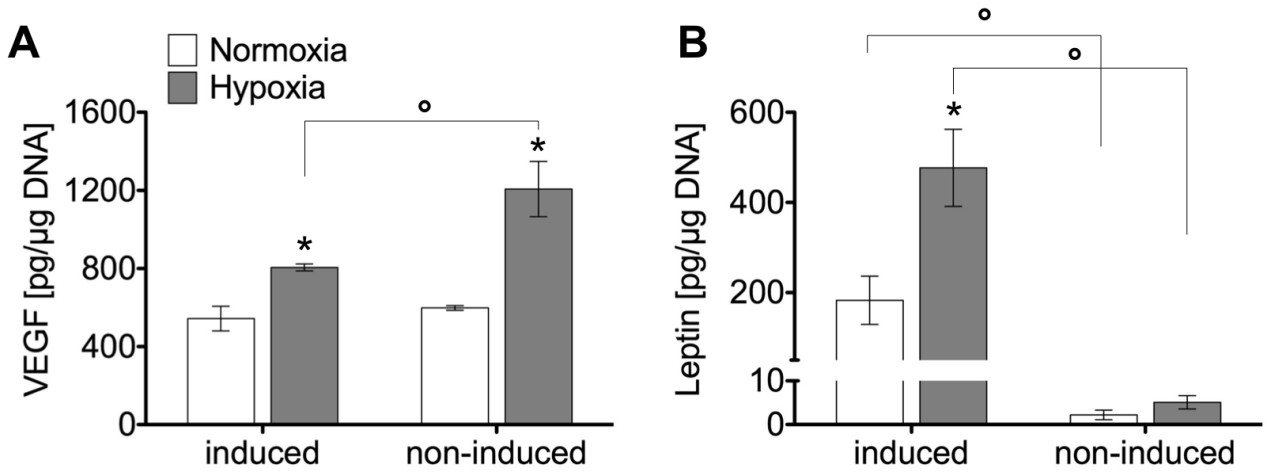

Figure 8. Quantification of VEGF and leptin secretion. VEGF (A) and leptin levels (B) were analyzed in cell culture supernatants of ASC-seeded stable fibrin gels collected between day 19 and 21 of culture; the obtained values were normalized to DNA content. * Statistically significant differences between normoxic and hypoxic treatment ($p < 0.05$). ° Statistically significant differences between adipogenic induction and non-induced conditions ($p < 0.05$).

Significantly increased production of VEGF was observed in the hypoxia-treated groups relative to normoxic conditions (Figure 8 A). The highest VEGF levels were detected in hypoxic non-induced specimen, which significantly exceeded VEGF secretion under induction. Equal amounts of VEGF were secreted by ASCs cultured at 21% pO_2 independent of adipogenic induction. Leptin levels were again increased under adipogenic treatment, especially in combination with hypoxic culture (Figure 8 B).

5.4 DISCUSSION

Upon *in vivo* implantation of engineered constructs, rapid oxygen supply is a major limiting factor deciding over the fate of the implanted cells. Effective prevascularization *in vitro* prior to implantation hence represents a valuable strategy to shorten hypoxic episodes to which transplanted cells are exposed *in vivo*, and to foster the maintenance and vascular integration of engineered tissue substitutes. Exploiting the fact that oxygen-limited conditions stimulate the production of pro-angiogenic effectors and potentially accustom implanted cells to the initially encountered hypoxic milieu *in vivo*, exposure to hypoxia was elucidated as a means of *in vitro* prevascularization. Thus, in the present work, the potential of hypoxic preconditioning to mediate angiogenic and pro-neovascular properties of ASCs, as well as the effect of low oxygen on adipogenic differentiation of ASCs in 2D culture and 3D stable fibrin gels, were explored.

In first 2D experiments, the response of ASCs towards reduced oxygen tension was evaluated in detail by expanding cells either under normoxia or hypoxia, and subsequently transferring them to normoxic or hypoxic conditions during the adipogenic differentiation phase. The respective recombinations of normoxia and hypoxia during expansion and differentiation led to the conclusion that the oxygen tension prevalent during the differentiation phase was decisive for adequate adipogenesis. This was specifically reflected by enhanced adipogenesis of ASCs cultured under normoxia/normoxia (NN) and hypoxia/normoxia (HN) relative to normoxia/hypoxia (NH) or hypoxia/hypoxia (HH) treatment. In a comparable setup, Valorani *et al.* similarly described the recovery of adipogenic differentiation of ASCs upon switching back to normoxia after exposure to hypoxic conditions [68].

On the protein level, leptin production in 2D culture under adipogenic stimulation was strongly regulated by hypoxia with leptin levels inversely correlating with adipogenesis. Wang *et al.* showed previously that preadipocytes under hypoxia markedly upregulate leptin [50], a finding that was also earlier reported in a study employing murine 3T3-F442A cells [26]. Leptin production is acknowledged to be hypoxia-sensitive and since leptin features both adipokine and non-adipokine functions [69,70], the above-described observations may be related to its role as a potent pro-angiogenic factor [7-9,71-76].

Notably in this study, cell numbers were not affected by hypoxic treatment indicating that low oxygen in the applied 2D and also 3D culture setups of ASCs neither resulted in altered proliferation rates nor cell death. In this context, differential observations have been reported by others, showing that hypoxia can either enhance [30,33,77], inhibit [12,78] or have no effect on the proliferative potential of ASCs and MSCs [42,79].

In accordance with results obtained for NN and HH conditions in 2D monolayer culture, it was demonstrated that in 3D adipose tissue constructs, exposure to hypoxic conditions equally impaired adipogenic differentiation by ASCs compared to normoxia. These findings were in line with previous work showing that generally, adipogenesis is reduced under hypoxia [33,59,80-82]. Likewise, in ASC-seeded stable fibrin gels, low pO_2 strongly provoked the secretion of great amounts of leptin under adipogenic induction, especially in longer-term culture after 21 days. Although not as pronounced, leptin levels were also increased in non-induced specimen under hypoxia relative to normoxic conditions, strengthening the aforementioned assumption that hypoxia-induced leptin secretion of ASCs in 3D constructs is rather pro-angiogenic than adipogenesis-associated. In support of this, Grosfeld *et al.* showed that hypoxia activates the leptin promoter and linked high leptin production in response to low oxygen with enhanced vessel development during adipose tissue growth [69,83]. Accordingly, the capacity of adipokines such as leptin and adiponectin to foster angiogenic processes by stimulation of VEGF expression and endothelial cell recruitment has been reported previously [7,71-74,84,85].

Histological analysis of 3D constructs after 21 days revealed further that endothelial and adipogenic differentiation of ASCs were inversely regulated under hypoxia. Likely owing to their multipotency, hypoxic treatment enforced CD31 expression of ASCs, whereas the amount of differentiating perilipin-positive ASCs upon adipogenic induction was distinctly reduced relative to normoxic conditions. Nevertheless, the fact that the simultaneous adipogenic and endothelial development of ASCs was facilitated upon hypoxic treatment was valued as a positive indicator since both processes are essential for adipose tissue development [86]. Elevated release of pro-angiogenic VEGF under hypoxia compared to normal pO_2 was in accordance with other studies [26,27] and additionally supported the notion that upon hypoxic treatment, the pro-angiogenic capability of ASCs was enhanced. Interestingly, the action of leptin in concert with VEGF and other pro-angiogenic growth

factors has early on been associated with the successful clinical use of adipose tissue in wound healing and revascularization of ischemic tissues [87,88].

With respect to the observed inverse correlation of adipogenic and pro-angiogenic properties of ASCs under hypoxia, and particularly the profound hypoxia-dependent secretion of leptin in the present study, further investigations are warranted. Exploring the mechanism by which hypoxia inhibits adipogenesis, Kim *et al.* discovered that low pO_2 blocks the clonal expansion step in 3T3-L1 preadipocytes [57] and in general, differentiation arrest under hypoxia is associated with the inhibition of PPARγ activation [57,89,90]. Impaired adipogenesis has further been linked with increased expression of hypoxia-inducible factor 1 alpha (HIF-1α) [89,90], which is the major transcription factor involved in oxygen sensing [89,91,92] and the activation of hypoxia-responsive genes [69,93]. HIF-1α is usually maintained at a low level in normoxic cells by proteasomal degradation and is stabilized by hypoxia [94-96]. It has been shown that HIF-1α transcription in the presence of low pO_2 controls the expression of VEGF and leptin [69,97-99]. Accordingly, analysis of PPARγ and HIF-1α, as well as VEGF and leptin on the transcriptional level, may provide more detailed insight concerning the regulation of adipogenic and pro-angiogenic capacities of ASCs in response to reduced oxygen tension.

In summary, it was demonstrated in this study that hypoxia plays a critical role in regulating adipogenic differentiation as well as angiogenic properites of ASCs on the cellular and molecular level. Specifically, hypoxic treatment of ASCs in engineered adipose tissue constructs provoked an increased secretion of leptin and VEGF in conjunction with enhanced CD31 expression which pointed towards the acquisition of enhanced pro-angiogenic properties by ASCs and the establishment of a microenvironment supportive of vascularization. Whether pre-exposure to hypoxia *in vitro* results in enhanced tolerance of ASCs towards hypoxic conditions and improved vascularization upon implanation *in vivo* remains to be determined. If this approach proved beneficial, preconditioned constructs may be employed as cell-based ‘delivery systems’ for endogenously produced pro-angiogenic factors in appropriate amounts and ratios for the induction of angiogenesis and neovascularization. Depending on the experimental approach, hypoxic treatment may represent a suitable precultivation strategy *in vitro* in addition to adipogenic induction and may be integrated with other (pre-)vascularization strategies such as co-seeding of

endothelial cells and microsurgical integration of a vascular supply. Finally, the adjustment of oxygenation to a more physiological tissue level, which commonly lies below 21% pO_2, may contribute to refining culture conditions for ASCs *in vitro* and to expanding current possibilities for construct precultivation. Overall, further advancing the understanding of the role of low oxygen tension on adipogenesis in 3D constructs was considered invaluable for the improvement of cell-based adipose tissue engineering approaches.

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Chapter 6

Investigation of the Stromal-Vascular Fraction as a Novel Cell Source for Adipose Tissue Engineering

6.1 INTRODUCTION

Growing and rebuilding adipose tissue requires a diversity of highly specialized cell types [1]. For approaches in tissue engineering and regenerative medicine, adipose tissue itself represents a valuable source of multipotent adipose-derived stem cells (ASCs) [2]. Traditionally, adipose tissue engineering strategies involve the minimally invasive harvest of autologous tissue, the isolation and expansion of the ASC subpopulation *in vitro*, seeding of tissue-specific scaffolds and reimplantation into the defected site [3,4]. Building upon this 'regenerative cycle', previous work by our own and other groups aimed at the reconstruction of adipose tissue based on ASCs *in vitro* and *in vivo* [5-11]. However, the effectiveness of ASCs differed greatly among studies and also the extent to which implanted ASCs contributed to adipose regeneration *in vivo* varied significantly [5,9,11-13].

In search for alternatives, uncultured stromal-vascular fraction (SVF) cells have been suggested as a novel cell source for adipose tissue engineering approaches [14]. Seminal studies using SVF cells for soft tissue regeneration have been conducted by Yoshimura *et al.*, who have supplemented lipoaspirates with SVF cells, improving volume retention and viability of grafted tissue [15,16]. Regarding the regenerative role of the stromal compartment in a physiological context [17,18], it is reasonable to assume that the application of the stromal cell pool may promote the formation and maintenance of functional adipose tissue. Indeed, the multicellularity of adipose tissue is particularly well represented by the SVF, which includes ASCs, vascular precursors and endothelial cells, pericytes, fibroblasts, smooth muscle cells and immune cells, among others [1,19-23].

Regarding the overall goal to engineer vascularized adipose tissue, apart from ASCs exhibiting adipogenic differentiation potential, endothelial cells and pericytes contained in the SVF were considered especially advantageous to improve vascular (self-)assembly and capillary formation in adipose tissue constructs. Current approaches to achieve prevascularization entail coculture setups combining ASCs or other cell types of interest with either human umbilical vein endothelial cells (HUVECs), endothelial progenitor cells (EPCs) or dermal microvascular endothelial cells (MVECs) [24-30]. In this context, utilizing the SVF as a source of endothelial cells, Koh *et al.* have demonstrated the effectiveness of SVF implants for therapeutic neovascularization [31], whereas

Scherberich *et al.* have previously identified the SVF as a relevant source of vasculogenic progenitors for bone regeneration [32]. Further, using the entire pool of cells concentrated in the SVF potentially allows the application of tissue-inherent ratios of the respective cell types [33], which may foster the reciprocal interaction of adipogenesis and angiogenesis as the fundamental basis of adipose tissue formation [34-36].

Despite various studies using SVF cells for bone and vascular tissue engineering approaches [31,32,37], for adipose tissue engineering, the verification of adipogenesis by SVF cells and some general *in vitro* characterization have currently only been addressed by Zimmerlin *et al.* in Tisseel fibrin spray [38] and Lin *et al.* using collagen and gelatin sponge scaffolds [14], whereas notably, no *in vivo* investigations have been undertaken so far. Indeed, the cellular heterogeneity and herewith associated ineligibility of some SVF subpopulations for expansion in 2D culture, as well as the difficulty of establishing suitable *in vitro* conditions that adequately support the nutritional and metabolic requirements of the respective SVF cell types, complicate the use of SVF cells for adipose tissue engineering applications. Consequently, since only limited experience exists concerning adipogenic culture of the SVF, the present study pursued the evaluation of this promising new cell source for adipose tissue engineering in preliminary experimental work *in vitro* in order to prepare the investigation of SVF cells in successive *in vivo* experiments.

Further, to more comprehensively investigate *in vivo* adipose tissue constructs after explantation, we additionally intended to introduce a method by which newly developed tissue structure could be efficiently visualized. Especially the interactions between adipocytes, ASCs, blood vessels and stromal cells during adipogenesis are difficult to grasp by conventional histology. Since the latter solely conveys a 2D impression of structural elements of the tissue, the development of a whole mount staining (WMS) technique, potentially allowing a thorough 3D interpretation of tissue architecture and dimensions, was approached. Nishimura and coworkers for example, have applied this cell imaging technique to study adipogenic development in a murine model of obesity [39] and Koh *et al.* employed WMS to analyse capillary formation by SVF cells [31], however, to date this technique has not been ultilized for the visualization of 3D tissue structure in engineered adipose tissue *in vivo*.

Altogether, this study aimed at the preliminary investigation of uncultured SVF cells *in vitro* for their suitability as cell source for adipose tissue engineering. Initial experiments seeding SVF cells in 3D stable fibrin gels were intended to serve as pre-evaluation for *in vivo* studies investigating the potential of SVF-seeded constructs for the generation of vascularized adipose tissue. Eminently, cellular response towards hormonal induction and different media compositions was evaluated, characterizing SVF cells in terms of adipogenic and endothelial development in comparison to ASCs as reference cells. Further, to facilitate the histological analysis of engineered adipose tissue *in vivo*, the stepwise development of a WMS technique was intended. Here, employing native adipose tissue as model tissue, different complementary staining procedures for structual analysis of adipocytes and blood vessels in a 3D context, were established.

6.2 MATERIALS AND METHODS

6.2.1 Cell isolation

SVF cells were isolated from subcutaneous adipose tissue obtained from abdominal depots of healthy female donors. The study was approved by the ethics committee of the University of Wuerzburg, Germany. Written informed consent was obtained from all patients. Patients were between 30 and 56 years of age and had a body mass index (BMI) ranging from 27 to 38.

For cell isolation, the tissue was minced and digested with 0.1% collagenase NB4 from Clostridium histolyticum (Serva Electrophoresis, Heidelberg, Germany) in collagenase buffer for 2 h at 37 °C on an orbital shaker. The digested tissue was filtered through a 100 µm nylon mesh and centrifuged at 300 g for 10 min. The pelleted SVF cells were washed with phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria) and resuspended in basal medium containing Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12) supplemented with 1% penicillin-streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 10% fetal bovine serum (FBS), all obtained from Invitrogen (Karlsruhe, Germany). For cryopreservation of the SVF in liquid nitrogen, 5% DMSO (Sigma-Aldrich, Steinheim, Germany) was added to the medium.

For seeding of fibrin gels, SVF cells were thawed and resuspended in prewarmed PBS (37 °C). After centrifugation, erythrocyte lysis buffer was added to the pelleted cells for 10 min until subsequent removal of the buffer by centrifugation. The cells were washed with PBS and stained with 0.4% trypan blue for counting.

For cell expansion and subsequent seeding of constructs with ASCs, cryopreserved SVF cells were thawed and plated on tissue culture-treated plastic flasks (175 cm^2). The ASC subpopulation was selected via plastic adherence in basal medium. The cells were cultured to a subconfluent level and expanded for 2 passages until seeding of the scaffolds. For passaging, trypsin-EDTA at 0.25% from Invitrogen (Karlsruhe, Germany) was used.

6.2.2 Preparation of stable fibrin gels

Stable fibrin gels were prepared as described previously [5,40] with a fibrinogen concentration of 25 mg/mL, 20 mM $CaCl_2$, and 2.5 U/mL thrombin. Bovine fibrinogen and aprotinin from bovine lung were obtained from Sigma-Aldrich (Steinheim, Germany).

20 µL of 50 mg/mL fibrinogen dissolved in an aprotinin solution [10,000 kallikrein inhibitory units (KIU)/mL] were mixed with an equal volume of a 5 U/mL thrombin solution and subjected to 45 min of gelation at 37 °C and 5% CO_2. The thrombin component was prepared using a 1:100 dilution of thrombin-S (500 U/mL, TissuCol® Kit Immuno 1.0; Baxter, Unterschleissheim, Germany).

6.2.3 3D cell culture

Stable fibrin gels (40 µL; height 2 mm, diameter 5 mm) were seeded with either 0.2 x 10^6 or 1.0 x 10^6 viable SVF cells or ASCs. Cells were suspended in the thrombin component. 20 µL of the thrombin-cell suspension were mixed with 20 µL fibrinogen (50 mg/mL) and transferred to sterile glass rings with an inner diameter of 5 mm. Constructs were removed from the glass rings after gelation and cultured dynamically on an orbital shaker at 50 rpm (37 °C, 5% CO_2).

For *in vitro* culture, constructs were either cultured in preadipocyte growth medium 2 (PGM-2) or in a 1:1 mixture of PGM-2 and endothelial cell growth medium 2 (EGM-2). PGM-2 consisted of preadipocyte basal medium 2 (PBM-2; Lonza, Walkersville, USA) supplemented with 1% penicillin-streptomycin and 10% FBS. For EGM-2 preparation, endothelial cell basal medium 2 (EBM-2; Lonza, Walkersville, USA) was supplemented with 5% FBS, gentamycin/amphotericin-B, insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), ascorbic acid (AA), hydrocortisone (HC) and fibroblast growth factor 2 (FGF-2) according to the manufacturer. For adipogenic induction, PGM-2 and PGM-2/EGM-2 respectively, were supplemented with 1.7 µM insulin, 1 µM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine (IBMX) and 200 µM indomethacin. For medium preparation, IBMX was obtained from Serva Electrophoresis (Heidelberg, Germany), bovine insulin from PromoCell (Heidelberg, Germany), and indomethacin as well as dexamethasone were purchased from Sigma-Aldrich (Steinheim, Germany). Cell-seeded stable fibrin gels in the non-induced control groups were cultured either in PGM-2 alone or in PGM-2/EGM-2 for the entire culture period without adipogenic induction. Medium exchange was performed every other day. Constructs were maintained *in vitro* for 10, 21 or 35 days as indicated for the respective experiments in the results section.

6.2.4 Oil Red O staining

Adipogenesis *in vitro* was histologically investigated by Oil Red O staining. Constructs were fixed in buffered formalin (3.7% in PBS) and dehydrated in increasing sucrose series (10-60%) prior to embedding in Tissue-Tek® O.C.T. compound (Sakura Finetek, Zoeterwonde, Netherlands). 8 µm sections were cut and in the following stained with Oil Red O solution (3 mg/mL in 60% isopropanol; Sigma-Aldrich, Steinheim, Germany). Counterstaining was performed with hematoxylin (Bio Optica, Milan, Italy) for visualization of nuclei. Slides were coverslipped with Glycergel Mounting Medium (Dako, Carpinteria, USA) and microscopically analyzed. The obtained images were processed with the Olympus cellSens™ Dimension Microscope Imaging Software (Olympus, Hamburg, Germany).

6.2.5 CD31 staining

Endothelial cells in SVF-seeded stable fibrin gels were analyzed by staining against CD31. 8 µm cryosections were prepared as described above. After rehydration in PBS, unspecific binding was blocked with blocking solution (1.5% BSA in PBS) prior to overnight incubation with a monoclonal mouse anti-human CD31 antibody (Clone JC70A; Dako, Carpinteria, USA). Slides were thoroughly washed with PBS and incubated with a rabbit anti-mouse HRP-conjugated secondary antibody for 30 min (Dako, Carpinteria, USA). After repeated washing, slides were developed upon addition of 3,3'-diaminobenzidine (DAB; Liquid DAB Substrate, Biogenex, Freemont, USA). Nuclei were counterstained with hematoxylin (Bio Optica, Milan, Italy). Slides were coverslipped with Glycergel Mounting Medium (Dako, Carpinteria, USA) prior to microscopic analysis.

6.2.6 BODIPY/CD31/DAPI staining

Maturing adipocytes and prevascular structures in long-term cultured SVF-seeded constructs *in vitro* were analyzed by BODIPY/CD31/DAPI staining. 8 µm cryosections were rehydrated in PBS and unspecific binding was blocked with blocking solution prior to incubation with a monoclonal mouse anti-human CD31 antibody (Clone JC70A; Dako, Carpinteria, USA) overnight. Slides were thoroughly washed with PBS and a Cy™3-conjugated AffiniPure donkey anti-mouse secondary antibody (Jackson Immuno Research, West Grove, USA) was added for 30 min in the dark. Lipid inclusions were

additionally stained by incubation with BODIPY® 493/503 (stock 1 mg/mL, 1:100 dilution in PBS; Invitrogen, Karlsruhe, Germany) for 15 min, and after thorough washing with PBS, nuclei were counterstained with IS Mounting Medium DAPI (Dako, Carpinteria, USA). Slides were coverslipped and viewed under a fluorescence microscope. Resulting images were overlaid using Olympus cellSens™ software.

6.2.7 Whole mount staining in native adipose tissue

Native adipose tissue was applied for the establishment of WMS. Tissue blocks sized approximately 4 x 4 mm were fixed in 3.7% buffered formalin overnight and washed twice with PBS for 1 h. Enzymatic digest for antigen retrieval was tested prior to the blocking step with Digest All-4 (Life Technologies, Frederick, USA) for 5 min at room temperature. Whole mounts were then incubated with blocking buffer (3% BSA in PBS/Triton X-100) overnight and afterwards rinsed with PBS/Triton X-100 for 2 h.

Immunostainings: For the immunolabeling of blood vessels, the respective primary antibodies against CD31 and von Willebrand factor were added overnight [mouse anti-human CD31, rabbit anti-human factor VIII related antigen (Diagnostic BioSystems, Pleasanton, USA)]. Accordingly, for perilipin and laminin staining, mouse anti-human perilipin (Creative Diagnostics, Shirley, USA) and polyclonal rabbit anti-laminin (Abcam, Cambridge, UK) antibodies were employed. Human vimentin-positive structures were stained with a monoclonal rabbit anti-human vimentin antibody (Clone SP20; Zytomed Systems, Berlin, Germany). After incubation with the primary antibodies, the tissue was washed with PBS/Triton X-100 for 2 h. Blocking buffer was again added for 2 h, then whole mounts were incubated with the according species-matched secondary antibodies [Alexa Fluor® 488 or 594-conjugated AffiniPure goat anti-rabbit, Cy™3-conjugated AffiniPure donkey anti-mouse secondary antibody (Jackson Immuno Research, West Grove, USA)] for 2 h.

Lectin staining: As an alternative staining method for the visualization of capillaries, whole mounts were incubated overnight with rhodamine-conjugated ulex europaeus agglutinin I (UEA I; Vector Laboratories, Burlingame, USA) directed against human endothelial cells. Prior to incubation, UEA I was diluted in antibody diluent (Dako, Carpinteria, USA) as indicated in the results section.

BODIPY staining: BODIPY staining of mature adipocytes was performed following either immuno- or lectin staining of blood vessels. Whole mounts were washed with PBS/Triton X-100 overnight and for another 2 h with PBS prior to incubation with a 1:100 dilution of BODIPY® 493/503 (stock 1 mg/mL; Invitrogen, Karlsruhe, Germany). After 1 h, the BODIPY solution was removed and whole mounts were rinsed for 2 h with PBS.
All staining steps were performed on an orbital shaker at 50 rpm and room temperature. For counterstaining of nuclei IS Mounting Medium DAPI (Dako, Carpinteria, USA) was employed. Whole mount-stained native tissue was mounted on coverslips and imaged using a fluorescence microscope. The resulting images were overlaid using cellSens™ software. Alternatively, a TCS-SP2 AOBS Leica confocal laser scanning microscope (CLSM) was employed together with the Leica LCS Lite Software (Leica, Wetzlar, Germany).

6.2.8 Real-time qRT-PCR analysis

Total RNA from SVF cells seeded in stable fibrin gels was harvested after 10 days of *in vitro* culture using TRIzol® reagent (Invitrogen, Karlsruhe, Germany). First-strand cDNA was synthesized with the ImProm-II™ Reverse Transcription System from Promega (Madison, USA). MESA GREEN qPCR MasterMix Plus (Eurogentec, Seraing, Belgium) was employed for real-time qRT-PCR analysis performed with Meteor*Taq* polymerase at the following cycling conditions: 95 °C for 15 min followed by 40 cycles at 95 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec. Melting curve analysis was carried out from 74 °C to 89 °C. Specific primer pairs for peroxisome proliferator-activated receptor gamma (PPARγ; QT00029841), adipocyte protein 2 (aP2; QT01667694) and endothelial CD31 (QT00081172) were purchased from Qiagen (Hilden, Germany). mRNA expression levels were normalized to β-actin (QT00079247). Fold increase in expression levels relative to non-induced constructs (mean ΔCT) was determined using the $2^{-\Delta\Delta CT}$ method.

6.2.9 Statistics

Results are presented as mean values ± standard deviation (SD). Statistical significance was assessed by analysis of variance followed by an unpaired two-way Student's t-test at the level of $p < 0.05$. Statistical analysis was performed using GraphPad Prism, Version 5.0 (GraphPad Software, La Jolla, USA). If not stated otherwise, the number of samples per group was n=3.

6.3 RESULTS

6.3.1 Establishment of culture conditions for SVF cells in 3D constructs

SVF cells were evaluated concerning their suitability for adipose construct development. Pelleted SVF cells were seeded directly in stable fibrin gels and investigated *in vitro* (Figure 1). For the experiments, stable fibrin gels with reduced stiffness (Chapter 3) were prepared at a fibrinogen concentration of 25 mg/mL.

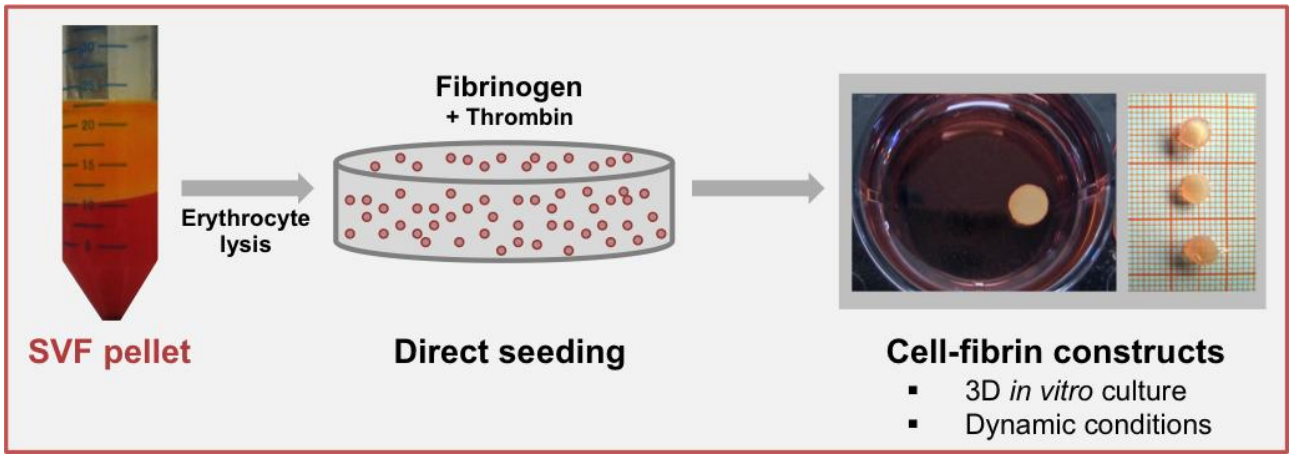

Figure 1. Outline of construct preparation and culture of SVF-seeded fibrin gels. Constructs were prepared by direct seeding of SVF cells in stable fibrin gels. SVF-seeded constructs were subjected to *in vitro* culture under dynamic conditions at 50 rpm on an orbital shaker.

After lysis of red blood cells, stable fibrin gels were seeded with 0.2 x 10^6 viable SVF cells per 40 µL construct. This comparatively low seeding density was employed for the establishment of SVF culture, since otherwise vast amounts of SVF cells, which cannot be expanded in 2D culture as commonly performed with ASCs, would have been required. The cells were resuspended in the thrombin component and, by addition of an equal volume of fibrinogen solution (50 mg/mL), fibrin gels were prepared as described before (Chapters 3-5). In the following, SVF-seeded fibrin gels were submitted to *in vitro* culture under dynamic conditions.

As illustrated in Figure 2, a modified induction and culture setup in comparison to the afore-applied conditions for ASC-seeded constructs were employed. To avoid the positive selection or loss of certain SVF subpopulations during the previously performed two-day proliferation phase prior to adipogenic induction, SVF-seeded constructs were now directly

after preparation subjected to the respective adipogenic or non-adipogenic culture conditions.

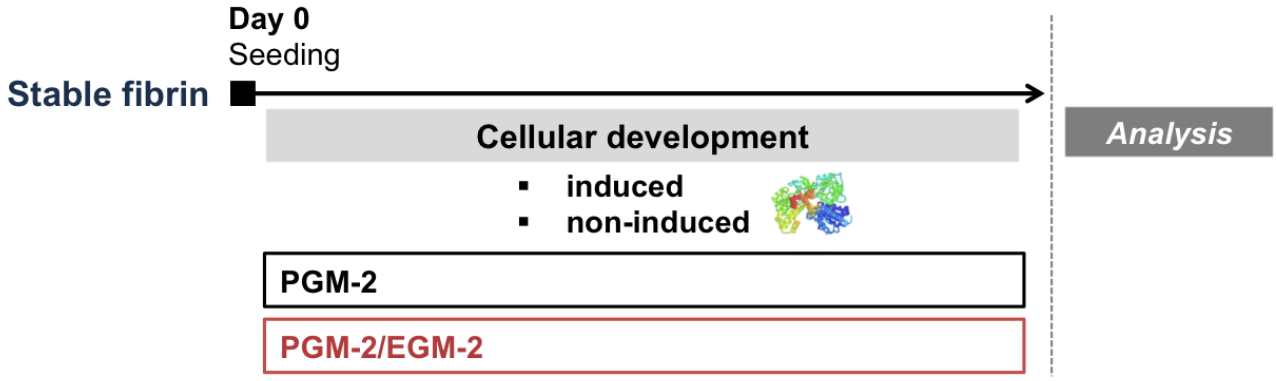

Figure 2. Experimental setup. Stable fibrin gels were seeded with SVF cells and adipogenic differentiation was induced directly after construct preparation (day 0). Different media compositions were tested for SVF cell culture. Preadipocyte growth medium 2 (PGM-2) was compared to a 1:1 mixture of PGM-2 and endothelial cell growth medium 2 (EGM-2). For adipogenic induction, the hormonal cocktail was added to the respective media at concentrations previously employed for the induction of ASCs. Non-induced constructs were cultured without the addition of adipogenic inducers. Constructs were analyzed at the cellular and molecular levels.

To better support cell survival and development of the heterogeneous cell types present in the SVF, different medium compositions were applied for *in vitro* culture. Constructs were initially subjected to culture conditions established for ASCs employing preadipocyte growth medium 2 (PGM-2). As an alternative, a 1:1 mixture of PGM-2 and endothelial cell growth medium 2 (EGM-2) was applied. EGM-2 contains pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2), as well as insulin-like growth factor 1 (IGF-1), ascorbic acid and hydrocortisone. Epidermal growth factor (EGF) as additional supplemental factor was not added to the culture medium due to inhibitory effects on adipogenic differentiation [41-43].

Adipogenic induction was accomplished by addition of the complete set of hormonal inducers (insulin, IBMX, dexamethasone, indomethacin) to PGM-2 or PGM-2/EGM-2, respectively. Non-induced culture was performed devoid of adipogenic inducers. Since the response of SVF cells towards stable fibrin as cell carrier had not been evaluated before, ASCs were included in the culture setup for comparison. Thus, ASCs were encapsulated in stable fibrin gels at the same seeding density and treated according to SVF cells in 3D constructs.

6.3.2 Differentiation of SVF cells in 3D culture

Cellular response of SVF cells in 3D culture in stable fibrin gels under adipogenic and non-induced conditions in PGM-2 and PGM-2/EGM-2 was investigated after a culture period of 21 days and compared to ASC-seeded constructs. Oil Red O staining of intracellular triglycerides in cross-sections of cryopreserved constructs was employed to assess adipogenic differentiation *in vitro* (Figure 3).

Upon general comparison of SVF cells and ASCs in stable fibrin gels, it was observed that cell numbers in the SVF group were considerably reduced, independent of the applied cell culture medium or adipogenic induction. Accordingly, the cell distribution in SVF-seeded gels was less homogeneous than in constructs prepared with ASCs. SVF-seeded constructs displayed irregularly shaped (induced group) and spindle-like, stretched cells (non-induced group), whereas ASCs under the respective conditions were more uniform in size and morphology. Adipogenic induction of SVF cells and ASCs in both PGM-2 and PGM-2/EGM-2 resulted in the formation of lipid-filled vacuoles as indicated by Oil Red O staining. In ASC-seeded constructs, cellular differentiation similar to previous experiments was observed, with distinct intracellular lipid droplets displaying a uniform size. SVF cells and ASCs cultured under non-induced conditions did not show any notable signs of lipid storage.

Concerning medium composition, differences in differentiation capacity and cell number were observed between PGM-2 alone and composite PGM-2/EGM-2 medium. In SVF-seeded constructs, adipogenic culture in PGM-2/EGM-2 resulted in enhanced adipogenic differentiation, especially with respect to droplet size in comparison to PGM-2 alone. Further, cell numbers were higher in SVF-seeded gels cultured in the 1:1 mixture, irrespective of induction. In constrast, an improved differentiation capacity and cell density was noted for induced ASCs in PGM-2.

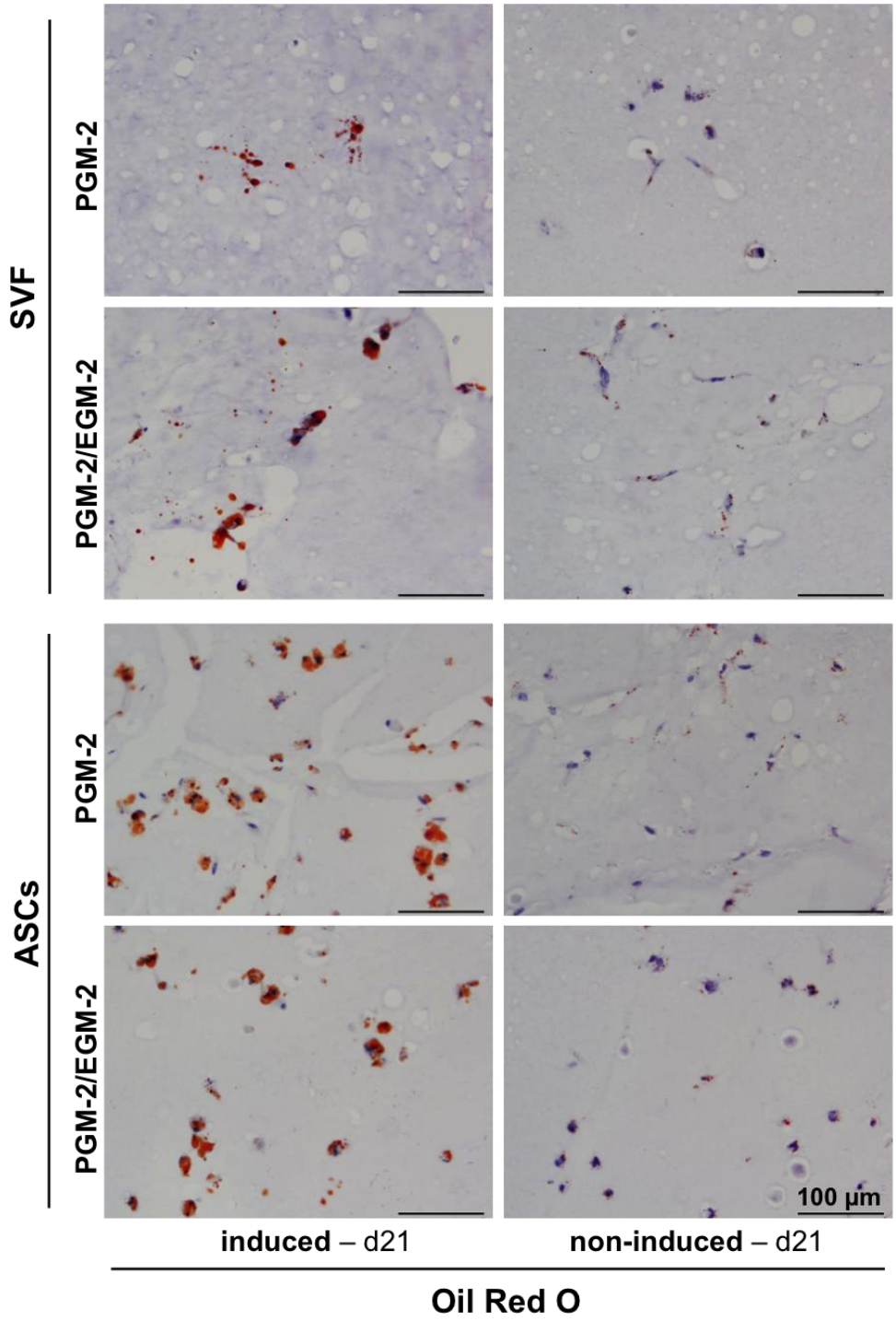

Figure 3. Adipogenic differentiation of SVF cells and ASCs in different cell culture media. Cells were seeded in stable fibrin gels and cultured in PGM-2 or a 1:1 mixture of PGM-2/EGM-2 under induced and non-induced conditions. Oil Red O staining was performed at day 21 of culture; nuclei were counterstained with hematoxylin. Scale bars represent 100 µm.

Apart from adipogenesis, endothelial differentiation capacity of SVF cells was investigated on the cellular level by staining against CD31 (Figure 4). Observations from the previous section with regard to cell shape, cell number and distribution were confirmed. SVF-seeded

constructs under induced conditions in PGM-2 were largely devoid of $CD31^+$ cells, however under non-induced conditions some isolated cells staining positive for CD31 were found.

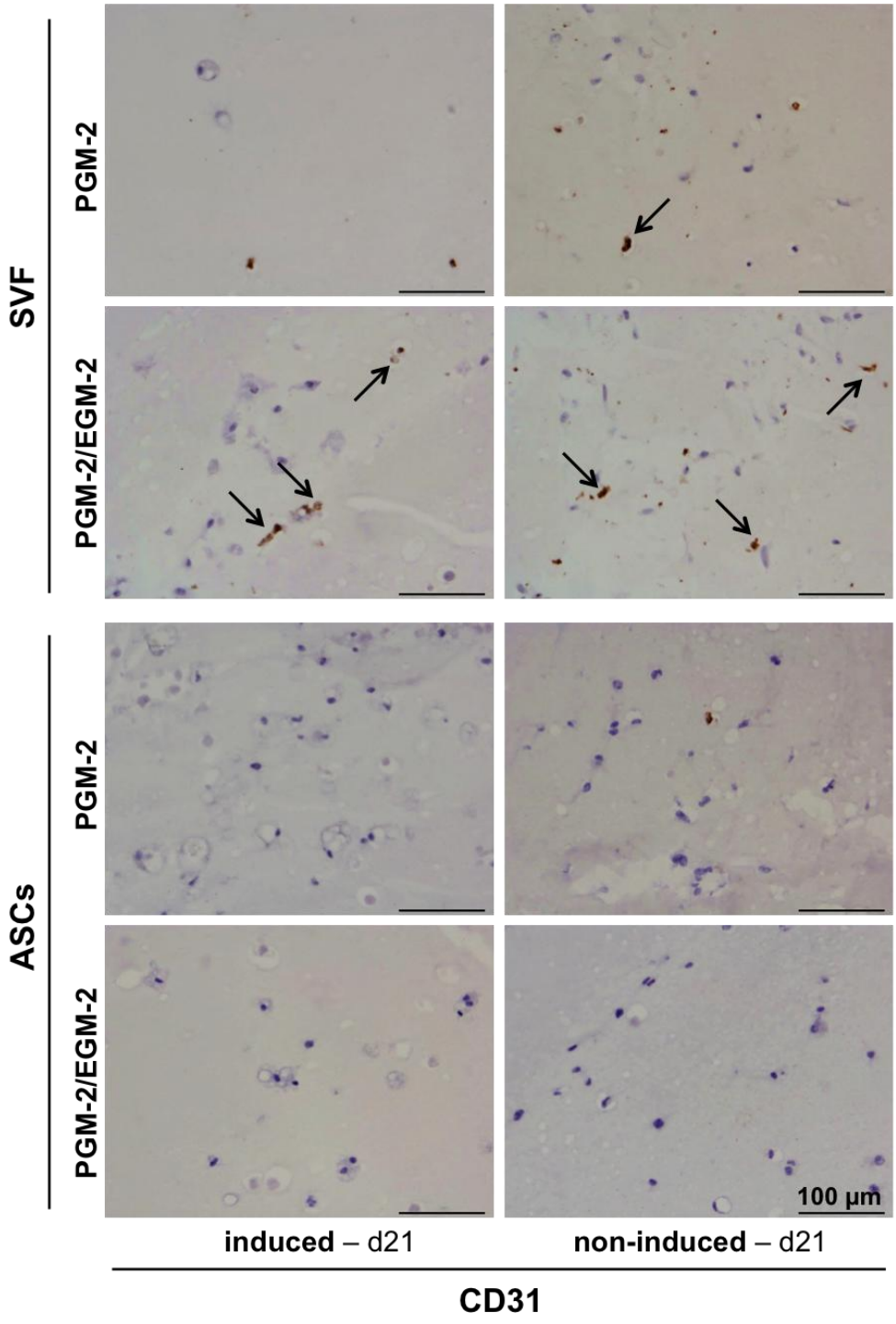

Figure 4. Endothelial development of SVF cells and ASCs in different cell culture media. Cells were seeded in stable fibrin gels and cultured in PGM-2 or a 1:1 mixture of PGM-2/EGM-2 under induced and non-induced conditions, respectively. Staining against CD31, as indicated by a brown precipitate, was performed at day 21 of culture; nuclei were counterstained with hematoxylin. Scale bars represent 100 µm; arrows indicate examples of CD31-positive cells.

In contrast, in constructs cultured in PGM-2/EGM-2, various $CD31^+$ cells were observed independent of induction. A slight tendency towards higher numbers of $CD31^+$ cells was found for non-induced SVF-seeded constructs in PGM-2/EGM-2. Irrespective of media composition, endothelial differentiation was not observed in constructs seeded with ASCs.

For further investigation of SVF cells on the molecular level and evaluation of long-term culture *in vitro*, PGM-2/EGM-2 was applied, since this medium composition appeared to more efficiently maintain SVF cells in stable fibrin gels. In addition, for continuative experiments, constructs were seeded at an elevated seeding density of 1.0×10^6 cells/construct.

To support the histological observations from the previous experiments, adipogenic and endothelial properties of SVF cells seeded in stable fibrin gels were evaluated on the transcriptional level by qRT-PCR after 10 days of culture in PGM-2/EGM-2 under induced and non-induced conditions (Figure 5).

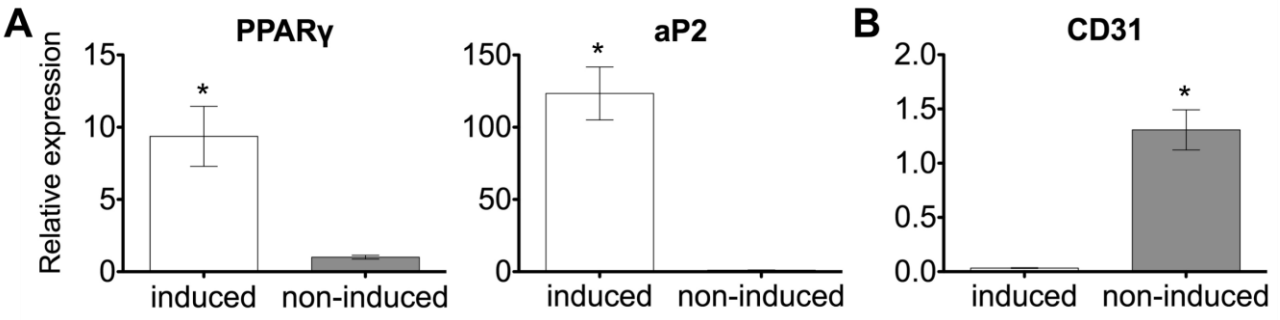

Figure 5. Gene expression analysis of SVF cells in stable fibrin gels cultured in PGM-2/EGM-2. Adipogenic (PPARγ, aP2) (A) and endothelial markers (CD31) (B) were analyzed after 10 days of *in vitro* culture; values were normalized to β-actin. * Statistically significant differences between the indicated groups ($p < 0.05$).

Adipogenic marker expression of SVF cells, as represented by the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) and adipocyte protein 2 (aP2), was upregulated upon adipogenic treatment relative to non-induced culture (Figure 5 A). In contrast, in non-induced specimen, endothelial CD31 marker expression was significantly increased compared to induced conditions (Figure 5 B).

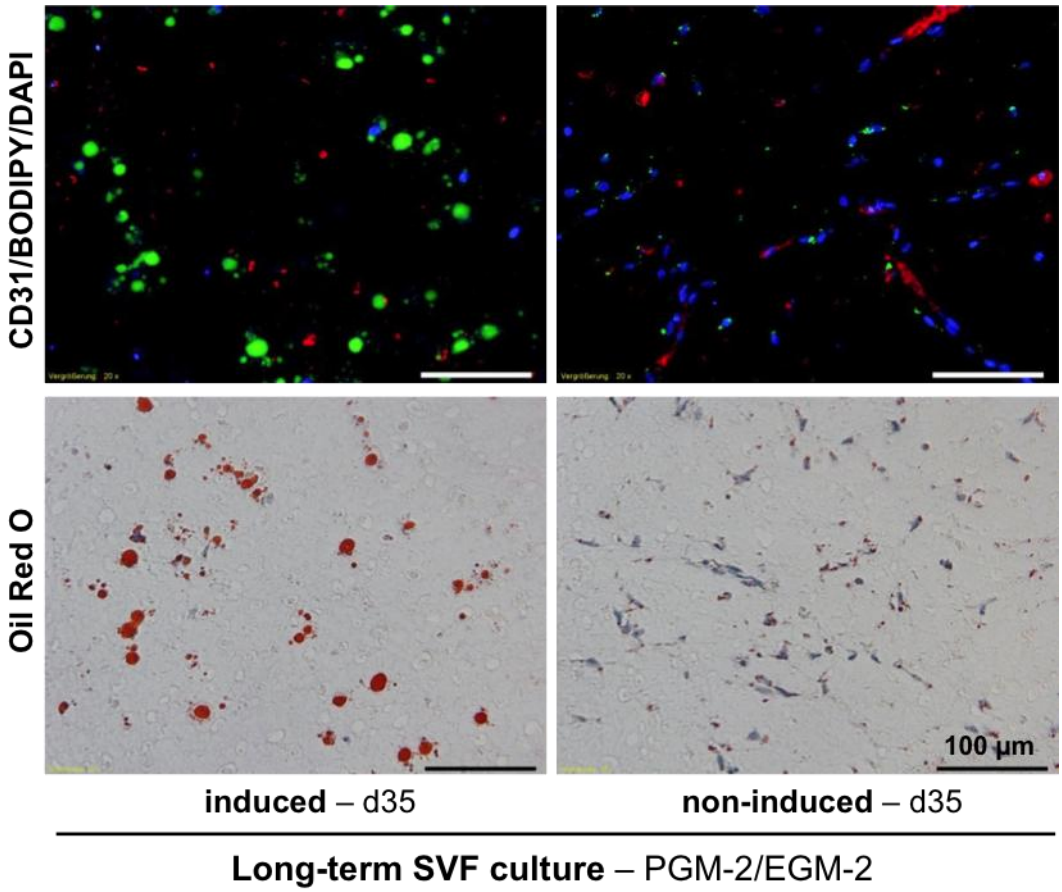

Figure 6. Long-term culture of SVF-seeded stable fibrin gels. Constructs were cultured in PGM-2/EGM-2 for 35 days under adipogenic and non-induced conditions. Endothelial differentiation was evaluated by staining against CD31 (red), lipid vacuoles were stained with BODIPY (green), and DAPI (blue) was used for counterstaining of nuclei. Adipogenic differentiation was additionally analyzed by Oil Red O staining; nuclei were counterstained with hematoxylin. Scale bars represent 100 µm.

To additionally investigate the long-term outcome of SVF cell culture in 3D constructs *in vitro*, SVF-seeded stable fibrin gels were cultured over a period of 35 days in PGM-2/EGM-2 and again analyzed in terms of adipogenic and endothelial differentiation (Figure 6). Cells were triple-stained against CD31, as well as with BODIPY and DAPI to simultaneously visualize endothelial structures, lipid vacuoles and nuclei, respectively. For comparison, SVF-seeded constructs were also subjected to conventional staining of intracellular triglycerides with Oil Red O. As reflected by CD31/BODIPY/DAPI staining, SVF cells distinctly accumulated intracellular triglycerides upon adipogenic induction, whereas under non-induced conditions lacking adipogenic stimuli, cells formed pronounced prevascular $CD31^+$ structures under long-term culture. In the non-induced group, the

absence of BODIPY^{+} lipid vacuoles was noted. Regarding lipid accumulation, the described observations were confirmed by Oil Red O staining.

6.3.3 Establishment of a whole mount staining technique for improved construct analysis

Despite the more laborious handling and less predictable results associated with SVF cells as cell source, the coimplantation of different cell types *in vivo* to reconstitute native adipose tissue was of particular interest. To facilitate the analysis of *in vivo* adipose tissue constructs after explantation, a whole mount staining technique (WMS) was developed. In contrast to conventional 2D histology, which entails the stepwise embedding, cross-sectioning and staining of the structures of interest, WMS may be performed in whole 3D tissues (Figure 7). Since this method had previously not been applied for the evaluation of engineered adipose tissue constructs, 3D staining protocols for the main structural components of adipose tissue, namely adipocytes and blood vessels, were separately established and optimized in experimental work described in the following.

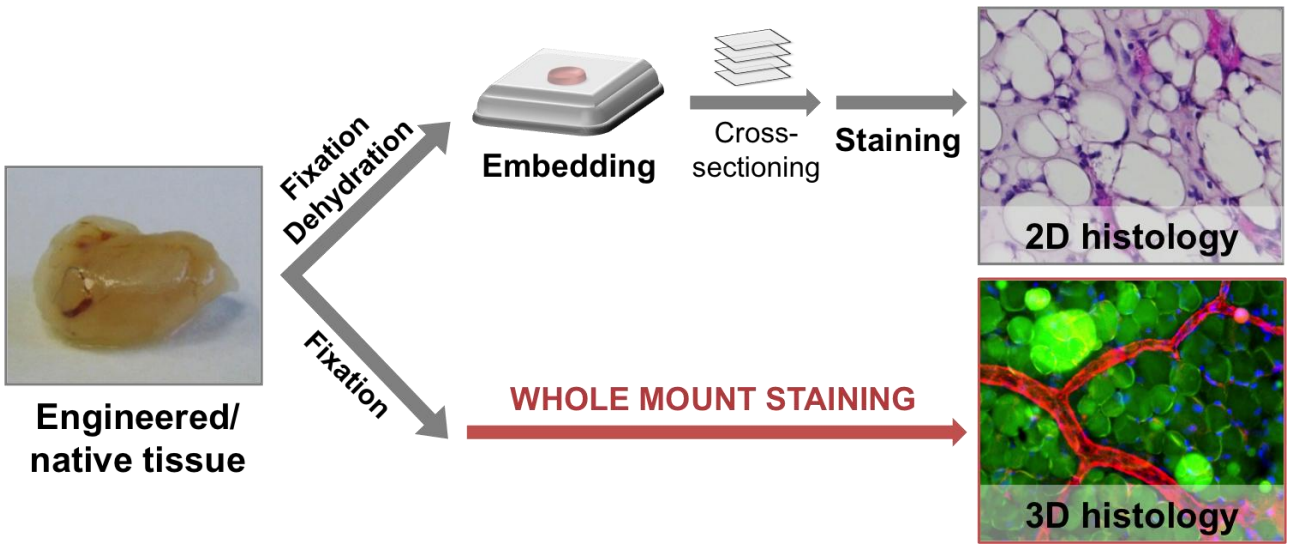

Figure 7. Comparison of conventional 2D histology and 3D whole mount staining.

Native human adipose tissue was employed for method development to afterwards translate the WMS technique to engineered constructs *in vivo*. The preparative steps for WMS, resulting in a triple staining of vascular structures (red), adipocytes (green) and nuclei (blue) are described in detail in Figure 8.

Fixation of *en-bloc* adipose tissue was carried out according to protocols for 2D histology using 3.7% buffered formalin. Enyzmatic digest using proteinase K (Digest All-4) for

antigen retrieval was tested, yet no beneficial effect on the staining result was observed between treated and untreated specimen (data not shown). An initial permeabilization step (PBS/Triton X-100) together with blocking of unspecific bindings sites (3% BSA in PBS/Triton X-100) was found essential prior to addition of the respective primary antibodies (e.g. rabbit anti-human von Willebrand factor (vWF) for vascular staining). To improve permeation of the staining solutions into the tissue, the staining steps were performed under constant motion on an orbital shaker.

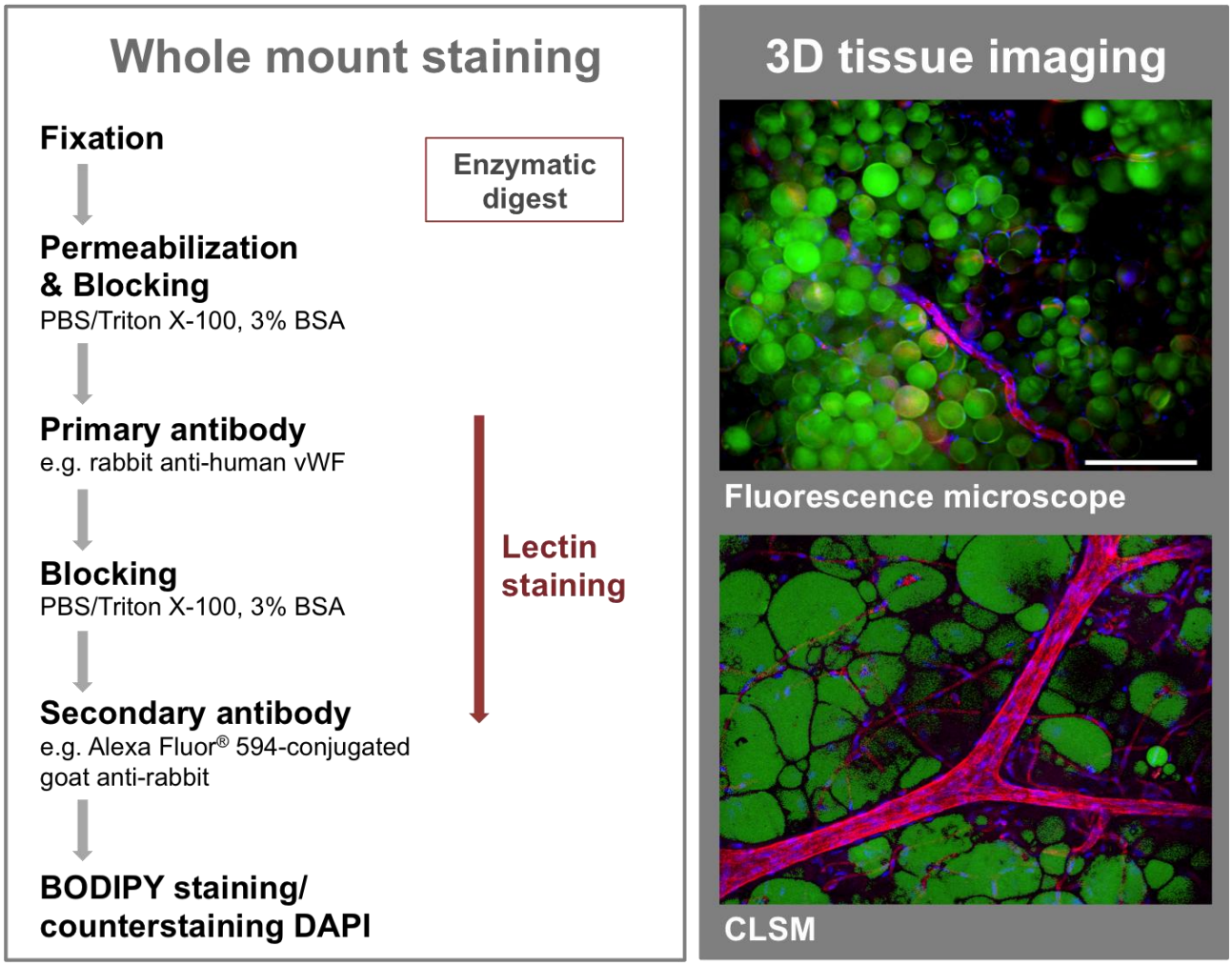

Figure 8. Whole mount staining procedure and suitable 3D imaging methods.

Each staining step was followed by intermittent thorough washing with either PBS or PBS/Triton X-100 to remove unbound antibody. An additional blocking step prior to addition of the secondary antibody was adapted according to the WMS protocol reported by Xue *et al.* [44]. The respective fluorescence-conjugated secondary antibodies (e.g. Alexa Fluor® 594-conjugated goat anti-rabbit) were added in a second step. As an alternative

capillary staining, a rhodamine-conjugated lectin directed against human endothelial cells was introduced as described in the following paragraph in detail. After labeling of vascular structures, mature adipocytes were visualized by addition of BODIPY, whereas nuclei were counterstained with DAPI. The structural assembly of capillaries within the bulk of BODIPY-stained adipocytes was analyzed by microscopy using either a conventional fluorescence microscope with the appropriate filters or a confocal laser scanning microscope (CLSM) (Figure 8).

In subsequent experiments, the staining procedures for vascular structures and mature adipocytes were optimized. Using an antibody directed against human CD31 (referred to as 'CD31 staining') and ulex europaeus agglutinin I (UEA I), a lectin which binds to carbohydrate moieties of glycoproteins or glycolipids on human endothelial cells [45] (here and in the literature [39,44] this staining is referred to as 'lectin staining'), different methods for the visualization of the capillary system were evaluated in detail (Figure 9).

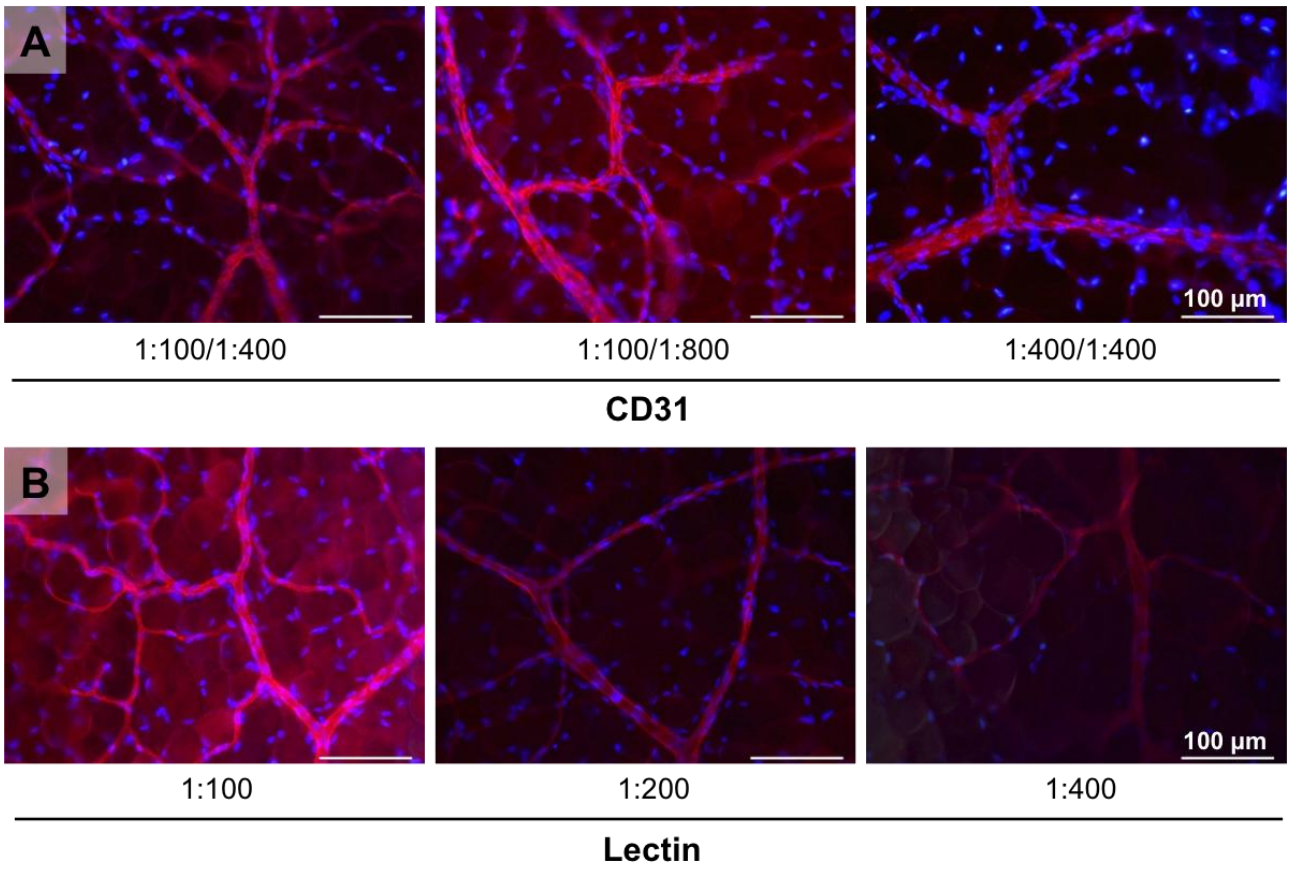

Figure 9. Optimization of vascular staining. Different dilutions of the applied primary and secondary antibodies were recombined to improve staining against CD31 in native adipose tissue (A). A more simplified method for the labeling of capillaries was investigated using dilutions of rhodamine-conjugated UEA I (B). Nuclei were stained with DAPI; scale bars represent 100 µm.

To enhance the quality of endothelial CD31 staining in native adipose tissue, the primary antibody was applied at two different dilutions, 1:100 and 1:400 respectively, and combined with 1:400 and 1:800 dilutions of the secondary antibody. A significantly enhanced staining result and a reduced background signal were obtained at a 1:400/1:400 combination of primary and secondary antibody, revealing the strongly branched adipose vasculature (Figure 9 A).

The WMS protocol was shortened considerably by application of a human-specific lectin (UEA I) as an alternative to the immunolabeling of $CD31^+$ structures. Optimization of the staining procedure was approached employing a range of different dilutions. A strong background signal was observed at a dilution of 1:100, whereas a 1:400 dilution did not satisfyingly label the capillaries. At an intermediate dilution (1:200), well distinguishable capillaries were observed and the extensive 3D organization of the blood vessels was nicely reflected (Figure 9 B). Most importantly, lectin staining was well in accordance with the results previously obtained for stainings against CD31 and vWF (Figure 10).

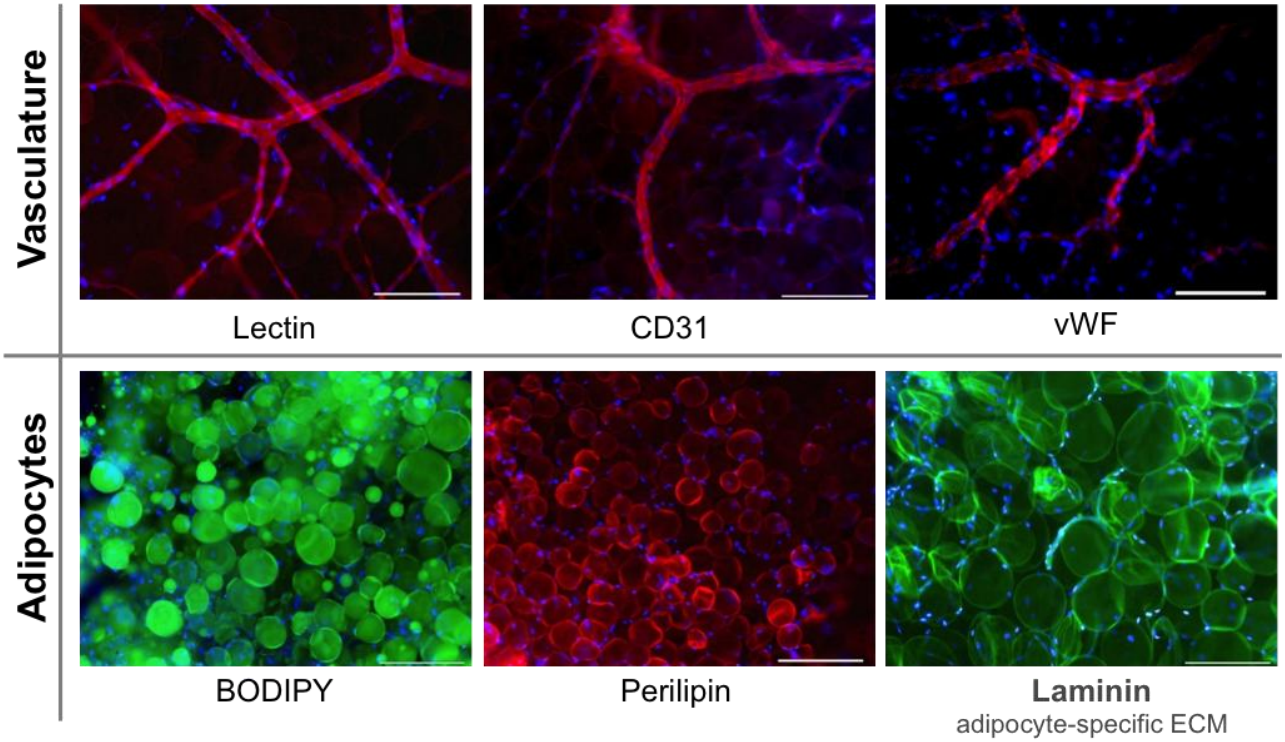

Figure 10. Whole mount staining options. Lectin staining and immunolabeling against CD31 or vWF were employed to label vascular structures. Adipocytes were visualized using BOPIPY or anti-perilipin staining, and adipocyte specific ECM was stained with an antibody directed against laminin.

Apart from using BODPIY as stain for mature adipocytes, an antibody directed against human perilipin, which is a stabilizing protein of the lipid vacuole [46,47], similarly revealed the characteristic morphology of lipid-laden fat cells. Staining against laminin as a major ECM component of the adipocytes' basement membrane [48] was approached as well, confirming the results obtained with BODIPY and perilipin staining (Figure 10).

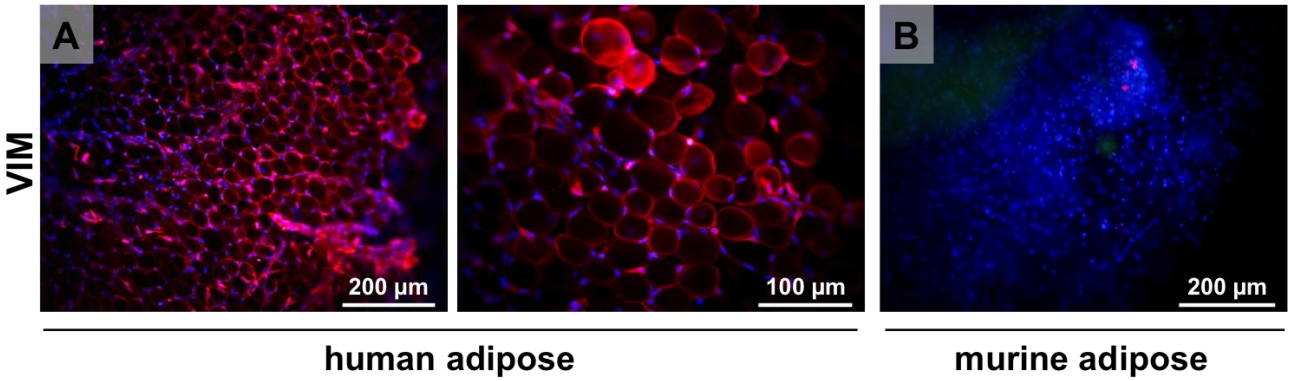

Figure 11. Establishment of a species-specific staining against human vimentin (VIM). Structural components of human adipose tissue were visualized at two different magnifications; murine adipose tissue served as negative control. Nuclei were counterstained with DAPI; scale bars represent 200 and 100 µm, respectively.

With respect to upcoming studies investigating SVF cells in a nude mouse model *in vivo*, it was further considered crucial to distinguish between human-derived and murine adipose tissue. Using a species-specific antibody against human vimentin, the structural components of human adipose tissue were visualized (Figure 11). Analogous to previously established stainings, species specificity was verified employing murine adipose tissue as negative control, showing no indication of a positive human vimentin signal, solely the counterstained blue nuclei were observed.

Overall, the evaluated staining options for the respective structural elements of adipose tissue using WMS are summarized in Table 1. The subsequent transfer of the established method to engineered tissue and the general applicability of WMS for the analysis of *in vivo* constructs were investigated in the context of an *in vivo* study in mice, which is described in detail in Chapter 7.

Vasculature	Adipocytes	Nuclei
Lectin	BODIPY	DAPI
CD31	Perilipin	
vWF	Laminin (ECM)	
Vimentin		

Table 1. Summary of whole mount staining options for the structural analysis of adipose tissue. Lectin staining and immunolabeling against CD31 and vWF were established for the visualization of vascular structures. Adipocytes were stained using BOPIPY or anti-perilipin antibody, and adipocyte-specific ECM was labeled with an antibody directed against laminin. Employing an anti-human vimentin antibody allowed the species-specific staining of human structures. DAPI was applied for staining of nuclei.

6.4 DISCUSSION

De novo adipogenesis is characterized by a tight spatial and temporal interplay between maturing adipocytes, stromal cells and vascular structures [34,36,49]. The stromal compartment as the non-adipocyte part of adipose tissue functions as a reservoir from which adipogenic and vasculogenic precursors can be recruited as needed [17,32,39]. Adipose tissue thus represents a unique and abundant source of different types of regenerative cells, available for direct application without the need for *in vitro* expansion [32,33,38,50]. Exploiting this profound trait of native adipose, the present study successfully explored the SVF *in vitro* as a potential cell source candidate for the development of vascularized adipose tissue constructs. In preparation of prospective *in vivo* studies using SVF cells, a novel WMS technique was established to promote the in-depth analysis of 3D tissue structure in engineered constructs.

Initially, uncultured SVF cells were shown suitable for the generation of 3D adipose tissue constructs according to the protocol previously introduced for ASC-seeded stable fibrin gels (Chapter 3). Direct seeding in 3D fibrin gels maintained the inherently contained cell types in the SVF, whereas upon 2D plastic-adherent culture solely the ASC subpopulation can be expanded [51]. Employing a mixture of preadipocyte and endothelial cell culture media (PGM-2/EGM-2), *in vitro* conditions were adjusted to the heterogeneous cellular composition of the SVF and especially tailored to preserve ASCs and endothelial cells, which are the cell types attributed a key role in adipose development [17,36,52,53]. Accordingly, the desired differentiation and maintenance of adipogenic and vasculogenic cell types *in vitro* was achieved as reflected on the cellular level by Oil Red O and CD31 staining, respectively, as well as on the molecular level by mRNA expression of the adipogenic markers PPARγ and aP2, and endothelial CD31.

In particular, comparison with conventional preadipocyte medium (PGM-2) for 3D SVF culture revealed the superiority of composite PGM-2/EGM-2 medium in terms of cell density as well as adipogenic and endothelial development. SVF cells were shown to react promptly to adipogenic stimuli by lipid accumulation and upregulation of adipocyte markers, whereas lower $CD31^+$ cell numbers, especially in long-term cultured gels (35 days), depicted a reduced vasculogenic potential under hormonal induction. In turn,

enhanced endothelial cell survival in SVF-seeded non-induced constructs may be attributed to the addition of the pro-angiogenic growth factors VEGF and FGF-2 to PGM-2/EGM-2. Eminently, VEGF and angiopoietin 1 (Ang-1) had afore been reported by Koh *et al.* to support the vascular assembly of SVF cells [31]. In this context, the maintenance of endothelial cells was considered essential as a direct involvement of these cells in tissue revascularization was expected. In contrast to SVF cells, compromised adipogenic development observed in ASC-seeded constructs in PGM-2/EGM-2 was attributed to a possible negative influence of the pro-angiogenic factors on the differentiation potential of *in vitro*-expanded monocultured ASCs. Hence, EGF as supplemental factor of PGM-2/EGM-2 had already been eliminated from the culture medium due to adverse effects on adipogenic differentiation [41-43].

The maintenance of prevascular structures and the formation of distinctly sized lipid vacuoles over a period of 35 days under the respective non-induced and induced conditions, further strongly supported the use of PGM-2/EGM-2 for long-term SVF culture *in vitro*. The fact that adipogenic and endothelial development was less pronounced in SVF-seeded constructs cultured for 21 days relative to 35 days, can on the one hand be explained with the lower seeding density and shorter culture period, but on the other hand, may be rooted in the application of cells from different donors for these preliminary investigations. In addition, distinctly fewer cells were found in SVF-seeded constructs in comparison to ASC-seeded fibrin gels upon culture, implying the need for higher seeding densities when employing SVF cells for construct preparation. Further, since the composition of the SVF, for example with respect to ASC content, may vary between donors and sites of harvest, and also depends on BMI and age [22,23,50], flow cytometric analysis of the cells prior to seeding is advocated in order to facilitate the interpretation of the experimental outcome.

Having demonstrated the suitability of SVF cells for 3D culture in fibrin gels and, in accordance with other previous reports, their adipogenic and prevascularization potential *in vitro* [14,38], a novel WMS technique was further established to in the future enable the 3D analysis of engineered constructs studied *in vivo*. Whereas conventional 2D histology provides only limited insight into the actual tissue structure [39], this technique was shown to result in the detailed visualization of adipocytes and blood vessels employing native adipose as model tissue. The observed structural arrangements were in correspondence to

previous studies using WMS for the illustration of tissue architecture in murine and human adipose tissue [39,54,55]. By applying complementary markers such as lectin, CD31 and vWF to label capillaries, as well as BODIPY, perilipin and laminin for adipocytes, the specificity of the stainings for the respective structures was confirmed. The translation of the WMS technique to engineered tissue *in vivo* will be approached in upcoming studies investigating SVF-seeded constructs *in vivo*. Thereby, histological analysis may be advanced applying the WMS technique in conjunction with methods for 2D histological analysis, for example lectin/BODIPY-WMS and conventional H&E staining. Regarding the non-disruptive and time-saving processing associated with WMS, this method may broadly be applied in successive work to monitor tissue quality in engineered constructs and to comprehensively recapitulate the kinetics of tissue development in pendency of influential parameters such as the applied cell source, choice of biomaterial or addition of growth factors. Co-staining of adipocytes and endothelial structures may facilitate the correlation of adipogenesis with the extent of vascularization, for example by quantification of vessel density relative to adipocyte numbers [44]. In the context of the differential contribution of implanted cells to tissue formation *in vivo* [20], the established species-specific WMS against human vimentin represents a valuable tool to verify the origin of developed adipose tissue and possibly, the specific interactions between grafted and host cells.

Overall, in the present study, the detailed analysis of SVF-seeded adipose tissue constructs *in vitro* was accomplished, demonstrating adipogenesis and endothelial development of the incorporated cells. On the basis of a suitable 3D environment provided by fibrin as cell carrier, culture conditions by which SVF cells could be appropriately preconditioned *in vitro* were established, providing an ideal starting point for the investigation of SVF-seeded constructs *in vivo*. Thus, upcoming work will further explore the SVF as concomitant supply of adipogenic and vasculogenic cells, and specifically address the *in vivo* investigation of SVF cells to foster adipose tissue formation and effective vascularization in engineered constructs. Here, the newly established WMS technique is considered a valuable tool for the study of adipose tissue structure in a 3D context, providing an efficient method for 3D histological analysis of engineered adipose tissue constructs.

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Chapter 7

Engineering Vascularized Adipose Tissue Using the Stromal-Vascular Fraction and Fibrin Hydrogels

The contents of this chapter have been submitted to Tissue Eng Part A.

Engineering Vascularized Adipose Tissue Using the Stromal-Vascular Fraction and Fibrin Hydrogels

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7.1 ABSTRACT

The development of vascularized and functional adipose tissue substitutes is required to improve soft tissue augmentation. Vascularized adipose tissue constructs were generated using uncultured cells from the stromal-vascular fraction (SVF) of adipose tissue as an alternative cell source to adipose-derived stem cells (ASCs). SVF cell behavior and tissue formation were compared in a stable fibrin formulation developed by our group and a commercial fibrin sealant (TissuCol, Baxter) upon direct subcutaneous implantation in a nude mouse model. Further, the effect of *in vitro* adipogenic induction on SVF cell development was investigated by implanting stable fibrin constructs after 1 week of precultivation (adipogenic vs. non-induced control). Constructs were thoroughly analyzed before implantation regarding adipogenic differentiation status, cell viability and distribution as well as the presence of endothelial cells. Prior to implantation, *in vitro* precultivation strongly promoted adipogenesis (under adipogenic conditions) and the formation of $CD31^+$ prevascular structures by SVF cells (under non-adipogenic conditions). Tissue development *in vivo* was determined after 4 weeks by histology (H&E, anti-human vimentin) and quantified histomorphometrically. In stable fibrin gels, adipogenic precultivation was superior to non-induced conditions, resulting in mature adipocytes and the formation of distinct vascular structures of human origin *in vivo*. Strong neovascularization by the implanted cells predominated in non-induced constructs. Without pretreatment, the SVF in stable fibrin gels displayed only a weak differentiation capability. TissuCol gels strongly supported the formation of coherent and well-vascularized adipose tissue of human origin, displaying large unilocular adipocytes. The developed native-like tissue architecture was highlighted by a whole mount staining (WMS) technique. Taken together, SVF cells from human adipose tissue were shown to successfully lead to adipose tissue formation in fibrin hydrogels *in vivo*. The results render the SVF a promising cell source for subsequent studies *in vitro* and *in vivo* with the aim to engineer clinically applicable soft tissue substitutes.

7.2 INTRODUCTION

Adipose tissue engineering seeks to provide novel solutions for the regeneration of soft tissue defects and pathologies. Clinical approaches use autologous flaps, enriched grafts or liposuctioned tissue as well as allogenic or synthetic fillers [1-3]. Despite improved surgical techniques, these treatments encompass substantial donor site morbidity and often yield limited success in terms of integration and maintenance of volume and shape [4-6], calling for alternatives to current reconstructive procedures [7-9].

Tissue engineering approaches join biomaterials and cells that exhibit a pronounced regenerative potential to functionally imitate or rebuild native tissues [1,10,11]. A suitable cell-biomaterial combination and, essentially, sufficient vascularization, are key parameters for successfully engineering adipose tissue. As a metabolic and endocrine organ, native adipose is densely vascularized and hence, adipocytes display a low tolerance to ischemia, explaining the need for rapid revascularization in transplanted and engineered adipose substitutes [12-14].

ASCs have widely been applied for tissue engineering approaches [5,7]. *In vitro*, hormonal induction initiates the cellular commitment of ASCs to the adipose lineage and subsequent full differentiation [15,16]. In a previous study by our group, we incorporated ASCs in volume-stable fibrin-polyurethane constructs and improved vascularization and adipogenesis by insertion of a femoral pedicle [17]. Similarly, other groups have reported adipose construct development based on ASC *in vivo* within a broad spectrum of biomaterials [18-22]. Yet the formation of coherent and long-term stable adipose tissue *in vivo*, for which intrinsic cellular differentiation potential and vascularization are the most fundamental requirements, remains challenging.

The present approach therefore elucidates the feasibility of the entire stromal-vascular fraction of adipose tissue instead of the isolated, plastic-adherent ASC subpopulation for adipose tissue engineering. Given the heterogeneity of the SVF including, among others, mesenchymal stem cells, endothelial cells, vascular smooth muscle cells and pericytes [23-25], which are all building blocks of adipose tissue, their coimplantation may promote tissue formation. Eminently, the vascularization potential of endothelial precursors

in the SVF is considered highly supportive of early network assembly to guarantee nutrient and oxygen supply in the constructs.

To date, only Lin *et al.* have specifically used uncultured SVF cells for adipose tissue engineering, investigating differentiation of the SVF *in vitro* in collagen and gelatin scaffolds [26], whereas in an approach utilizing Tisseel fibrin spray for wound healing, adipogenic differentiation of SVF cells was also demonstrated previously [27]. Further, aspirated fat enriched with SVF cells in order to enhance volume maintenance and long-term survival of fat grafts, was investigated by Condé-Green *et al.* [28]. Employing SVF cells for other regenerative purposes, such as bone and cartilage engineering, also yielded promising results [29-31]. However, as of yet, SVF cells have not been investigated for adipose tissue engineering *in vivo*.

Consequently, it was the aim of this study to generate vascularized adipose tissue *in vivo* using SVF cells seeded in fibrin gels and to analyze cellular development in the engineered constructs. Prior to implantation, adipogenic and prevascular development were characterized. The potential of adipogenic pretreatment *in vitro* to foster adipose conversion *in vivo* was explored. Further, different fibrin formulations, a long-term stable fibrin gel developed by our group [32] and a commercially available fibrin sealant (TissuCol) exhibiting a faster degradation rate [17], were elucidated concerning their impact on tissue development. To our knowledge, this is the first study demonstrating adipose tissue formation by SVF cells *in vivo*.

7.3 MATERIALS AND METHODS

7.3.1 Cell isolation

SVF cells were isolated from subcutaneous adipose tissue obtained from abdominal depots of healthy female donors. Written informed consent was obtained from all patients. The study was approved by the ethics committee of the University of Wuerzburg, Germany. Patients were between 30 and 56 years of age and had a body mass index (BMI) ranging from 27 to 32.

For cell isolation, the tissue was minced and digested with 0.1% collagenase NB4 from Clostridium histolyticum (Serva Electrophoresis, Heidelberg, Germany) in collagenase buffer for 2 h at 37 °C on an orbital shaker. The digested tissue was filtered through a 100 µm nylon mesh and centrifuged (300 g, 10 min). The pelleted SVF cells were washed with phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria) and resuspended in basal medium containing Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12) supplemented with 1% penicillin-streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 10% fetal bovine serum (FBS), all obtained from Invitrogen (Karlsruhe, Germany). For cryopreservation of the cells in liquid nitrogen, 5% DMSO (Sigma-Aldrich, Steinheim, Germany) was added to the medium.

For construct preparation, SVF cells from three different donors were used. The cells were thawed and resuspended in prewarmed PBS (37 °C). After centrifugation, erythrocyte lysis buffer was added to the pelleted cells for 10 min until subsequent removal of the buffer by centrifugation. The cells were washed with PBS, stained with 0.4% trypan blue for counting and pooled in equal parts prior to seeding of the constructs.

7.3.2 Flow cytometry

After thawing and erythrocyte lysis, SVF cells were resuspended in flow cytometry (FC) buffer (0.3% BSA in PBS) and viable cells were counted (trypan blue exclusion). 1×10^5 cells per tube were transferred to FC tubes, washed twice with 1 mL FC buffer and centrifuged (400 g, 4 °C, 5 min). Fc receptor blocking solution (5% mouse serum, FC buffer) was added for 20 min at 4 °C. After washing with FC buffer, cells were incubated with the respective monoclonal antibodies against CD14, CD34, CD31, CD45, CD73, CD90 and CD105 (Biolegend, London, UK); corresponding isotype controls for

mouse IgG_1 were employed. Dead cells were excluded by 7-AAD viability staining. Stained cells were washed twice prior to analysis using a FACSCanto flow cytometer (BD Biosciences, Palo Alto, USA). Data analysis was carried out using FlowJo v.10.0.6 software (Treestar, San Carlos, USA).

7.3.3 Preparation of TissuCol gels

TissuCol® Kit 1.0 Immuno was obtained from Baxter (Unterschleissheim, Germany). 20 µL thrombin-L solution and an equal volume of the TissuCol fibrinogen component were mixed and transferred to sterile glass rings in 12-well culture dishes. TissuCol gels were subjected to 45 min of gelation at 37 °C and 5% CO_2 in an incubator.

7.3.4 Preparation of stable fibrin gels at 25 mg/mL fibrinogen

Stable fibrin gels were prepared as described previously [17,32], with a fibrinogen concentration of 25 mg/mL, 20 mM $CaCl_2$ and 2.5 U/mL thrombin. Bovine fibrinogen and aprotinin from bovine lung were obtained from Sigma-Aldrich (Steinheim, Germany). 20 µL of 50 mg/mL fibrinogen dissolved in an aprotinin solution [10,000 kallikrein inhibitory units (KIU)/mL] were mixed with an equal volume of a 5 U/mL thrombin solution and subjected to 45 min of gelation at 37 °C and 5% CO_2. The thrombin component was prepared using a 1:100 dilution of the thrombin-S component (500 U/mL) provided in the TissuCol® Kit.

7.3.5 Seeding and in vitro culture

Stable fibrin and TissuCol gels (40 µL; heigt 2 mm, diameter 5 mm) were seeded with 1.0×10^6 viable SVF cells. Cells were suspended in the thrombin component of the respective fibrin formulation. For construct preparation, 20 µL of the thrombin-SVF suspension were combined with 20 µL fibrinogen and transferred to sterile glass rings with an inner diameter of 5 mm. Two groups, SVF-seeded stable fibrin and TissuCol gels, each without precultivation, were implanted immediately after construct preparation.

For *in vitro* precultivation, SVF-seeded stable fibrin gels were cultured in a 1:1 mixture of preadipocyte growth medium 2 (PGM-2) and endothelial cell growth medium 2 (EGM-2) under dynamic conditions on an orbital shaker (37 °C, 5% CO_2). PGM-2 consisted of preadipocyte basal medium 2 (PBM-2; Lonza, Walkersville, USA) supplemented with 10% FBS and 1% penicillin-streptomycin. For EGM-2 preparation, endothelial cell basal

medium 2 (EBM-2; Lonza, Walkersville, USA) was supplemented with 5% FBS, gentamycin/amphotericin-B, insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), ascorbic acid (AA), hydrocortisone (HC) and fibroblast growth factor 2 (FGF-2) according to the manufacturer. Precultivated stable fibrin constructs were maintained *in vitro* for 7 days prior to implantation and medium exchange was performed every other day. For adipogenic induction, constructs were cultured in induction medium [1:1 PGM-2/EGM-2 with 1.7 µM insulin, 1 µM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine (IBMX) and 200 µM indomethacin]. For medium preparation, IBMX was obtained from Serva Electrophoresis (Heidelberg, Germany), bovine insulin from PromoCell (Heidelberg, Germany) and indomethacin and dexamethasone were purchased from Sigma-Aldrich (Steinheim, Germany). SVF-seeded stable fibrin gels in the non-induced control group were cultured in 1:1 PGM-2/EGM-2 for the entire culture period without adipogenic induction.

7.3.6 Live/dead assay in vitro

A live/dead cell staining kit from PromoKine (Heidelberg, Germany) was employed for viability testing. After washing with PBS, whole constructs were stained by incubation in 0.5 mL of staining solution [4 µM ethidium bromide homodimer III (EthD-III), 2 µM calcein acetoxymethyl ester (calcein-AM) in PBS] for 1 h. Constructs were washed with PBS and cut transversely in half to view the middle sections under a fluorescence microscope The resulting images were overlaid using cellSens™ Dimension Microscope Imaging Software (Olympus, Hamburg, Germany).

7.3.7 Oil Red O staining in vitro

In stable fibrin and TissuCol gels, adipogenic development of SVF cells *in vitro* was histologically investigated by Oil Red O staining. Constructs were fixed in 3.7% buffered formalin and dehydrated in increasing sucrose series (10-60%) prior to embedding in Tissue-Tek® O.C.T. compound (Sakura Finetek, Zoeterwonde, Netherlands). 8 µm sections were stained with Oil Red O solution, counterstained with hematoxylin (Bio Optica, Milan, Italy) and coverslipped with Glycergel Mounting Medium (Dako, Carpinteria, USA).

7.3.8 CD31 staining in vitro

Endothelial structures at the time points of construct preparation and implantation were analyzed by staining against CD31. 8 µm cryosections were prepared as described above. After rehydration in PBS, unspecific binding was blocked with blocking solution (1.5% BSA in PBS) prior to overnight incubation with monoclonal mouse anti-human CD31 (Clone JC70A; Dako, Carpinteria, USA). Slides were thoroughly washed with PBS and a Cy™3-conjugated AffiniPure donkey anti-mouse secondary antibody (Jackson Immuno Research, West Grove, USA) was added for 30 min in the dark. Lipid inclusions were additionally stained with BODIPY® 493/503 (stock 1 mg/mL, 1:100 dilution in PBS; Invitrogen, Karlsruhe, Germany) and nuclei were counterstained with IS Mounting Medium DAPI (Dako, Carpinteria, USA). Slides were coverslipped and viewed under a fluorescence microscope. The resulting images were overlaid using Olympus cellSens™ software.

7.3.9 Implantation of SVF-seeded fibrin gels in a subcutaneous nude mouse model

For the *in vivo* study, female NMRI-*Foxn1*^{nu}/*Foxn1*^{nu} mice (Janvier Labs, St Berthevin Cedex, France), 6-8 weeks of age, were used. The experiments were approved by the ethics board of the local authorities. Constructs were implanted in subcutaneous pockets in the shoulder region of the animals. Prior to surgery, mice were treated with rimadyl (Zoetis, Berlin, Germany) and anaesthesized with 3.5 vol% isoflurane (CP Pharma, Burgdorf, Germany). A small cut was made along the scapulae, constructs were inserted into prepared subcutaneous pockets (5 constructs per group) and fixed at the muscle fasciae with a non-resorbable prolene suture (Ethicon Prolene 6-0 P-1; Johnson & Johnson GmbH, Neuss, Germany). The incision was closed with four interrupted sutures and covered with antibiotic fucidin ointment 20 mg/g (LEO Pharma AS, Ballerup, Denmark). Animals had free access to food and water and were weighed daily. After 4 weeks, the mice were anaesthesized and implants were removed. Animals were sacrificed after explantation.

7.3.10 Analysis of tissue development in vivo

After explantation, implants were washed in PBS and fixed in 3.7% buffered formalin. Constructs were dehydrated in increasing ethanol concentrations, embedded in paraffin and cross-sectioned. 3 µm sections were deparaffinized and stained with hematoxylin and

eosin (H&E; Bio Optica, Milan, Italy). Microscopic images of middle sections were taken to evaluate tissue formation.

Immunohistochemistry against human vimentin was employed to identify cells of human origin in the constructs. Paraffin cross-sections were deparaffinized and antigen retrieval was performed using pepsin (Digest All™-3; Invitrogen, Karlsruhe, Germany). Slides were washed with wash buffer (Zytomed-Systems, Berlin, Germany) and incubated with a monoclonal rabbit anti-human vimentin antibody (Clone SP20; Zytomed-Systems, Berlin, Germany) overnight. After rinsing with wash buffer, anti-rabbit AP-Polymer (ZUC031-006; Zytomed-Systems, Berlin, Germany) was added for 30 min. For visualization, Permanent AP Red (Zytomed-Systems, Berlin, Germany) was added after thorough washing. Nuclei were counterstained with hematoxylin.

7.3.11 Histomorphometric quantification in vivo

Adipocyte formation and the extent of vascularization were quantified in middle sections (H&E staining) of SVF-seeded fibrin gels in all groups after 4 weeks *in vivo*. For adipocytes, a minimum diameter of 20 µm of the lipid vacuole was defined for counting. High-resolution images of histological cross-sections were acquired at 20-fold magnification. Construct areas were calculated with cellSens™ software. Adipocytes and blood vessels were counted in whole cross-sectional areas and expressed as counts/mm^2.

7.3.12 Whole mount staining

Engineered SVF-seeded TissuCol gels displaying a mature adipose tissue appearance and native adipose tissue as reference were whole mount-stained for visualization of the tissue architecture. TissuCol constructs and 4 x 4 mm native human adipose tissue blocks were fixed in 3.7% buffered formalin for 20 h, washed twice with PBS for 1 h and incubated with blocking buffer (3% BSA, PBS/Triton X-100) overnight. The blocking buffer was removed and whole mounts were rinsed with PBS/Triton X-100 for 2 h. Rhodamine-conjugated ulex europaeus agglutinin I (UEA I; Vector Laboratories, Burlingame, USA) was diluted in antibody diluent (Dako, Carpinteria, USA) and added overnight to stain blood vessels of human origin. To subsequently stain mature adipocytes, whole mounts were washed with PBS/Triton X-100 overnight and for 2 h with PBS prior to incubation with a 1:100 dilution of BODIPY (stock 1 mg/mL) in PBS. After 1 h, the BODIPY solution was removed and

whole mounts were rinsed with PBS for 2 h and counterstained with DAPI.

For labeling of human vimentin-positive structures in TissuCol, constructs were incubated with a monoclonal rabbit anti-human vimentin antibody (Clone SP20; Zytomed Sytems) overnight and washed with PBS/Triton X-100 for 2 h. Blocking buffer was added for 2 h, then whole mounts were incubated with Alexa Fluor® 594-conjugated AffiniPure goat anti-rabbit (Jackson Immuno Research, West Grove, USA) for another 2 h.

All staining steps were performed on an orbital shaker at 50 rpm and room temperature. Whole mount-stained constructs and native tissue were mounted on coverslips and microscopically analyzed. The resulting images were overlaid using cellSens™ software.

7.3.13 Statistics

Quantitative results are expressed as mean values ± standard deviation (SD). Statistically significant differences between the experimental groups were assessed using a non-parametric two-tailed Mann-Whitney U rank-sum test at the level of $p < 0.05$. Statistical analysis was performed using GraphPad Prism, Version 5.0 (GraphPad Software, La Jolla, USA). For the *in vitro* experiments, if not stated otherwise, the number of samples per group was n=3.

7.4 RESULTS

7.4.1 Design of the study

The formation of vascularized adipose tissue by SVF cells in engineered constructs was investigated upon *in vivo* implantation in a nude mouse model. An outline of the study is displayed in Figure 1.

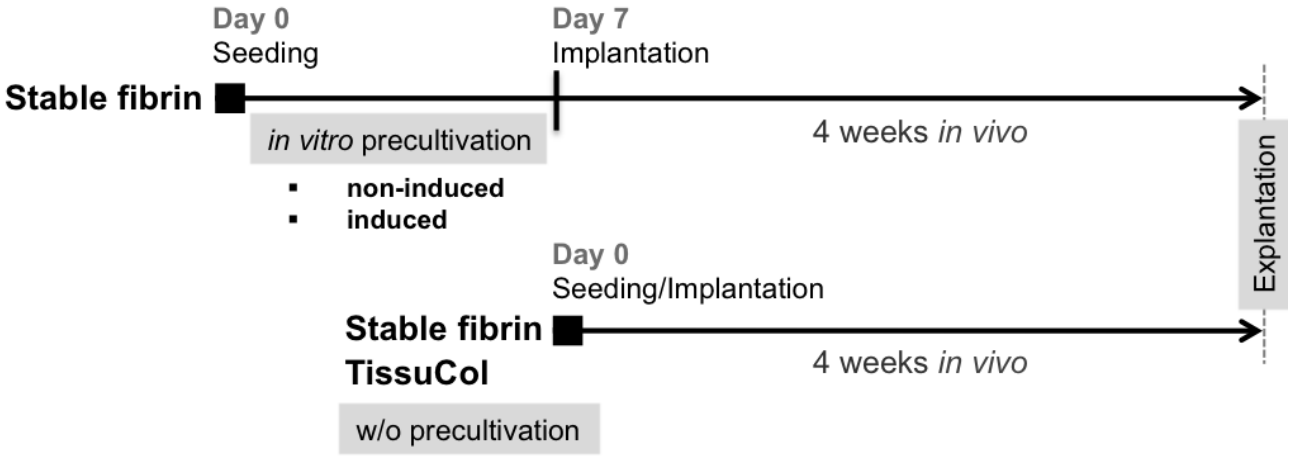


Figure 1. Experimental setup of construct preparation and implantation. Adipogenic precultivation *in vitro* prior to implantation was assessed in SVF-seeded stable fibrin gels. Stable fibrin and TissuCol gels were compared by direct implantation without (w/o) pretreatment. Engineered constructs were excised after 4 weeks *in vivo*.

SVF-seeded stable fibrin gels were implanted after precultivation *in vitro* to investigate the effect of adipogenic induction. Different fibrin formulations were compared by directly implanting SVF-seeded stable fibrin and TissuCol gels without (w/o) precultivation. Engineered constructs were placed in subcutaneous pockets in female, immunodeficient NMRI nu/nu mice and excised after an *in vivo* time span of 4 weeks.

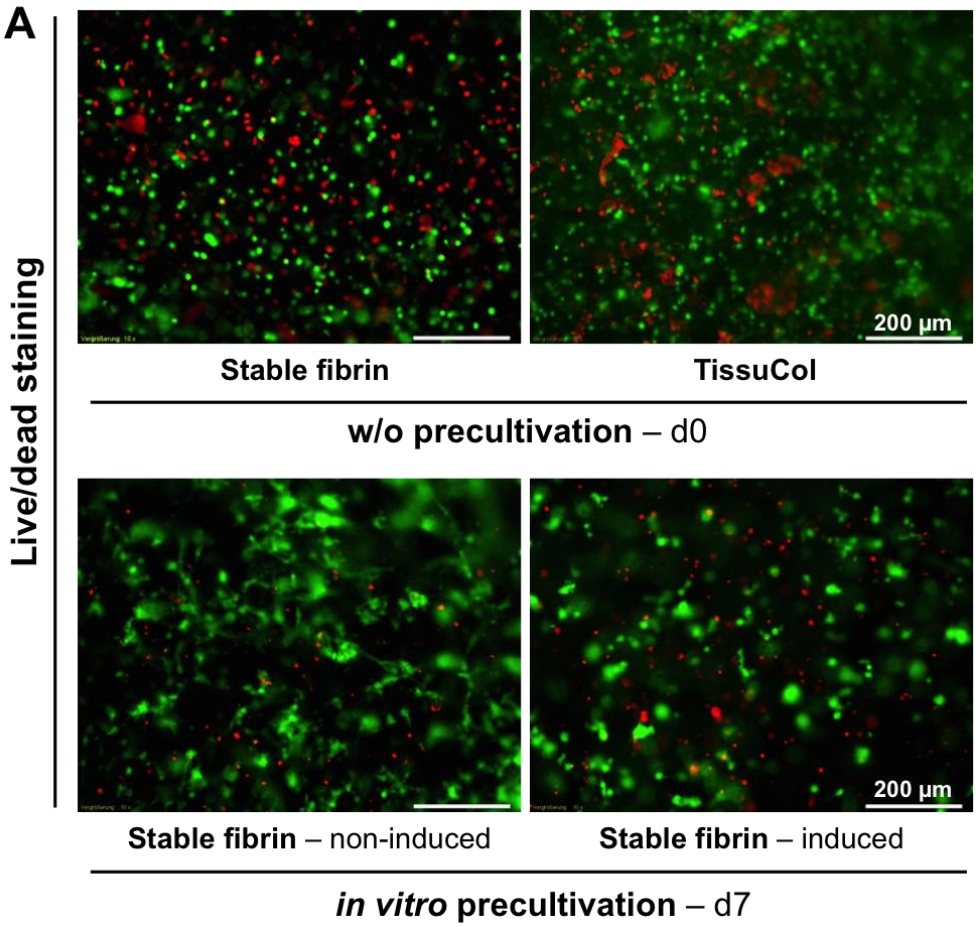
7.4.2 SVF surface marker analysis and cell viability in seeded fibrin gels

Flow cytometry-based analysis of freshly isolated SVF cells revealed high marker levels of CD14, CD34, CD73 and CD90 as well as the expression of CD31, CD45 and CD105, implying the presence of mesenchymal stem cells, endothelial cells, monocytes and cells of the hematopoietic lineage prior to seeding (Table 1).

	CD73	CD90	CD105	CD14	CD45	CD31	CD34
[%]	57.87 ± 13.13	73.62 ± 2.99	19.11 ± 8.93	59.55 ± 2.78	31.42 ± 8.44	43.85 ± 25.47	68.73 ± 6.87

Table 1. Flow cytometric analysis of surface markers. SVF cells were analyzed for stem cell-associated markers (CD73, CD90, CD105), hematopoietic markers (CD14, CD45), the endothelial marker CD31, and additionally CD34. Results were calculated from three independent donors.

To determine the cell viability at the time point of implantation, either immediately after construct preparation (day 0) in stable fibrin and TissuCol constructs or after 7 days of *in vitro* precultivation in stable fibrin, a live/dead assay was performed (Figure 2 A).



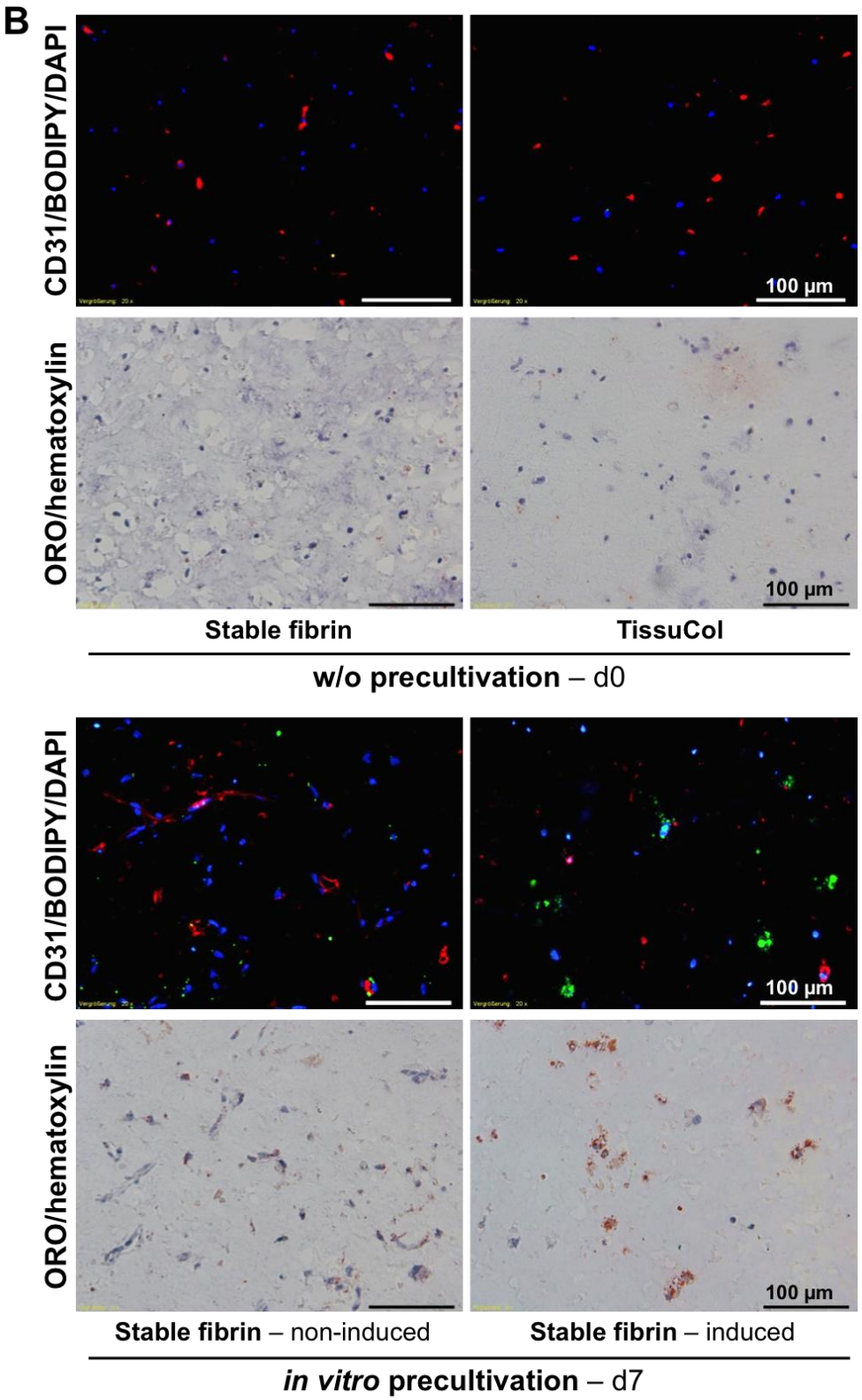


Figure 2. Analysis of SVF-seeded fibrin gels at the time point of implantation. Cell viability in SVF-seeded stable fibrin and TissuCol gels implanted without precultivation (d0) and of *in vitro* precultivated (non-induced, induced; d7) stable fibrin constructs. Viable cells were labeled green with calcein acetoxymethyl ester (calcein-AM) and dead cells red with ethidium bromide homodimer III (EthD-III); scale bars represent 200 µm (A). Histology of stable fibrin and TissuCol

gels implanted without precultivation (d0) and of *in vitro* precultivated (non-induced, induced; d7) stable fibrin constructs. Endothelial differentiation was demonstrated by CD31/BOPIPY/DAPI (red/green/blue) staining and adipogenic development by staining with Oil Red O (ORO) and hematoxylin; scale bars represent 100 μm (B).

In all groups, viable cells were contained in the constructs upon implantation. In constructs without precultivation, the presence of dead cells and cellular debris was attributed to the isolation procedure of the SVF. Precultivated SVF-seeded stable fibrin gels apparently displayed more viable cells than constructs implanted without precultivation, likely due to proliferation of the viable cell fraction within 7 days of *in vitro* culture. This especially applied to the non-induced group, where SVF cells adopted a fibroblastoid morphology and network-like organization. The cell distribution in all fibrin gels appeared homogeneous, irrespective of pretreatment or fibrin formulation.

7.4.3 Histological assessment of SVF cells in fibrin gels in vitro prior to implantation

SVF-seeded stable fibrin and TissuCol gels were analyzed histologically at the time points of implantation. According to the study design, stable fibrin constructs were examined after 7 days of precultivation (adipogenic vs. non-induced control) and constructs without precultivation were analyzed immediately after construct preparation (day 0).

Cryosections were stained against CD31 to estimate the fraction of cells with an endothelial phenotype. BODIPY and Oil Red O were applied to stain for intracellular triglycerides. At the time point of construct preparation, $CD31^+$ endothelial cells were homogeneously distributed in both stable fibrin and TissuCol gels, whereas no differentiating adipocytes positive for BODIPY or Oil Red O were initially present (d0; Figure 2 B).

Precultivated stable fibrin constructs were investigated after 7 days *in vitro*, again by staining cryosections for CD31/BODIPY/DAPI and Oil Red O. In the non-induced group, $CD31^+$ cells had aligned to form prevascular structures, whereas BODIPY and Oil Red O-stained lipid vacuoles were rarely found. In sharp contrast, in induced constructs, cells displayed lipid vacuoles positive for BODIPY and Oil Red O. Interestingly, fewer $CD31^+$ cells and less distinct prevascular structures were found in the adipogenic group (d7; Figure 2 B).

7.4.4 Adipose tissue formation and vascularization in SVF-seeded fibrin gels in vivo

The impact of different fibrin formulations and adipogenic precultivation on SVF cell development *in vivo* were addressed in an animal study using immunodeficient mice. Stable fibrin gels cultured under adipogenic and non-induced conditions were implanted after 7 days *in vitro*, whereas without pretreatment, stable fibrin and TissuCol constructs were directly implanted after preparation (Figure 3 A).

After 4 weeks *in vivo*, paraffin cross-sections of the dissected implants revealed different degrees of tissue formation (Figure 3 B and C). SVF-seeded stable fibrin gels precultivated under non-induced conditions displayed a dense vascular network with maturing capillaries that were found to be of human origin by immunohistochemisty (Figure 3 B, first row). In contrast, adipogenically induced SVF-cells in stable fibrin gels formed mature adipocytes of which many were in direct contact to blood vessels. Again, these structures were shown to derive from the implanted human cells by anti-human vimentin (VIM) staining (Figure 3 B, second row).

With no precultivation applied, SVF-seeded stable fibrin gels after 4 weeks *in vivo* revealed some mature fat cells as well as few vascular structures, however, cellular development did not appear as uniform as in the precultivated groups. Interestingly, many incorporated adipocytes as well as cells in vessel structures were negative for human vimentin (Figure 3 C, first row). The formation of coherent adipose tissue *in vivo* occurred solely in TissuCol gels, with large areas of the engineered tissue staining positive for human vimentin (Figure 3 C, second row). Constructs made from TissuCol were almost completely filled with adipose tissue leaving only small patches of residual hydrogel (blue arrows). Importantly, the tissue very closely resembled the native tissue architecture, displaying a close interaction between mature fat cells and blood vessels (black arrows).

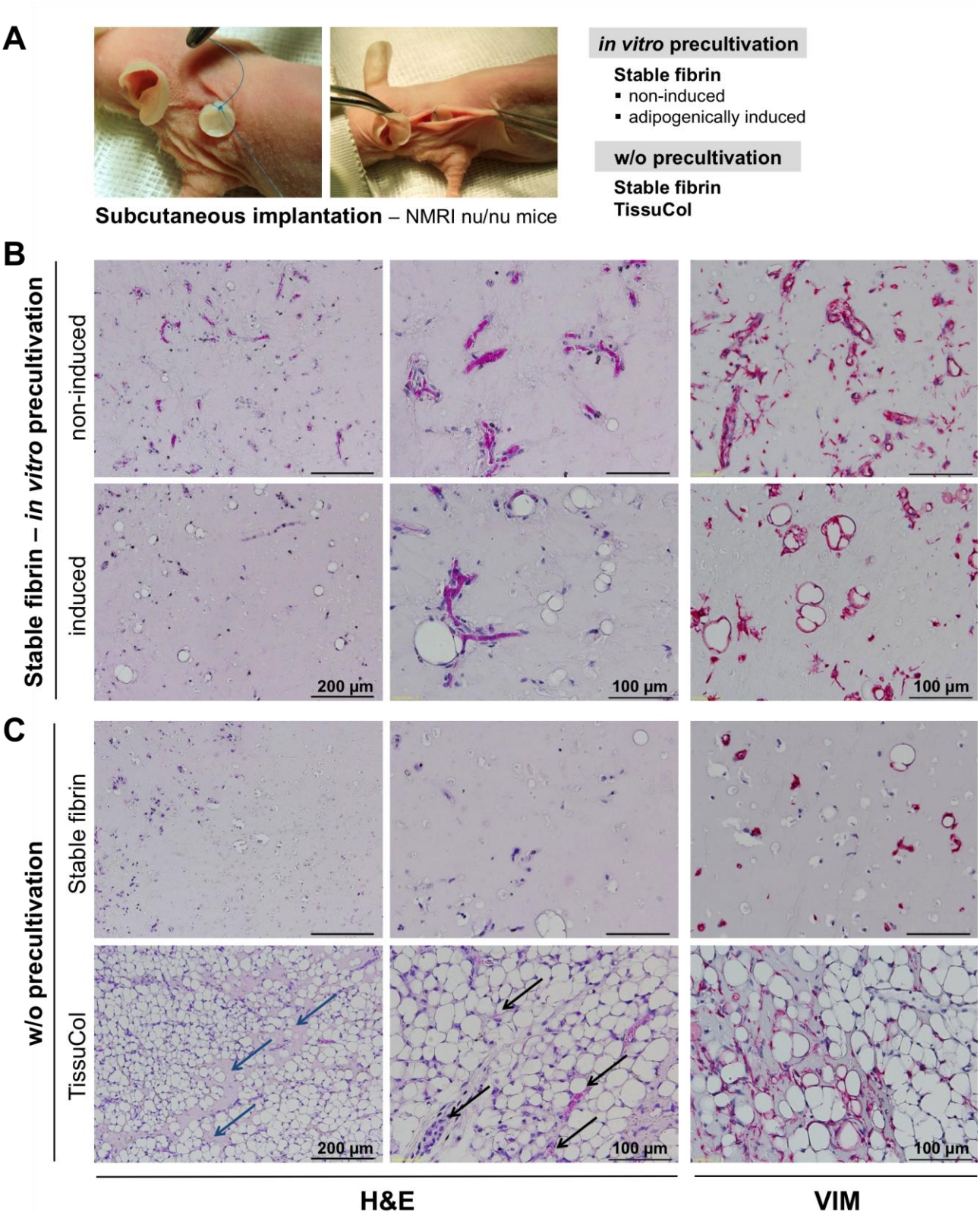


Figure 3. Investigation of tissue formation *in vivo*. Subcutaneous implantation of SVF-seeded fibrin gels in immunodeficient NMRI nu/nu mice (A). Precultivated SVF-seeded stable fibrin gels (non-induced, induced; 7 days prior to implantation) (B) and stable fibrin gels and TissuCol implanted without precultivation (C) were stained with hematoxylin and eosin (H&E; two different

magnifications are shown) and against human vimentin (VIM, red); scale bars represent 200 and 100 µm, respectively. Blue arrows indicate remaining fibrin gel; black arrows indicate vascular structures. n=5 constructs were evaluated; representative results are shown.

Tissue formation was additionally quantified by histomorphometry (Figure 4). Adipocytes and vascular structures were counted in middle sections of five specimen per group. Construct size of TissuCol compared to stable fibrin gels was reduced at the time point of explantation. Histomorphometric measurements were normalized to the overall area of the respective middle sections expressed as counts/mm^2.

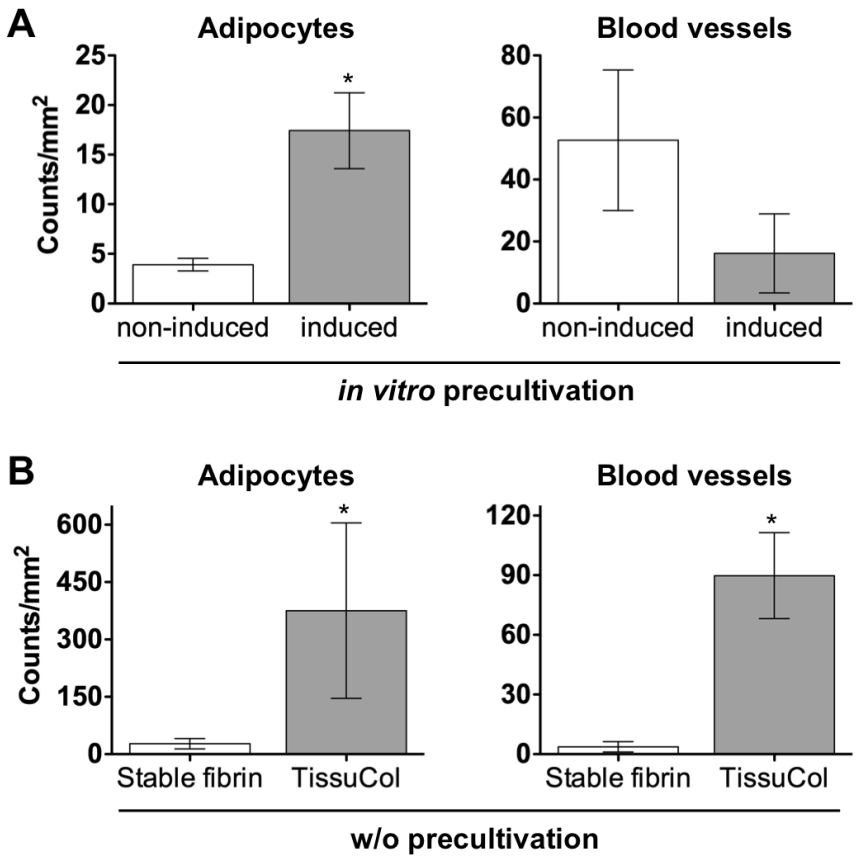


Figure 4. Histomorphometric quantification of adipose tissue development and vascularization. Adipocytes and blood vessels were counted in middle sections (H&E staining) of precultivated stable fibrin gels (non-induced, induced) (A) and in stable fibrin and TissuCol gels implanted without precultivation (B); results are expressed in counts/mm^2; n=5 constructs were evaluated. * Statistically significant differences between the respective groups ($p < 0.05$).

In precultivated stable fibrin gels, the number of adipocytes per area was significantly elevated in induced constructs compared to the non-induced counterparts. In turn, blood vessel density was enhanced under non-induced conditions (Figure 4 A). TissuCol gels

displayed superior tissue forming qualities, and adipose development by far exceeded that in stable fibrin gels seeded with SVF cells. Mature adipocytes as well as blood vessels were most abundant in TissuCol gels (Figure 4 B).

7.4.5 Visualization of tissue architecture in engineered adipose tissue

To visualize the tissue architecture and demonstrate adipocyte and blood vessel interaction in the coherent tissue formed in SVF-seeded TissuCol gels *in vivo*, a whole mount staining technique was applied (Figure 5). For comparison, native adipose tissue was treated accordingly. Species-specific staining against human vimentin was further used to identify the incorporated human cells in the engineered tissue.

Branched human lectin^{+} blood vessels assembled within TissuCol, surrounding cells with BODIPY-stained lipid vacuoles (Figure 5 A). Although whole mount-stained human fat tissue displayed more densely packed adipocytes (Figure 5 A, box), the structural resemblance of the engineered tissue with its native counterpart was striking.

Staining with a human-specific antibody against vimentin revealed the dense organization of human cells in the engineered constructs, including stretched vascular and fibroblast-like structures as well as the rounded morphology of mature adipocytes. Human vimentin^{+} adipocytes were colocalized with BODIPY stained lipid vacuoles (white arrows, Figure 5 B).

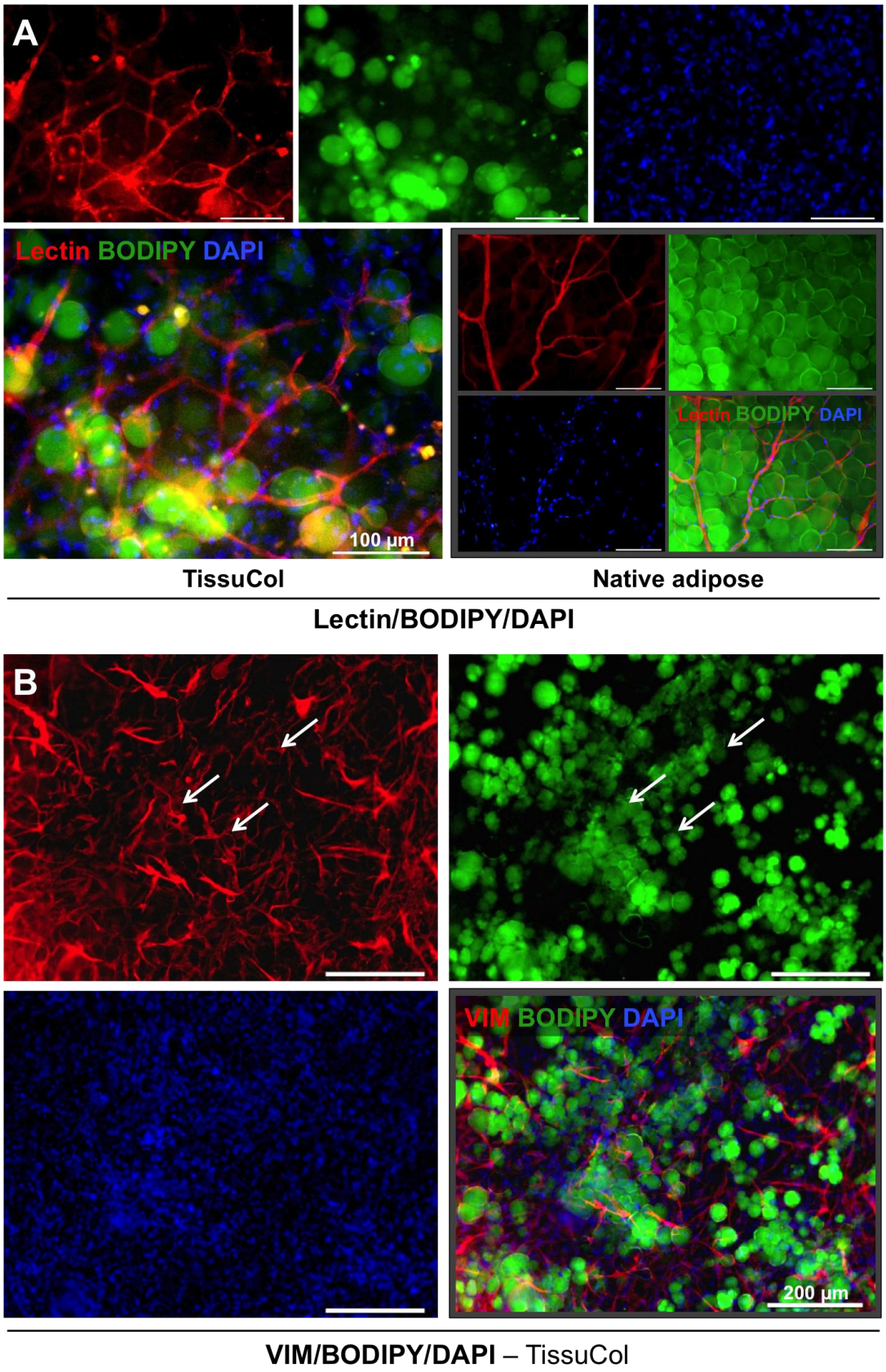


Figure 5. Visualization of tissue formation *in vivo* by whole mount staining. Interaction of blood vessels and adipocytes in engineered constructs (TissuCol) was demonstrated by Lectin/BODIPY/DAPI (red/green/blue) staining and compared to native adipose tissue (box);

individual fluorescent images were overlaid; scale bars represent 100 μm (A). Human vimentin (VIM)/BODIPY/DAPI (red/green/blue) staining revealed the contribution of SVF cells to tissue formation; individual fluorescent images were overlaid; scale bars represent 200 μm. Arrows indicate rounded adipocytes staining positive for human vimentin and BODIPY (B).

7.5 DISCUSSION

The discovery of multipotent mesenchymal stem cells residing in the adipose stromal compartment and in bone marrow has fueled bioengineering and clinical research for many years [1,33]. ASCs have extensively been studied for adipose regeneration, however, their adipogenic potential *in vitro* does not necessarily translate into fat development *in vivo* [34,35]. Clinical needs related to improved soft tissue reconstruction hence remain unresolved. We herein report adipose tissue development on the basis of human SVF cells and fibrin gels.

Uncultured SVF cells represent an alternative cell source to plastic-adherent, cultured ASCs. Owing to its heterogeneous, tissue-inherent cellular composition, the SVF offers a selection of cells that may act synergistically in the tissue building process. Apart from multipotent ASCs, the abundance of vascular precursors and pericytes in the SVF [23,35] has been considered conducive to vascularization as a prerequisite of adipogenesis [36].

In the present study, the SVF characteristically resembled a flow cytometric profile positive for stem cell-associated, hematopoietic and endothelial markers, which is in general accordance to other recent reports [37,38].

To enhance the differentiation potential of adipose precursor cells, adipogenic pretreatment *in vitro* prior to implantation was proven beneficial in various studies using ASCs [7]. In the present approach, this pretreatment was translated to SVF cells in order to commit precursors within the cell pool to the adipogenic lineage. SVF culture in 3D constructs avoided the positive selection or loss of certain cell types associated with plastic adherence in 2D culture. Precultivation was conducted by combining preadipocyte and endothelial cell media to meet the needs of the respective cell types considered essential for tissue development. Adipogenic pretreatment *in vitro* successfully induced adipogenesis of SVF cells in stable fibrin gels, which was accompanied by a negative regulation of endothelial CD31 possibly indicating a negative effect of the adipogenic hormonal cocktail on endothelial cells. In non-induced cultures, the formation of prevascular $CD31^+$ structures suggested a strong vasculogenic environment. Apart from fibrin as a blood clotting component, which is acknowledged to support vascular assembly [39-41], vasculogenic conditions were presumably further supported by pro-angiogenic factors such as FGF-2 and

VEGF contained in the endothelial cell medium added during *in vitro* culture.

Pretreatment *in vitro* had distinct effects on the *in vivo* outcome. Adipogenic induction strongly guided SVF cell fate in stable fibrin gels *in vivo* as reflected by the significant increase of adipocyte numbers relative to non-induced gels. Adipogenic differentiation was accompanied by vascular development, supporting the interdependence of adipogenesis and angiogenesis *in vivo* [36,42,43]. Having adopted an endothelial phenotype *in vitro*, SVF cells in non-induced constructs developed distinct, branched capillary structures *in vivo*. The finding that non-adipogenic precultivation significantly enhanced blood vessel formation compared to induced conditions in stable fibrin accentuates the enormous vascularization capability inherent to the SVF. This may further promote the use of SVF-seeded hydrogels for wound repair [27] and tissue vascularization, as shown by Klar *et al.* for the prevascularization of dermo-epidermal skin substitutes using endothelial cells derived from the SVF [44]. Taken together, the correlation between predetermination of the SVF *in vitro*, namely by culture under adipogenic induction or under non-induced conditions, and cellular behavior *in vivo* was proven.

Apart from the evaluation of precultivation conditions, the SVF was also investigated directly without precultivation. Here, two different fibrin formulations were compared and it was evident that the choice of fibrin formulation had a tremendous impact on tissue development. In TissuCol gels, a nearly coherent fat pad was formed featuring mature, unilocular adipocytes surrounded by a network of fine capillaries. Adipocyte and blood vessel counts significantly surpassed histomorphometric evaluation of stable fibrin gels.

The suitability of fibrin as a biomaterial that supports adipogenesis has been established in studies by various groups including our own [17,20,40,45]. Individual biomaterial properties play an important role in mimicking the extracellular matrix (ECM), simultaneously transducing mechanical and chemical cues [46]. Here, different fibrin formulations were shown to affect the cellular response *in vivo*. Despite their advantageous volume stability, stable fibrin gels *in vivo* were less supportive of adipogenesis than TissuCol gels. The faster degradation kinetics in TissuCol gels [17] as well as different gel compliance and enhanced remodeling, likely facilitated cellular commitment and coherent adipose formation within a short time span of merely 4 weeks *in vivo*. To exploit the advantages of TissuCol gels with regard to adipogenesis and still be able to generate

volume-stable constructs in future studies, a combination with mechanically stable support structures, e.g., biodegradable polyurethane (PU) scaffolds, may be advantageous, as previously evaluated with other cell types [17,19].

Human-specific staining in TissuCol constructs demonstrated the coexistence of human and murine adipose tissue in the developing tissue. Apart from human vimentin-positive adipocytes, the prevalence of vascular structures from human origin was striking. These findings imply that the implanted SVF strongly contributed to the tissue building process by providing precursors with an intrinsic potential for both adipogenesis and angiogenesis.

Whole mount staining illustrated the native-like architecture of the developing tissue in the constructs. Blood vessels in close proximity to large fat cells were observed, demonstrating the reciprocal interaction between angiogenesis and adipogenesis [47,48].

Noteworthily, SVF cells implanted in TissuCol *in vivo* provoked the spontaneous reorganization of adipose tissue structure even in the absence of adipogenic stimuli. Clinical translation of this cell-biomaterial combination is potentially facilitated, since TissuCol is an FDA-approved fibrin sealant and the SVF is harvested as a minimally manipulated cell source. Intraoperative approaches using autologous SVF cells in a one-step surgical procedure have already been suggested, reducing the risk of immune reactions and *in vitro* culture-associated contamination [49-51].

In conclusion, the development of vascularized adipose tissue *in vivo* by SVF cells seeded in fibrin gels was successfully demonstrated. With the SVF, an alternative route to engineering adipose tissue was chosen, applying a mixture of different cell types and precursors that constitute the native tissue and, as shown here, have the ability to rebuild it. Given that various factors influence the *in vivo* outcome, the precise mechanisms underlying tissue morphogenesis of SVF cells have yet to be explored.

On the basis of SVF cells and TissuCol gels, future studies may address the impact of *in vitro* precultivation as well as selected SVF subpopulations on adipogenesis *in vivo*. Moreover, detailed insights on the beneficial effects of SVF cells in comparison to ASCs for adipose tissue engineering applications may be explored in order to further contribute to our understanding of the tissue formation process *in vivo*.

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Chapter 8

Summary and Perspectives

8.1 SUMMARY

Adipose tissue defects and related pathologies still represent major challenges in reconstructive surgery. Based on to the paradigm ‘replace with alike’, adipose tissue is considered the ideal substitute material for damaged soft tissue [1-3]. Yet the transfer of autologous fat, particularly larger volumes, is confined by deficient and unpredictable long-term results, as well as considerable operative morbidity at the donor and recipient site [4-6], calling for innovative treatment options to improve patient care.

With the aim to achieve complete regeneration of soft tissue defects, adipose tissue engineering holds great promise to provide functional, biologically active adipose tissue equivalents. Here, especially long-term maintenance of volume and shape, as well as sufficient vascularization of engineered adipose tissue represent critical and unresolved challenges [7-9]. For adipose tissue engineering approaches to be successful, it is thus essential to generate constructs that retain their initial volume *in vivo*, as well as to ensure their rapid vascularization to support cell survival and differentiation for full tissue regeneration [9,10]. Therefore, it was the ultimate goal of this thesis to develop volume-stable 3D adipose tissue constructs and to identify applicable strategies for sufficient vascularization of engineered constructs. The feasibility of the investigated approaches was verified by translation from *in vitro* to *in vivo* as a critical step for the advancement of potential regenerative therapies.

For the development of volume-stable constructs, the combination of two biomaterials with complementary properties was successfully implemented. In contrast to previous approaches in the field using mainly non-degradable solid structures for mechanical protection of developing adipose tissue [11-13], the combination of a cell-instructive hydrogel component with a biodegradable porous support structure of adequate texture was shown advantageous for the generation of volume-stable adipose tissue. Specifically, stable fibrin hydrogels previously developed in our group [14] served as cell carrier and supported the adipogenic development of adipose-derived stem cells (ASCs) as reflected by lipid accumulation and leptin secretion. Stable fibrin gels were thereby shown to be equally supportive of adipogenesis compared to commercial TissuCol hydrogels *in vitro*. Using ASCs as a safe source of autologous cells [15,16] added substantial practicability to the

approach. To enhance the mechanical strength of the engineered constructs, porous biodegradable poly(ε-caprolactone)-based polyurethane (PU) scaffolds were introduced as support structures and shown to exhibit adequately sized pores to host adipocytes as well as interconnectivity to allow coherent tissue formation and vascularization. Low wettability and impaired cell attachment indicated that PU scaffolds alone were insufficient in retaining cells within the pores, yet cytocompatibility and differentiation of ASCs were adequately demonstrated, rendering the PU scaffolds suitable as support structures for the generation of stable fibrin/PU composite constructs (**Chapter 3**).

Volume-stable adipose tissue constructs were generated by seeding the pre-established stable fibrin/PU composites with ASCs. Investigation of size and weight *in vitro* revealed that composite constructs featured enhanced stability relative to stable fibrin gels alone. Comparing stable fibrin gels and TissuCol as hydrogel components, it was found that TissuCol gels were less resilient to degradation and contraction. Composite constructs were fully characterized, showing good cell viability of ASCs and strong adipogenic development as indicated by functional analysis via histological Oil Red O staining of lipid vacuoles, qRT-PCR analysis of prominent adipogenic markers (PPARγ, C/EBPα, GLUT4, aP2) and quantification of leptin secretion. In a pilot study *in vivo*, investigating the suitability of the constructs for transplantation, stable fibrin/PU composites provided with a vascular pedicle gave rise to areas of well-vascularized adipose tissue, contrasted by insufficient capillary formation and adipogenesis in constructs implanted without pedicle. The biomaterial combination of stable fibrin gels and porous biodegradable PU scaffolds was thereby shown highly suitable for the generation of volume-stable adipose tissue constructs *in vivo*, and in addition, the effectiveness of immediate vascularization upon implantation to support adipose tissue formation was demonstrated (**Chapter 4**).

Further pursuing the objective to investigate adequate vascularization strategies for engineered adipose tissue, hypoxic preconditioning was conducted as a possible approach for *in vitro* prevascularization. In 2D culture experiments, analysis on the cellular level illustrated that the adipogenic potential of ASCs was reduced under hypoxic conditions when applied in the differentiation phase, irrespective of the oxygen tension encountered by the cells during expansion. Hypoxic treatment of ASCs in 3D constructs prepared from stable fibrin gels similarly resulted in reduced adipogenesis, whereas endothelial CD31

expression as well as enhanced leptin and vascular endothelial growth factor (VEGF) secretion indicated that hypoxic treatment indeed resulted in a pro-angiogenic response of ASCs. Especially the observed profound regulation of leptin production by hypoxia and the dual role of leptin as adipokine and angiogenic modulator were considered an interesting connection advocating further study. Having confirmed the hypothesis that hypoxia may generate a pro-angiogenic milieu inside ASC-seeded constructs, faster vessel ingrowth and improved vascularization as well as an enhanced tolerance of hypoxia-treated ASCs towards ischemic conditions upon implanatation may be expected, but remain to be verified in rodent models *in vivo* (**Chapter 5**).

Having previously been utilized for bone and cartilage engineering [17-19], as well as for revascularization and wound healing applications [20-22], stromal-vascular fraction (SVF) cells were investigated as a novel cell source for adipose tissue engineering. Providing cells with adipogenic differentiation as well as vascularization potential, the SVF was applied with the specific aim to promote adipogenesis and vascularization in engineered constructs *in vivo*. With only basic *in vitro* investigations by Lin *et al.* addressing the SVF for adipose repair to date [23], the present work thoroughly investigated SVF cells for adipose tissue construct generation *in vitro*, and in particular, pioneered the application of these cells for adipose tissue engineering *in vivo.*

Initial *in vitro* experiments compared SVF- and ASC-seeded stable fibrin constructs in different medium compositions employing preadipocyte (PGM-2) and endothelial cell culture medium (EGM-2). It was found that a 1:1 mixture of PGM-2 and EGM-2, as previously established for co-culture models of adipogenesis [24], efficiently maintained cells with adipogenic and endothelial potential in SVF-seeded constructs in short and long-term culture setups. Observations on the cellular level were supported by analysis of mRNA expression of characteristic adipogenic and endothelial markers. In preparation of the evaluation of SVF-seeded constructs under *in vivo* conditions, a whole mount staining (WMS) method, facilitating the 3D visualization of adipocytes and blood vessels, was successfully established and optimized using native adipose tissue as template (**Chapter 6**).

In a subcutaneous nude mouse model, SVF cells were, for the first time *in vivo*, elucidated for their potential to support the functional assembly of vascularized adipose tissue. Investigating the effect of adipogenic precultivation of SVF-seeded stable fibrin constructs

in vitro prior to implantation on the *in vivo* outcome, hormonal induction was shown beneficial in terms of adipocyte development, whereas a strong vascularization potential was observed when no adipogenic inducers were added. Via histological analysis, it was proven that the developed structures were of human origin and derived from the implanted cells. Applying SVF cells without precultivation *in vitro* but comparing two different fibrin carriers, namely stable fibrin and TissuCol gels, revealed that TissuCol profoundly supported adipose formation by SVF cells *in vivo*. This was contrasted by only minor SVF cell development and a strong reduction of cell numbers in stable fibrin gels implanted without precultivation. Histomorphometric analysis of adipocytes and capillary structures was conducted to verify the qualitative results, concluding that particularly SVF cells in TissuCol were highly suited for adipose regeneration *in vivo*. Employing the established WMS technique, the close interaction of mature adipocytes and blood vessels in TissuCol constructs was impressively shown and via species-specific human vimentin staining, the expected strong involvement of implanted SVF cells in the formation of coherent adipose tissue was confirmed (**Chapter 7**).

With the development of biodegradable volume-stable adipose tissue constructs, the application of ASCs and SVF cells as two promising cell sources for functional adipose regeneration, as well as the thorough evaluation of strategies for construct vascularization *in vitro* and *in vivo*, this thesis provides valuable solutions to current challenges in adipose tissue engineering. The presented findings further open up new perspectives for innovative treatments to cure soft tissue defects and serve as a basis for directed approaches towards the generation of clinically applicable soft tissue substitutes.

8.2 PERSPECTIVES

As postulated by Langer and Vacanti already in 1993, successful tissue regeneration needs to recapture central processes governing tissue formation, vascularization as well as appropriate dimensionality [25]. This is still valid today, specifically with volume stability, tissue functionality and vascularization of engineered constructs being the central challenges in adipose tissue engineering [8,9]. The results of this thesis offer valuable solutions and novel directions with regard to these challenges. To promote the development of applicable constructs for future clinical use, a refinement and optimization of the presented approaches as well as their integration within a broader scientific perspective are neccessary.

First of all, with the development of clinically applicable constructs at the center of tissue engineering efforts, caution must be exercised against the design of constructs that lack translational potential since the clinical application of complex biomaterial scaffolds and *ex vivo* cultured cells faces immense safety concerns and regulatory hurdles [16,26]. Therefore, the use of minimally manipulated SVF cells instead of culture-expanded ASCs for the generation of adipose tissue constructs may represent an important advancement. As a possible next step towards the generation of translatable volume-stable adipose tissue constructs, SVF cells may be applied together with TissuCol as FDA-approved material, and combined with the established biodegradable PU scaffolds as structural support. Thereby, volume-stable adipose tissue constructs with a strong potential for full tissue regeneration and vascularization by use of SVF cells as cellular component may be provided. In addition, this setup may be optimized towards a one-step clinical procedure, by which the extracted cells are directly incorporated into the scaffold and reimplanted without *in vitro* manipulation or exposure to xenogenic reagents. Recently introduced automated devices that digest harvested tissue in a closed system and isolate the stromal cell population for subsequent use, would further facilitate the development of an intraoperative approach [16].

Despite the regulatory concerns related with cultured ASCs, these cells still represent one of the most promising cell sources for translational clinical research, having found their way into preclinical studies and experimental trials in the fields of gastroenterology, neurology, orthopedics, reconstructive surgery and related disciplines [15,16,27]. International tissue

engineering societies and regulatory agencies therefore work towards the establishment of standardized protocols and guidelines for good manufacturing practice (GMP) to allow a more precise definition of both clinical-grade human ASCs and SVF cells [15]. Minor setbacks to the substantial progress made with regenerative cells obtained from adipose tissue are associated with cellular heterogeneity as well as donor- and depot-dependent variability of proliferation and differentiation [28-30]. Since the latter may be decisive influential factors for the experimental outcome [31-33], routine characterization of the cells, for example via flow cytometric analysis, prior to seeding is advocated to facilitate interpretation of results. In light of the still limited understanding of the mechanisms underlying tissue formation in engineered adipose tissue constructs [34], detailed characterization and also tracking of the implanted cells by fluorescent labeling may prove valuable [5], especially with regard to the possible dual role of ASCs and SVF cells as both building blocks and sources of throphic factors modulating the host response [8,35,36].

In analogy to the applied cells, the effective application of the introduced vascularization strategies requires directed optimiziation and more detailed knowledge on the fundamental mechanisms guiding construct vascularization. First of all, to better estimate the efficiency of the respective methods *in vivo*, it is essential to introduce techniques for routine oxygen monitoring and likewise, imaging techniques that can be consulted to provide tangible information on the actual required time frame for construct (re-)perfusion. Regarding the vascularization of larger-volume constructs after implantation, the chances of success may be increased by combining complementary methods [34], for example SVF cell culture or growth factor administration with the integration of a vascular pedicle. Thereby, the strengths of the investigated concepts may be fully exploited to synergistically promote construct vascularization and hence substantially shorten the interval needed for complete vascularization *in vivo*.

Finally, the suitability of engineered adipose tissue constructs for clinical use needs to be verified in animal models [38]. Despite substantial advancements in adipose construct generation, engineered tissue volumes in a majority of approaches do not reach clinically relevant sizes [8,39]. In the present work, the translation of the generated constructs from *in vitro* to *in vivo* and the thereby provided basic proof-of-principle in nude mouse models

represents a central strength. However, to prove the efficiency of the developed approaches for the engineering of larger-volume adipose tissue constructs and similarly, to validate the long-term maintenance of the engineered tissues *in vivo* for up to several months or even years, the use of larger animal models is a step that needs to be taken for potential clincial translation [7,38,39].

With regard to the above, it is eminent that adipose tissue engineering approaches for soft tissue repair may be effectively implemented through the integration of standardized, highly regenerative cells, biocompatible volume-stable carriers and practical vascularization techniques that ensure implant survival and integration. Together with the long-standing clinical experience in fat grafting and soft tissue reconstruction [8,40], newly established approaches and innovative impulses provided by adipose tissue engineering research have the ability to accelerate the development of patient-specific soft tissue substitutes and their successful clinical application.

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Zusammenfassung

In der rekonstruktiven Chirurgie besteht ein ständig wachsender Bedarf an geeigneten Implantaten, um Weichteildefekte nach Tumorresektionen, Traumata, oder aufgrund von kongenitalen Missbildungen adäquat ersetzen zu können [1]. Hierbei stellt körpereigenes Fettgewebe als Weichteilersatz das ideale Substitutionsmaterial dar [2-4]. Derzeit angewandte Wiederherstellungsmethoden verwenden frei transplantierbare und gestielte Lappenplastiken aus autologem Fettgewebe oder greifen auf künstliche Kollagen- und Silikonimplantate zurück [5]. Diese Ansätze sind jedoch zum Teil mit gravierenden Nachteilen behaftet, wie Absorption und Nekrotisierung bei transplantiertem körpereigenem Fettgewebe, sowie Fremdkörperreaktionen und fibrotischen Verkapselungen bei Kollagen und Silikon. Insbesondere die Versorgung großvolumiger Defekte ist mit komplexen chirurgischen Eingriffen verbunden und geht häufig mit Komplikationen wie Infektionen, Narbenbildung und Volumenverlust, sowie Defiziten an der Hebe- und Empfängerstelle einher [1,5-8]. Es besteht daher ein großer Bedarf an innovativen Methoden und der Entwicklung neuer Materialien, die einen dauerhaften körpereigenen Weichteilersatz ermöglichen.

Das interdisziplinäre Feld des Tissue Engineerings von Fettgewebe zielt auf die Entwicklung neuer Ansätze zur Regeneration von Weichteildefekten und der Bereitstellung von biologisch äquivalentem Gewebeersatz, vor allem für die Rekonstruktion großvolumiger Defekte. Verringerte Volumenstabilität und unzureichende Blutgefäßversorgung stellen jedoch auch bei durch Tissue Engineering hergestelltem Gewebe zentrale Limitationen dar [5,8,9]. Für die erfolgreiche Substitution von Weichteildefekten mit Methoden des Tissue Engineerings ist es daher essenziell, Gewebekonstrukte mit ausreichender Volumenstabilität bereitzustellen, um auch nach Implantation *in vivo* langfristig zu bestehen, sowie eine adäquate Blutgefäßversorgung zu gewährleisten, um Zellüberleben und Differenzierung für eine vollständige Geweberegeneration zu garantieren [5,10].

Folglich war es Ziel dieser Arbeit, volumenstabile Fettgewebekonstrukte zu entwickeln und neue Strategien zur Vaskularisierung der generierten Konstrukte zu evaluieren. Als wichtiger Schritt in Bezug auf eine potenzielle klinische Anwendbarkeit wurden außerdem vielversprechende *In-vitro*-Ansätze auf den *In-vivo*-Kontext in etablierten Mausmodellen übertragen.

Für die Entwicklung volumenstabiler Fettgewebekonstrukte wurde die Kombination zweier Biomaterialien mit komplementären Eigenschaften verfolgt. So wurden für die Konstruktherstellung Fibrinhydrogele als Zellträger mit hochporösen bioabbaubaren Scaffolds als mechanische Schutzstrukturen kombiniert. Im Gegensatz zu bisherigen Ansätzen zur Verbesserung der Volumenstabilität, in denen hauptsächlich nicht abbaubare, rigide Gerüst- oder Hohlkörperstrukturen zum mechanischen Schutz des entstehenden Gewebes appliziert wurden [11-13], wurden hier ausschließlich bioabbaubare und Gewebe-kompatible Materialien verwendet. Dabei konnte auf bereits zuvor entwickelte stabile Fibringele [14] zurückgegriffen werden, die in dieser Arbeit erstmals für das Fettgewebe-Engineering als Zellträger für mesenchymale Stammzellen aus dem Fettgewebe (adipose-derived stem cells; ASCs) verwendet wurden. Mittels sich ergänzender Analysemethoden auf zellulärer (Oil Red O-Färbung) und molekularer Ebene (Leptin-Sekretion; ELISA) konnte erfolgreich die adipogene Differenzierung der in den Fibringelen inkorporierten ASCs nachgewiesen werden. Im Vergleich zu kommerziell erhältlichem Fibrin (TissuCol, Baxter) zeigten ASCs in den stabilen Fibringelen eine mit TissuCol vergleichbare, gute adipogene Differenzierbarkeit. Durch die Verwendung von ASCs als sichere und autologe Zellquelle [15,16] für die Konstruktherstellung wurde zudem die potenzielle klinische Anwendbarkeit der generierten Zell-Biomaterial-Konstrukte erhöht.

Zur Verbesserung der Volumenstabilität wurden bioabbaubare Poly(ε-caprolacton)-basierte Polyurethan-Scaffolds als zusätzliche Gerüststruktur evaluiert. Aufgrund ihrer hohen Porosität und Interkonnektivität stellten sich die Scaffolds als besonders geeignet für die Differenzierung von Adipozyten sowie für die Generierung von kohärentem Fettgewebe heraus. Bei direkter Besiedelung mit ASCs wiesen die PU-Scaffolds zwar eine geringe Zelladhäsion und inhomogene Zellverteilung auf, die adipogene Differenzierung der Zellen war jedoch nicht beeinträchtigt. Daraufhin wurde die Generierung von Fibrin/PU-Kompositkonstrukten durch Kombination der PU-Scaffolds mit den zuvor untersuchten stabilen Fibringelen angestrebt (**Kapitel 3**).

Durch Zusammenführung der stabilen Fibringele als Zellträger für ASCs mit den PU-Scaffolds als zusätzlicher Gerüststruktur konnten in folgenden Arbeiten erfolgreich homogene und mechanisch stabile Fettgewebekonstrukte hergestellt werden. Die detaillierte

Evaluation von Größe und Gewicht zeigte, dass in den Kompositkonstrukten durch die zusätzliche poröse PU-Scaffoldstruktur eine erhöhte Stabilität im Vergleich zu den stabilen Fibringelen als alleinigem Zellträger erreicht werden konnte. Der Vergleich der stabilen Fibringele mit TissuCol als Hydrogelkomponente zeigte, dass TissuCol-Gele unter *In-vitro*-Kulturbedingungen stärker kontrahierten und schneller abgebaut wurden. Die in den Kompositkonstrukten inkorporierten ASCs zeigten gute Viabilität sowie deutliche adipogene Differenzierung auf histologischer (Oil Red O-Färbung) als auch auf molekularer Ebene (qRT-PCR; ELISA). In einer *In-vivo*-Pilotstudie wurden die Kompositkonstrukte auf ihre Transplantierbarkeit hin überprüft und durch mikrochirurgische Insertion eines Durchflussgefäßes bei der Implantation unmittelbar vaskularisiert. In stabilen Fibrin/PU-Konstrukten mit integriertem Gefäßstiel wurde so die Entwicklung von vaskularisiertem Fettgewebe im Vergleich zu ungestielten Konstrukten entschieden verbessert. Mittels der erfolgreichen *In-vivo*-Implantation der Kompositkonstrukte konnte die Anwendbarkeit der Biomaterialkombination aus stabilem Fibrin und porösen PU-Scaffolds für die Generierung volumenstabiler Fettgewebekonstrukte demonstriert und gleichzeitig der positive Effekt einer direkten Vaskularisierung durch Integration eines Gefäßstiels gezeigt werden (**Kapitel 4**).

Im Rahmen der weiteren Evaluation potenzieller Vaskularisierungsstrategien wurden im Anschluss Ansätze zur Prävaskularisierung *in vitro* untersucht. Hierbei stellte die hypoxische Vorkultur von mittels Tissue Engineering generierten Fettgewebekonstrukten einen möglichen Ansatz zur Schaffung eines pro-angiogenen, vaskularisierungsfördernden Milieus innerhalb der Konstrukte dar. Ebenso von Interesse waren in diesem Zusammenhang die Auswirkungen von Hypoxie auf die adipogene Differenzierung von ASCs.

Erste Versuche im 2D-Kulturformat mit ASCs zeigten, dass das adipogene Potenzial der Zellen unter Hypoxie in der Differenzierungsphase stark vermindert war, wobei der während der Expansionsphase der Zellen bestehende Sauerstoffpartialdruck keinen Einfluss auf die Fettentwicklung hatte. Auch in 3D-Konstrukten basierend auf stabilen Fibringelen konnte eine verringerte adipogene Differenzierung von ASCs unter hypoxischer Kultur nachgewiesen werden, dabei wurden im Gegenzug endotheliale Marker (CD31) und pro-angiogene Wachstumsfaktoren, wie z.B. vaskulärer endothelialer Wachstumsfaktor

(VEGF), aber auch das Adipokin Leptin, stark hochreguliert. Insbesondere die deutliche Veränderung der Leptinsekretion unter hypoxischen Kulturbedingungen und die duale Rolle von Leptin als adipogener und pro-angiogener Faktor ergeben interessante Perspektiven für weiterführende Untersuchungen. Basierend auf den gezeigten Ergebnissen konnte insgesamt bestätigt werden, dass die hypoxische Vorkultur *in vitro* zur Entstehung eines pro-angiogenen und potenziell vaskularisierungsfördernden Milieus beitragen kann. Es gilt nun in Folgestudien das Potenzial der hypoxischen Vorkultur zur Verbesserung der Vaskularisierung *in vivo*, sowie eine erhöhte Toleranz der implantierten Zellen gegenüber hypoxischen Bedingungen nach der Implantation in etablierten *In-vivo*-Mausmodellen zu verifizieren (**Kapitel 5**).

Ein weiterer Ansatz zur Generierung von vaskularisiertem Fettgewebe *in vitro* und *in vivo* wurde durch den Einsatz der stromalen-vaskulären Fraktion (SVF) als neue Zellquelle für das Fettgewebe-Engineering verfolgt. Bisher wurde die SVF hauptsächlich für das Tissue Engineering von Knochen- und Knorpelgewebe [17-19] oder für Vaskularisierungs- und Wundheilungsansätze [20-22] untersucht. In der SVF enthalten sind sowohl Fettvorläuferzellen als auch Endothelzellen, Perizyten, Fibroblasten und Immunzellen [8]. Durch Verwendung dieses heterogenen Zellgemisches sollte die simultane Entwicklung von Fettzellen und vaskulären Strukturen erreicht werden, und damit eine schnellere und effizientere Fettgewebeentwicklung *in vivo*. Da sich bisher nur eine *In-vitro*-Studie explizit dem Tissue Engineering von Fettgewebe mit SVF-Zellen widmet [23], wurden in dieser Arbeit SVF-besiedelte Fettgewebekonstrukte basierend auf Fibringelen als Zellträger zunächst umfassend *in vitro* charakterisiert und erstmals die Fettgewebeentwicklung der Zellen im Mausmodell *in vivo* untersucht.

In vorbereitenden *In-vitro*-Arbeiten wurden SVF-besiedelte stabile Fibringele mit den bisher verwendeten ASC-basierten Konstrukten verglichen. Dabei wurde zunächst die adipogene und endotheliale Differenzierbarkeit der SVF in unterschiedlichen Zellkulturmedien untersucht. Eine 1:1-Mischung aus Präadipozytenmedium (PGM-2) und Endothelzellmedium (EGM-2), die zuvor schon für Kokulturexperimente von ASCs und Endothelzellen verwendet worden war [24], stellte sich als besonders geeignet für die Kurz- und Langzeitkultur der SVF in stabilen Fibringelen heraus. Umfassende histologische Untersuchungen zeigten, dass mit Hilfe dieser Medienkomposition insbesondere das

adipogene und endotheliale Differenzierungspotenzial der verschiedenen Zelltypen in der SVF innerhalb der generierten 3D-Konstrukte erhalten werden kann. Die auf zellulärer Ebene gewonnenen Erkenntnisse konnten mittels qRT-PCR-Analyse von adipogenen und endothelialen Markern (PPARγ, aP2, CD31) auf mRNA-Ebene bestätigt werden. Um in Zukunft die *In-vivo*-Untersuchung der generierten Fettgewebekonstrukte zu erleichtern, sowie eine strukturelle Analyse des Gewebeverbands und insbesondere die Interaktion von Adipozyten und Blutgefäßen zu ermöglichen, wurde zusätzlich eine 3D-Färbetechnik (Whole Mount Staining), zunächst unter Verwendung von nativem humanem Fettgewebe, etabliert (**Kapitel 6**).

In einer anschließenden umfassenden Studie in immundefizienten Nacktmäusen (NMRI-*Foxn1*^{nu}/*Foxn1*^{nu}) wurden SVF-Zellen zum ersten Mal *in vivo* für das Engineering von vaskularisiertem Fettgewebe untersucht. Hierbei wurden sowohl der Effekt der *In-vitro*-Vorkultur der SVF-basierten Konstrukte als auch der Einfluss des Trägermaterials auf die Gewebeentwicklung *in vivo* evaluiert. Die adipogene Vorkultur der SVF-besiedelten Konstrukte *in vitro* über einen Zeitraum von 7 Tagen vor Implantation wirkte sich positiv auf die Fettdifferenzierung *in vivo* aus, wohingegen die Vorkultur unter nicht-induzierten Bedingungen ohne adipogene Induktion verstärkt zur Bildung von vaskulären Strukturen führte. Durch Spezies-spezifische Färbung gegen humanes Vimentin konnte gezeigt werden, dass die beobachteten Strukturen humanen Ursprungs waren und daher von den implantierten SVF-Zellen stammten.

Der Einfluss des Trägermaterials auf die Gewebebildung *in vivo* wurde durch Besiedelung stabiler Fibringele und TissuCol-Gele mit SVF-Zellen verglichen. Die Konstrukte wurden ohne *In-vitro*-Vorkultur direkt nach der Herstellung implantiert. Hier zeigte sich in stabilen Fibringelen nach 4 Wochen *in vivo* keine nennenswerte Gewebeentwicklung, wobei auch der Anteil an humanen Zellen innerhalb der Konstrukte zum Zeitpunkt der Explantation stark verringert war. Im Gegensatz dazu konnte in TissuCol-Gelen die Entwicklung von kohärentem und maturem Fettgewebe nachgewiesen werden von dem große Teile humanen Ursprungs waren. Die histologischen Ergebnisse wurden mittels histomorphometrischer Quantifizierung von Adipozyten und Blutgefäßstrukturen verifiziert, wodurch das herausragende Potenzial der SVF für das Fettgewebe-Engineering *in vivo* nochmals verdeutlicht wurde. Unter Verwendung der zuvor etablierten 3D-Färbetechnik (Whole

Mount Staining) konnten anschließend Adipozyten und Blutgefäße innerhalb des entstandenen kohärenten Gewebeverbands in TissuCol-Gelen visualisiert werden. Mit Hilfe einer humanspezifischen Färbung in 3D konnte zusätzlich die weitreichende Beteiligung der implantierten SVF-Zellen bei der Gewebeentwicklung nachgewiesen werden (**Kapitel 7**).

Die in der Dissertation entwickelten bioabbaubaren volumenstabilen Fettgewebekonstrukte, die Untersuchung von ASCs und SVF-Zellen als vielversprechende regenerative Zellquellen für die Generierung funktioneller Konstrukte, sowie die Evaluation unterschiedlicher Vaskularisierungsstrategien *in vitro* und *in vivo* leisten einen wichtigen Beitrag zu neuen und innovativen Ansätzen im Bereich des Tissue Engineerings von Fettgewebe. Die Ergebnisse stellen eine Grundlage für die zielgerichtete Entwicklung regenerativer Implantate dar und eröffnen neue Perspektiven für die Generierung klinisch anwendbarer Fettgewebekonstrukte als Weichteilersatz.

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Appendix

1. LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
AA	ascorbic acid
7-AAD	7-aminoactinomycin D
ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AFN	atipamezol/flumazenil/naloxone
AM	acetoxymethyl ester
Ang-1/2	angiopoietin 1/2
ANOVA	analysis of variance
aP2	adipocyte protein 2
ASC	adipose-derived stem cell(s)
ASP	acylation stimulating protein
ASPS	American Society of Plastic Surgeons
AV	arteriovenous
BAT	brown adipose tissue
BCA	bicinchoninic acid
BMI	body mass index
BMP-2/4	bone morphogenic protein 2/4
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
BM-MSC	bone marrow-derived mesenchymal stem cell(s)
BSA	bovine serum albumin
CAL	cell-assisted lipotransfer
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cDNA	copy/complementary deoxyribonucleic acid
C/EBPα,β,δ	CCAAT-enhancer-binding protein alpha, beta, delta
CLSM	confocal laser scanning microscope
DAB	3,3’-diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole
DMEM/F-12	Dulbecco’s Modified Eagle’s Medium/Ham’s F-12
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBF	early B cell factor(s)
ECM	extracellular matrix
EBM-2	endothelial cell basal medium 2

EGF	epidermal growth factor
EGM-2	endothelial cell growth medium 2
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cell(s)
ESC	embryonic stem cell(s)
FBS	fetal bovine serum
FDA	Food and Drug Administration
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acid(s)
FGF-2	fibroblast growth factor 2
EthD-III	ethidium bromide homodimer III
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT-4	glucose transporter 4
GM-CSF	granulocyte macrophage colony-stimulating factor
GMP	good manufacturing practice
GPDH	glycerol-3-phosphate dehydrogenase
H&E	hematoxylin and eosin
HC	hydrocortisone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES-1	hairy and enhancer of split 1
HGF	hepatocyte growth factor
Hh	hedgehog
HIF-1α	hypoxia-inducible factor 1 alpha
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell(s)
IBMX	3-isobutyl-1-methylxanthine
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
IL-6	interleukin 6
iPSC	induced pluripotent stem cell(s)
KIU	kallikrein inhibitory unit(s)
KLF	Krueppel-like factor(s)
LPL	lipoprotein lipase
MMF	meditomidine/midazolam/fentanyl
MMP	matrix metalloproteinase(s)
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell(s)
MVEC	microvascular endothelial cell(s)
NPY	neuropeptide Y

ORO	Oil Red O
PAI-1	plasminogen activator inhibitor 1
PBM-2	preadipocyte basal medium 2
PBS	phosphate-buffered saline
PEG	poly(ethylene glycol)
PEGDA	PEG-diacrylate
PGA	poly(glycolic acid)
PGM-2	preadipocyte growth medium 2
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PlGF	placental growth factor
pO_2	partial oxygen pressure
PPARγ	peroxisome proliferator-activated receptor gamma
PU	polyurethane
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SDF-1	stem cell-derived factor 1
SEM	scanning electron microscopy
SET	stem cell-enriched tissue
SREBP-1/ADD-1	sterol regulatory element binding protein 1/adipocyte determination and differentiation-dependent factor 1
SVF	stromal-vascular fraction
T3	tyronine
TCF/LEF	T-cell factor/lymphoid enhancer factor
TF	tissue factor
TG	triglyceride(s)
TGF-β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase(s)
TNF-α	tumor necrosis factor alpha
TSP	thrombospondin
UEA I	ulex europaeus agglutinin I
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor 2
VIM	vimentin
vWF	von Willebrand factor
WAT	white adipose tissue
WMS	whole mount staining

2. BUFFERS, SOLUTIONS AND INSTRUMENTS

If not stated otherwise in the Materials and methods sections of the respective chapters, all chemicals and reagents applied for the preparation of buffers and solutions were obtained from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), B. Braun AG (Melsungen, Germany) or Applichem (Darmstadt, Germany).

Buffers and solutions	
Collagenase buffer	0.1 M HEPES, 0.12 M NaCl, 0.05 M KCl, 0.001 M $CaCl_2$, 0.005 M Glucose, 1.5% BSA
Thrombin dilution buffer	40 mM $CaCl_2$, 171 mM NaCl, 40 mM glycine (pH 7.4)
Phosphate-saline buffer	50 mM phosphate buffer, 2 mM $Na_2EDTA*2\ H_2O$, 2 M NaCl
Erythrocyte lysis buffer	0.156 M NH_4Cl, 0.013 M Na_2CO_3, 0.001 M EDTA (pH 7.4)
FC buffer	0.3% BSA, PBS
PBS/Triton X-100	0.3% Triton X-100, PBS
Blocking buffer (WMS)	3% BSA, PBS/Triton X-100
Blocking solution	1.5% BSA, PBS
Oil Red O solution	3 mg/mL Oil Red O, 60% isopropanol
BODIPY stock solution	1 mg/mL BODIPY 493/503, DMSO
Buffered formalin	3.7% formalin, PBS
Hoechst stock solution	0.1 mg/mL Hoechst 33258, ddH_2O

Adipogenic inducers (stock solutions)	
Insulin (1.7 mM)	10 mg/mL, 30 mM HCl
IBMX (25 mM)	5.55 mg/mL, ddH_2O, Na_2CO_3
Dexamethasone (10 mM)	3.925 mg/mL, abs. Ethanol
Indomethacin (50 mM)	17.89 mg/mL, abs. Ethanol

Instruments and Materials		
Olympus BX51 microscope/DP71 camera	Olympus	Hamburg, Germany
Olympus IX51 microscope/XC30 camera	Olympus	Hamburg, Germany
TCS-SP2 AOBS Leica confocal laser scanning microscope	Leica	Wetzlar, Germany
Philips SEM 525 M	Philips	Eindhoven, Netherlands
Z010 AllroundLine Materials Testing Machine	Zwick GmbH & Co. KG	Ulm, Germany
Centrifuge Rotina 420 R	Hettich	Tuttlingen, Germany
Cryostat CM 3050S	Leica	Wetzlar, Germany
Analytical balance	Ohaus	Zurich, Switzerland
Orbital shaker	Heidolph	Schwabach, Germany

CO_2 incubator	IBS Integra Biosciences	Fernwald, Germany
CO_2 incubator (hypoxia)	Binder GmbH	Tuttlingen, Germany
Laminar flow box	Heraeus	Hanau, Germany
Water bath	Memmert	Schwabach, Germany
pH meter HI2210	Hanna Instruments	Kehl am Rhein, Germany
FACSCanto flow cytometer	BD Biosciences	Palo Alto, USA
Tecan GENios pro spectrofluorometer	Tecan Deutschland GmbH	Crailsheim, Germany
NanoDrop 2000c spectrophotometer	Thermo Fisher Scientific	Waltham, USA
MRX microplate reader	Dynatech Laboratories	Chantilly, Virginia
TissueLyser	Qiagen	Hilden, Germany
Mastercycler® Gradient	Eppendorf	Hamburg, Germany
Real-Time PCR Detection System CFX96T™	Bio-Rad	Munich, Germany
Tissue culture flasks T175	Greiner Bio-One	Frickenhausen, Germany
Falcon cell strainers 100 µm	BD Biosciences	Heidelberg, Germany
Multi-well culture dishes	Greiner Bio-One	Frickenhausen, Germany
Super Frost Microscope slides	R. Langenbrinck	Emmendingen, Germany
Microscope cover glasses	Marienfeld GmbH & Co. KG	Lauda-Koenigshofen, Germany
Microtome blades	Feather	Osaka, Japan

3. CURRICULUM VITAE

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10/2006 – 04/2007	Student research assistant at the Chair of Pharmaceutical Chemistry, Ludwig-Maximilians-University, Munich

Scholarships and awards

12/2011 – 05/2013	MENTORING med Program Julius-Maximilians-University, Wuerzburg
03/2007 – 10/2011	Stipendiary of the Max Weber-Program of the State of Bavaria
12/2008	Herbert-Marcinek-Award Ludwig-Maximilians-University, Munich

4. LIST OF PUBLICATIONS

Publications

1. **Wittmann, K.**, Storck, K., Muhr, C., Mayer, H., Regn, S., Staudenmaier, R., Wiese, H., Maier, G., Bauer-Kreisel, P., Blunk, T. Development of volume-stable adipose tissue constructs using polycaprolactone-based polyurethane scaffolds and fibrin hydrogels. J Tissue Eng Regen Med 2013; published online in Wiley Online Library doi: 10.1002/term1830.
2. **Wittmann, K.**, Dietl, S., Ludwig, N., Berberich, O., Hoefner, C., Storck, K., Blunk T., Bauer-Kreisel, P. Engineering vascularized adipose tissue using the stromal-vascular fraction and fibrin hydrogels. Submitted to: Tissue Eng Part A.
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Conference contributions

1. **Wittmann, K.**, Dietl, S., Ludwig, N., Berberich, O., Hoefner, C., Storck, K., Blunk, T., Bauer-Kreisel, P. Engineering vascularized adipose tissue using the stromal-vascular fraction and fibrin hydrogels *(Oral presentation).* TERMIS-EU 2014, Genova, Italy.
2. Hoefner, C., **Wittmann, K.**, Muhr, C., Bauer-Kreisel, P., Becker, M., Blunk, T. Elucidating extracellular matrix dynamics during adipogenic differentiation of human adipose-derived stem cells: comparison of 3D and 2D culture conditions *(Poster presentation)*. TERMIS-EU 2014, Genova, Italy.
3. Hoefner, C., **Wittmann, K.**, Muhr, C., Bauer-Kreisel, P., Becker, M., Blunk, T. Elucidating extracellular matrix dynamics during adipogenic differentiation in a 3D environment *(Poster presentation)*. GSMB Annual Meeting 2014, Regensburg, Germany.
4. **Wittmann, K.**, Storck, K., Dietl, S., Berberich, O., Wiese, H., Blunk, T., Bauer-Kreisel, P. Engineering vascularized adipose tissue using the stromal-vascular fraction and fibrin hydrogels *in vivo (Poster presentation).* WCRM 2013, Leipzig, Germany.

5. Hoefner, C., **Wittmann, K.**, Muhr, C., Bauer-Kreisel, P., Becker, M., Blunk, T. Extracellular matrix development during adipogenic differentiation of adipose-derived stem cells: Comparison of 3D and 2D culture conditions *(Poster presentation)*. WCRM 2013, Leipzig, Germany.

6. **Wittmann, K.**, Storck, K., Dietl, S., Berberich, O., Mayer, H., Regn, S., Wiese, H., Maier, G., Bauer-Kreisel, P., Blunk, T. Volume-stable constructs made from porous scaffolds and fibrin hydrogels for adipose tissue engineering *in vitro* and *in vivo (Oral presentation).* TERMIS-EU 2013, Istanbul, Turkey.

7. **Wittmann, K.**, Storck, K., Dietl, S., Mayer, H., Regn, S., Wiese, H., Maier, G., Bauer-Kreisel, P., Blunk, T. Engineering volume-stable adipose tissue using composite constructs made from biodegradable porous scaffolds and hydrogels *(Oral presentation)*. ISPRES 2013, Berlin, Germany.

8. **Wittmann, K.**, Storck, K., Staudenmaier, R., Wiese, H., Maier, G., Muhr, C., Bauer-Kreisel, P., Blunk, T. Development of volume-stable adipose tissue constructs by combining fibrin gels and porous support structures *(Poster presentation).* TERMIS World Congress 2012, Vienna, Austria.

9. **Wittmann, K.**, Storck, K., Staudenmaier, R., Wiese, H., Maier, G., Muhr, C., Bauer-Kreisel, P., Blunk, T. Development of volume-stable adipose tissue constructs *(Poster presentation).* 3rd International Conference "Strategies in Tissue Engineering" 2012, Wuerzburg, Germany.

10. Dietl, S., **Wittmann, K.**, Muhr, C., Blunk, T., Bauer-Kreisel, P. Engineering adipose tissue constructs using different fibrin gel modifications *(Poster presentation).* 3rd International Conference "Strategies in Tissue Engineering" 2012, Wuerzburg, Germany.

11. Lenertz, A., **Wittmann, K.**, Blunk, T., Bauer-Kreisel, P. Differentiation of adipose-derived mesenchymal stem cells towards the endothelial lineage by combination of hypoxic treatment and angiogenic growth factors *(Poster presentation)*. 3rd International Conference "Strategies in Tissue Engineering" 2012, Wuerzburg, Germany.

12. **Wittmann, K.**, Muhr, C., Storck, K., Staudenmaier, R., Wiese, H., Maier, G., Bauer-Kreisel, P., Blunk, T. Combination of long-term stable fibrin gels and porous scaffolds for engineering of volume-stable adipose tissue *(Poster presentation).* WCRM 2011, Leipzig, Germany.

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