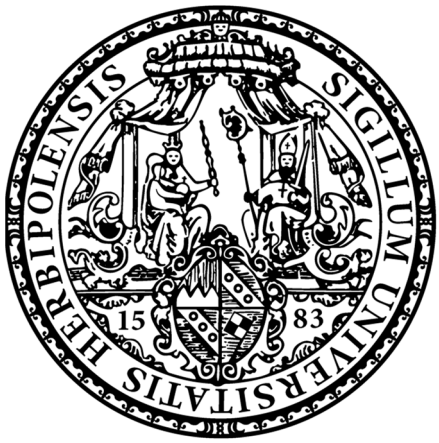

Role of Adipose-Derived Stromal/Stem Cells in Cell-Assisted Lipotransfer – Characterization of their Secretory Capacity under Ischemia-Like Stress Conditions and Establishment of a 3D Adipose Tissue-ASC Co-Culture

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Bedeutung von mesenchymalen Stammzellen aus dem Fettgewebe für den zellassistierten Lipotransfer – Charakterisierung der Sekretionskapazität unter Ischämie-artigen Stressbedingungen und Etablierung einer 3D Fettgewebe-ASC-Kokultur

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In Liebe und Dankbarkeit für meine Eltern

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Summary

The use of human adipose-derived mesenchymal stem cells (ASCs) for cell-based therapeutic approaches, in terms of repair and regeneration of various tissues and organs, offers an alternative therapeutic tool in the field of regenerative medicine. The ability of ASCs to differentiate along mesenchymal lineages is not the only property that makes these cells particularly attractive for therapeutic purposes. Their promising functions in promoting angiogenesis, reducing inflammation as well as in functional tissue restoration are largely related to the trophic effects of a broad panel of secreted cytokines and growth factors. However, in cell-based approaches, the cell-loaded construct often is exposed to an ischemic microenvironment characterized by severe oxidative and nutritional stress after transplantation due to the initial lack of vascular connection, resulting in reduced cell viability and altered cell behaviour. Therefore, the effective use of ASCs in regenerative medicine first requires a comprehensive characterization of the cells in terms of their viability, differentiation capacity and especially their secretory capabilities under ischemia-mimicking conditions in order to better understand their beneficial role. Accordingly, in the first part of this work, ASCs were investigated under different ischemic conditions, in which cells were exposed to both glucose and oxygen deprivation, with respect to viability and secretory function. Using mRNA gene expression analysis, significantly higher expression of selected angiogenic, anti-apoptotic and immunomodulatory factors (IL-6, VEGF, STC-1) could be demonstrated under harsh ischemic conditions. These results were reflected at the protein expression level by a significantly increased secretion of these factors. For stanniocalcin-1 (STC-1), a factor not yet described in ASCs, a particularly high expression with significant secreted amounts of the protein could be demonstrated under harsh ischemic conditions. Thus, the first part of this work, in addition to the characterization of the viability, provided first insights into the secretory response of ASCs under ischemic conditions.

The response of ASCs to glucose deficiency in combination with severe hypoxia has been little explored to date. Thus, the focus of the second part of this work was on a more detailed investigation of the secretory response of ASCs under glucose and oxygen deprivation. For a more comprehensive analysis of the secretion profile, a cytokine antibody array was performed, which allowed the detection of a broad panel of secreted angiogenic factors (IL-8, ANG), matrix-regulating proteins (TIMP-1, TIMP-2), chemokines (MCP-1/CCL2, IP-10/CXCL 10) and other factors under ischemic conditions. To verify these results, selected factors were examined using ELISA. The analysis revealed that the secretion of individual factors (e.g., STC-1, VEGF) was significantly upregulated by the combination of glucose and oxygen deprivation compared to oxygen deprivation alone.

In order to investigate the impact of the secretome of ischemic ASCs on cell types involved in tissue regeneration, the effect of conditioned medium of ischemia-challenged ASCs on both endothelial cells and fibroblasts was investigated in subsequent experiments. Significantly increased viability and tube formation of endothelial cells as well as activated migration of fibroblasts by the secreted factors of ischemic ASCs could be demonstrated. A direct correlation of these effects to STC-1, which was significantly upregulated under ischemic conditions and has been described as a regulator of key cellular functions, could not be verified.

The particular secretory capacity of ASCs provides a valuable tool for cell-based therapies, such as cell-assisted lipotransfer (CAL), where by enriching fat grafts with isolated ASCs, a significantly improved survival rate of the transplanted construct is achieved with less resorption of the fat tissue as well as a reduction in adverse implications, such as fibrosis and cyst formation. In order to better understand the function of ASCs in CAL, an autologous transwell-based lipograft-ASC co-culture was established in the last part of this work, in which first investigations showed a markedly increased secretion of VEGF compared to lipografts without added ASCs. As the stability rate of the fat tissue and thus the success of CAL is presumably also dependent on the preparation of the tissue before transplantation, the conventional preparation method of fat tissue for vocal fold augmentation in laryngoplasty was additionally evaluated *in vitro* in a pilot experiment. By analyzing the viability and tissue structure of the clinically prepared injection material, a large number of dead cells and a clearly damaged tissue structure with necrotic areas could be demonstrated. In comparison, the preparation method of the fat tissue established in this work as small tissue fragments was able to provide a clearly intact, vital, and vascularized tissue structure. This type of adipose tissue preparation represents a promising alternative for clinical vocal fold augmentation.

In conclusion, the results of this work contribute to a comprehensive characterization of ASCs under ischemic conditions, such as those prevalent at the transplantation site or in tissue regeneration. The results obtained, especially on the secretory capacity of ASCs, provide new insights into how ASCs mediate regenerative effects in an ischemic milieu and why their use for therapeutic purposes is highly attractive and promising.

Zusammenfassung

Der Einsatz von humanen mesenchymalen Stammzellen aus dem Fettgewebe (ASCs) für zell-basierte Therapieansätze zur Reparatur und Regeneration von verschiedenen Geweben und Organen bietet eine alternative therapeutische Lösung im Bereich der regenerativen Medizin. Die Fähigkeit der ASCs zur Differenzierung in verschiedene mesenchymale Zelltypen ist jedoch nicht die einzige Eigenschaft, die diese Zellen für therapeutische Zwecke besonders attraktiv macht. ASCs sezernieren vielmehr ein breites Spektrum an Zytokinen und Wachstumsfaktoren, die z.B. durch Förderung der Angiogenese oder der Reduktion von Entzündungsprozessen eine wichtige Rolle bei regenerativen Therapien spielen können. Allerdings ist in zellbasierten Ansätzen, das zellbeladene Konstrukt nach der Transplantation – durch den anfänglich fehlenden Gefäßanschluss und die damit einhergehende mangelnde Versorgung des implantierten Gewebes – starkem oxidativem und ernährungsbedingtem Stress, einem ischämischen Milieu, ausgesetzt, was zu einer reduzierten Zellviabilität und einem veränderten Zellverhalten führt. Der effektive Einsatz der ASCs in der regenerativen Medizin erfordert demnach zunächst eine umfassende Charakterisierung der Zellen in Bezug auf deren Lebensfähigkeit, Differenzierungsfähigkeit und insbesondere die sekretorischen Fähigkeiten unter simulierten ischämischen Bedingungen, um ihren therapeutischen Effekt besser verstehen und optimieren zu können. Dazu wurden im ersten Teil dieser Arbeit die ASCs unter verschiedenen ischämischen Bedingungen, bei denen die Zellen sowohl einem Glukose- als auch Sauerstoffmangel ausgesetzt waren, hinsichtlich der Viabilität und der sekretorischen Funktion *in vitro* untersucht. Durch mRNA Genexpressionsanalysen konnte für ausgewählte angiogene, anti-apoptotische und immunmodulatorische Faktoren (IL-6, VEGF, STC-1) eine signifikant höhere Expression unter stark ischämischen Bedingungen gezeigt werden. Diese Ergebnisse spiegelten sich gleichermaßen auf Proteinebene durch eine signifikant erhöhte Sekretion der Faktoren wider. Für Stanniocalcin-1 (STC-1), einen Faktor, dessen Rolle bislang im Zusammenhang mit ASCs noch nicht beschrieben ist, konnte eine besonders hohe Expression mit signifikanten sezernierten Mengen des Proteins bei hoher ischämischer Belastung der Zellen gezeigt werden. Somit konnten im ersten Abschnitt der Arbeit neben einer ersten Charakterisierung der ASCs auch erste Erkenntnisse über das sekretorische Verhalten der Zellen in einem ischämischen Milieu gewonnen werden.

Die Reaktion von ASCs auf Glukosemangel in Kombination mit Hypoxie ist bislang wenig untersucht. Somit lag der Fokus im zweiten Teil dieser Arbeit auf der detaillierteren Untersuchung des Sekretionsverhaltens von ASCs unter Glucose- und Sauerstoffdeprivation. Für eine umfassende Analyse des Sekretionsprofils wurde ein Zytokin-Antikörper-Array durchgeführt, mit welchem die Sekretion eines breiten Panels von angiogenen Faktoren (IL-8, ANG), matrixregulierenden Proteinen (TIMP-1, TIMP-2), Chemokinen (MCP-1/CCL2,

IP-10/CXCL 10) sowie weiterer Faktoren unter ischämischen Bedingungen nachgewiesen werden konnte. Zur Verifizierung dieser Ergebnisse wurden ausgewählte Faktoren mittels ELISA untersucht. Durch diese Analyse konnte gezeigt werden, dass die Sekretion einzelner Faktoren (z.B. STC-1, VEGF) durch die Kombination von Glukose- und Sauerstoffentzug deutlich hochreguliert wird, z.B. gegenüber nur dem Entzug von Sauerstoff.

Um die Wirkung des Sekretoms von ischämischen ASCs auf Zelltypen, die in der Regeneration von Geweben eine Rolle spielen, zu untersuchen, wurde in nachfolgenden Experimenten die Wirkung von konditioniertem Medium ischämischer ASCs sowohl auf Endothelzellen als auch auf Fibroblasten untersucht. Dabei konnte sowohl eine deutlich gesteigerte Röhrenbildung („tube formation“) von Endothelzellen als auch eine aktivierte Migration von Fibroblasten durch die sezernierten Faktoren der ischämischen ASCs nachgewiesen werden. Ein direkter Zusammenhang dieser Effekte mit dem unter ischämischen Bedingungen signifikant hochregulierten Faktor STC-1, welcher als Regulator zellulärer Schlüsselfunktionen beschrieben wird, konnte hingegen nicht nachgewiesen werden.

Die besondere Sekretionsfähigkeit von ASCs stellt ein wertvolles Werkzeug für zellbasierte Therapien dar, wie z.B. den zellassistierten Lipotransfer (CAL), bei dem durch die Anreicherung von Fetttransplantaten mit isolierten ASCs eine deutliche Verbesserung der Überlebensrate des transplantierten Konstrukts mit einer geringeren Resorption des Fettgewebes sowie einer Verringerung von unerwünschten Folgen, wie Fibrosen und Zystenbildung, erzielt wird. Um die Funktion der ASCs im CAL besser charakterisieren zu können, wurde im letzten Teil dieser Arbeit eine autologe Transwell-basierte Lipograft-ASC-Kokultur etabliert, in welcher durch erste Untersuchungen eine signifikant erhöhte Sekretion von VEGF im Vergleich zu den Lipografts ohne Zusatz von isolierten ASCs gezeigt werden konnte. Da die Stabilitätsrate des Fettgewebes und damit der Erfolg des CAL mutmaßlich auch von der Aufbereitung des Gewebes vor der Transplantation abhängig ist, wurde in einem Pilot-Experiment die konventionelle Präparationsmethode von Fettgewebe für die Stimmlippenaugmentation in der Laryngoplastik *in vitro* evaluiert. Durch Analysen zur Viabilität und Gewebestruktur konnte bei dem klinisch aufbereiteten Injektionsmaterial eine große Anzahl abgestorbener Zellen sowie eine deutlich geschädigte Gewebestruktur mit nekrotischen Arealen nachgewiesen werden. Im Vergleich dazu konnte mit der in dieser Arbeit etablierten Präparationsmethode des Fettgewebes als kleine Gewebsfragmente eine deutlich intakte, vitale und vaskularisierte Gewebestruktur erhalten werden. Damit bietet diese Art der Aufbereitung von Fettgewebe eine vielversprechende Alternative für die klinische Stimmlippenaugmentation.

Zusammengefasst tragen die Ergebnisse dieser Arbeit zu einer umfassenden Charakterisierung von ASCs unter ischämischen Bedingungen bei, wie sie beispielsweise am Transplantationsort oder in der Geweberegeneration vorliegen können. Die gewonnenen Ergebnisse, insbesondere zu den sekretorischen Fähigkeiten der ASCs, liefern neue Erkenntnisse darüber, wie ASCs regenerative Effekte in einem ischämischen Milieu vermitteln und weshalb deren Verwendung für therapeutische Zwecke besonders attraktiv und vielversprechend ist.

1 Introduction

1.1 Adipose tissue

Adipose tissue (AT) is considered one of the largest endocrine organs in the body and regulates a wide range of cellular reactions and metabolic homeostasis in humans [1–3]. In addition to its role as an energy reserve and insulating substance with mechanical support for important body structures, it influences a variety of physiological and pathophysiological processes [4].

The average amount of AT mass varies among genders, with healthy males having an average body fat between 10 and 20%, and females approximately 20 to 30% [5]. AT can generally be divided into subcutaneous and intra-abdominal AT, with subcutaneous AT making up about 80% of total AT [5,6]. In females, the subcutaneous fat is most abundant in the gluteofemoral regions as well as in the mammary area, whereas in males the main subcutaneous depots can be found in the neck, the area over the deltoid and triceps muscles, as well as in the lumbosacral region [5]. The remaining 20% of AT are located around organs and include the visceral AT around the digestive organs (mesenteric and omental) and the retroperitoneal depot (kidney) [5,7,8].

In recent years much progress has been made in understanding AT dysfunctionality that is clearly associated to the onset of important pathologies including obesity, type 2 diabetes, dyslipidemia, or nonalcoholic fatty liver [9–12]. The mechanisms underlying AT dysfunctionality may have different origins such as adipocyte hypertrophy, impaired lipid metabolism, AT inflammation, impaired extracellular matrix remodeling, inadequate vascularization as well as an altered secretion of adipokines. In 1997, obesity was declared as a chronic disease by the World Health Organization (WHO) [13]. In recent decades, the prevalence of obesity has increased steadily and has already reached epidemic dimensions with an increasing number of children and young adults in particular being significantly above normal weight [5,14]. This involves the diagnosis of diseases that previously only appeared in adulthood [15]. Genetic reasons as well as ethnic background and the gender also have an important impact in this context, but the imbalance in energy expenditure and intake represents the most common reason [16–18]. Apart from obesity and the resulting proportion of too much fat, there is also the condition of too little fat, which is relevant in connection with genetic or acquired lipodystrophies, aging, trauma, and tumor resections [19]. In the field of cosmetic and reconstructive surgery, autologous fat grafting is an established method to fill subcutaneous tissue loss [20,21]. This involves improving the loss or damage of AT by transferring AT to the impaired site with minimal risk of immune rejection or transmission of viral pathogens [22]. Furthermore, it is a minimally invasive procedure, with little scarring at the defective site with

recovery time for the patient being less than 48 h [23]. Overall, AT represents an important, multifaceted organ that can have a major impact on metabolic disorders, but also offers great potential for clinical applications in the field of tissue repair and regeneration.

1.1.1 Adipose tissue structure

AT is a dynamic and highly vascularized connective tissue consisting mainly of mature, lipid-filled adipocytes (35-70%) that are closely arranged within the tissue in a hexagonal configuration. In addition, AT contains a variety of other components, including the stromal vascular fraction (SVF) with blood cells, endothelial cells, pericytes, adipose-derived stem cells (ASCs), adipose progenitor cells, fibroblasts, immune cells as well as blood vessels [24–26] (Figure 1.1). There are two types of AT in mammals, brown adipose tissue (BAT) and white adipose tissue (WAT), which vary in their abundance and function [5]. BAT is mainly found only in infancy and is responsible for heat production through the adaptive, non-shivering thermogenesis to protect newborns against hypothermia [4,27]. With age, the amount of BAT decreases progressively and only remaining brown fat deposits can be found surrounding the vertebrae, above the clavicles, in the upper back, and in the mediastinum [28–30].

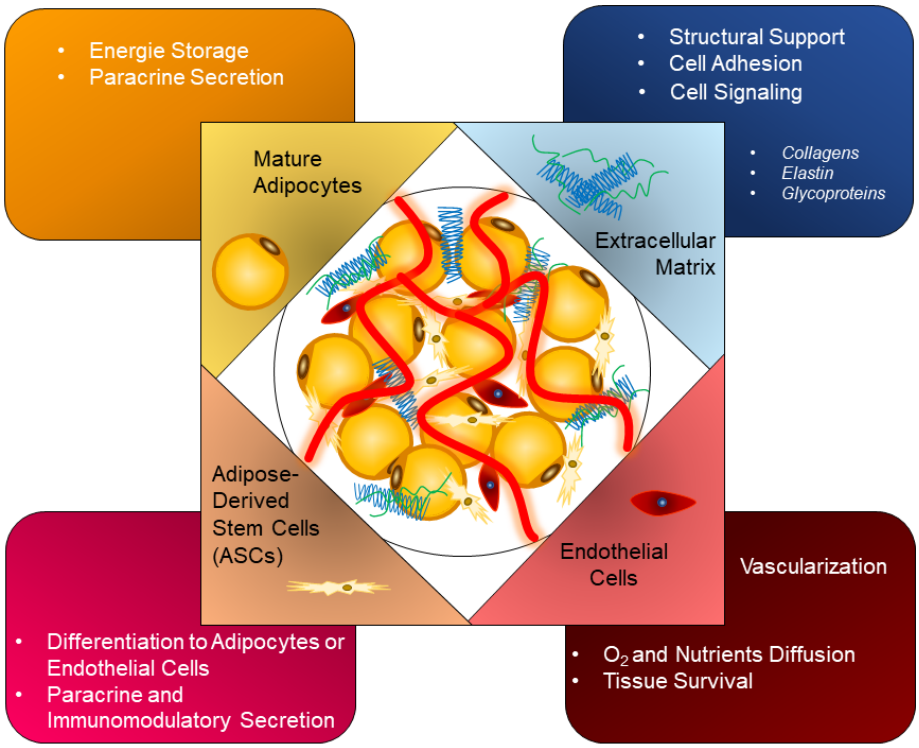

Figure 1.1: Adipose tissue: The most important components and their characteristics.
Adipose tissue consisting mainly of mature adipocytes, ASCs and endothelial cells surrounded by their dynamic extracellular matrix. All components are involved with their specific functions in the modulation of overall tissue homeostasis. Modified from Louis et al. 2020 [31].

In adult humans, WAT is the predominant form of AT and is divided into several depots in the body. The total fat mass can be divided into subcutaneous (~ 80%) and visceral and bone marrow (~ 20%) fat compartments [5]. WAT serves as an energy reservoir by storing triglycerides, which are located in intracellular lipid droplets in the adipocytes, when there is an energy overload, and releasing them into free fatty acids during an energy shortage. The increase in WAT mass in response to changes in the nutrient supply results from hypertrophy (enlarged adipocytes), the increase in size of already existing adipocytes due to increased lipid accumulation, and from hyperplasia (increased numbers of adipocytes), in which the differentiation of precursor cells into mature adipocytes leads to an increased number of cells in the tissue [25,32]. These two modes of AT expansion are regulated by environmental and genetic factors. Adipocytes can range in size from 20 to 200 µm in diameter [5]. Mature unilocular adipocytes contain a single large fat droplet that forms 90% of the cell volume. This is surrounded by a thin rim of cytoplasm and a peripherally located nucleus, giving the cell its typical signet ring form [5,8]. Smaller and multiple lipid droplets, in contrast, are found in developing adipocytes or when triglycerides are mobilized during nutrient deprivation. AT has a dynamic extracellular matrix (ECM), a composite of cell-secreted molecules generated during tissue development that includes the non-cellular component of the tissue and acts as a scaffold to provide structural support to the cells. The ECM, produced by both adipocytes and SVF cells, is fundamentally composed of water, proteins and polysaccharides and is involved in biochemical and biophysical interactions in the cellular microenvironment. Each adipocyte is surrounded by a specific ECM, the basal lamina, which consists mainly of collagen (COL) IV, laminin and heparan sulphate proteoglycan [33–36]. This ECM takes over numerous functions such as tissue architecture, cell adhesion, polarity, and migration, as well as functions in signal transduction between the individual cells [37].

1.1.2 Adipose tissue function

The traditional role attributed to the AT was thermal insulation, mechanical protection of important body structures and, above all, the function of a specialized energy storage organ [5,38–40]. A radical change in perspective of AT followed the discovery of leptin in 1994 [41], the first adipocyte-derived cytokine involved in modulating energy homeostasis indicated WAT as one of the largest endocrine organs of the human body [11,42]. The highly active metabolic and endocrine function is attributed to the secretion of a variety of biologically active factors, collectively termed as adipokines, including e.g., hormones, growth factors, enzymes, cytokines, complement factors as well as matrix proteins that contribute to many different functions in physiological and metabolic processes [5,43]. Thus, they are reported to be involved in energy homeostasis, food intake and satiety, insulin sensitivity, lipid metabolism, complement system, vascular homeostasis, and blood pressure regulation [4,5,44].

Based on the diverse physiological processes controlled by AT, there is already considerable evidence of associations between the increased production of some adipokines and the pathogenesis of obesity and type 2 diabetes mellitus and other metabolic and cardiovascular dysfunctions [11,12,42,45]. Obesity is defined as excess body fat and is attributed to a dysfunction in leptin signaling [46–48]. Leptin, that is primarily synthesized and secreted by mature adipocytes, corresponds with the proportion of AT mass [46,49], which is why it is increased in obesity [11,42,50]. Obesity is a critical risk factor for the development of type 2 diabetes mellitus and cardiovascular diseases caused by insulin resistance (IR) in WAT [5,51,52]. Another adipocyte-secreted adipokine, adiponectin, found in the blood [53,54], exerts anti-obesity and anti-diabetic actions, and soothes IR by stimulating lipid oxidation and anti-inflammatory responses [55–58]. In addition to these important factors, there are many other factors involved in lipid metabolism such as cholesteryl ester transfer protein (CETP), lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), apolipoprotein E (ApoE), and retinol binding protein-4 (RBP-4) [11]. In the regulation of vascular homeostasis are factors involved such as angiotensinogen, angiotensin II, angiotensin-converting enzyme (ACE), plasminogen activator inhibitor (PAI-1), prostaglandin E2 (PGE2) and apelin, whereby the modulation of angiogenesis and tissue repair is regulated by factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin (Ang)-1, Ang-2, among others [5,11,35]. Severe obesity leads to a chronic inflammatory state in which numerous pro-inflammatory secreted cytokines such as interleukins (IL) (e.g., IL-4, IL-6, IL-8, IL-10, IL-12, IL-18), tumor necrosis factor alpha (TNF-alpha) and C-reactive protein (CRP) negatively affect metabolic status [5,43,46,59]. Overall, the main function of AT is no longer primarily to store fats, but rather it functions as an endocrine organ that secretes various regulatory factors and is of great interest for several therapeutic approaches.

1.1.3 Adipose tissue vascularization

Native AT is a highly vascularized tissue in which each adipocyte is associated with one or more capillaries [60,61]. The density of blood vessels is important for the regulation of adipocyte function and maintenance, providing the transport of nutrients, oxygen, growth factors, cytokines, and hormones [62,63]. It is reported that there exists a complex crosstalk between angiogenesis, the formation of new blood vessels from pre-existing vessels, which involves the migration, proliferation, and differentiation of endothelial cells (ECs), and adipogenesis, including the differentiation of (pre-)adipocytes into adipocytes and their subsequent growth in size through lipogenesis, with each regulating the other [61,64,65]. The growth and expansion of AT by increasing of preformed adipocytes in size (hypertrophy), followed by an increase in adipocyte cell numbers (hyperplasia) is reported to be angiogenesis dependent [32,64]. For angiogenesis, in which new vessels are formed from existing ones,

various triggers can be responsible, such as metabolic signals related to the enlargement of adipocytes. In the presence of angiogenic and metabolic signals, vessels that are lined by a single monolayer of resting ECs in state of quiescence can rapidly switch to the angiogenic/proliferative state by activating angiogenesis-promoting proteins [66]. In contrast, vasculogenesis that is characterized by the *de novo* formation of blood vessels during embryonic development but is also reported in adults, involves the differentiation of distinct progenitor cells into ECs [67,68]. Apart from the expansion of AT associated with vascular growth, weight loss is associated with the regression of blood vessels whereby the underlying mechanisms of vessel regression are not yet fully elucidated [62].

The regulation of angiogenesis and the vascular structure in AT depends on the local balance between secreted pro-angiogenic and anti-angiogenic factors, the “angiogenic switch”, that indicates an autoregulatory function for angiogenesis in AT [69]. The functional communication between (pre-)adipocytes and capillary ECs seems to be via autocrine/paracrine signaling pathways, the interaction with extracellular components and by direct cell-cell contact [60,64]. Moreover, studies revealed that besides adipocytes also other cell types contribute to the modulation of AT angiogenesis like resident macrophages and other inflammatory and stromal cells [70]. Actively growing adipocytes mediate vascularity by producing and secreting many different types of pro-angiogenic factors, such as VEGF-A and hepatocyte growth factor (HGF) as key angiogenic factors produced by adipocytes as well as other angiogenesis-related factors including Ang-1, Ang-2, fibroblast growth factor 2 (FGF-2), placental growth factor (PIGF), leptin, estrogen, IL-6, transforming growth factor (TGF)-α, TGF-β, among others [60–62,64,65,71,72]. In addition to these secreted factors, adipocytes release several matrix metalloproteinases (MMPs) that are responsible for the modulation of the ECM and allow matrix-bound vascular growth factors to induce angiogenesis in AT [60]. However, the secreted paracrine factors are not only attributed to developing and mature adipocytes but also to ASCs that secrete high amounts upon appropriate molecular stimulation [71,73]. In this context, hypoxia has been shown to be a potent stimulus of angiogenesis by triggering the expression of hypoxia-inducible factor 1 alpha (HIF-1α) that stimulates especially VEGF and leptin with a resulting angiogenic response [60,65,74]. VEGF, which is expressed in the SVF and in mature adipocytes, is reported as the most potent stimulator that induces migration, survival, and proliferation of ECs and acts primarily via vascular endothelial growth factor receptor 2 (VEGFR2) signaling [64,75,76]. Leptin has also been shown to have pro-angiogenic effects via binding to leptin receptors that are expressed by ECs. In this context, Sierra-Honigmann et al. [77] revealed human umbilical vein endothelial cell (HUVEC) migration, proliferation and tube formation supported by leptin. Moreover, leptin has been reported to promote neovascularization by upregulating VEGF expression [78,79] as well as to induce matrix remodeling through the activation of MMP-2 and MMP-9 which, together with

other matrix remodeling enzymes, play an important role in angiogenic sprouting and vascular maturation by modulated ECM. The resulting release of the matrix-bound VEGF contributes to induced angiogenesis, thus the ECM itself also represents a key role in the development of vascularized AT [60,61].

Overall, the regulatory mechanisms and pathways that induce AT angiogenesis, and the role of the vasculature in AT function need a more detailed exploration regarding the temporal and spatial interplay between the different cell types during AT vascularization and vessel remodeling as well as the effects of these interactions on metabolic functions of the cell types [63,80,81]. A better understanding of the mechanisms involved in AT angiogenesis would be of great importance for, among other things, therapeutic purposes, such as vascularization following adipose tissue augmentation in the context of tissue regeneration.

1.2 Adipose-derived mesenchymal stromal/stem cells (ASCs)

AT is generally abundant in the human body, characterized by its easy accessibility and is comprised of adipocytes and a rich source of ASCs that are becoming of increasing relevance for therapeutic use [82–85]. ASCs can be easily obtained from either liposuction aspirates or subcutaneous AT fragments from reconstructive surgeries, which are minimal harvesting procedures compared to conventional stem cell sources such as bone marrow (BM) and can be easily expanded *in vitro* [84,86–88]. As the number of obtained stem cells is 100 to 500-fold higher than in BM, ASCs represent one of the most popular adult stem cells populations in the field of stem cell research and for applications in regenerative medicine [89,90].

ASCs are commonly isolated by enzymatic digestion of AT with collagenase whereby cellular components are separated by centrifugation steps. The supernatant contained mature adipocytes and the resulted pellet, a heterogeneous set of cell populations, is termed SVF [82,84,91]. The SFV contains besides ASCs also cells from the microvasculature, such as vascular endothelial cells and their progenitors, vascular smooth muscle cells, and leukocytes [92,93]. The subsequent cultivation of the cells allows ASCs to adhere quickly to the surface of tissue culture-treated flasks with an average doubling time between 2 to 5 days, depending on passage number as well as on culture medium [94,95]. Thereby non-adherent cells are removed. The spindle-shaped morphology of ASCs is very similar to bone marrow mesenchymal stem cells (BM-MSCs) [96]. Since the availability of fresh AT for isolation of cells is not always guaranteed, ASCs can be very easily cryopreserved in media containing serum and dimethylsulfoxide (DMSO), which does not impair proliferation and differentiation capability of the cells [97]. Commercial systems are now available for isolating ASCs non-enzymatically and directly to allow clinicians the immediate application within the same

operation [98,99]. Nevertheless, in terms of the number of ASCs collected, the enzymatic isolation method proved to be more effective with 25.9% compared to 5% [98].

The characterization of ASCs *in vitro* is based on the expression of cell surface CD (cluster of differentiation) antigens and can be examined by flow cytometry analysis [90]. There exists a general consensus that ASCs positively express typical mesenchymal markers such as $CD34^+$, $CD90^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD49d^+$, and $CD105^+$, and negatively express hematopoietic and endothelial antigens such as $CD14^-$, $CD31^-$, $CD45^-$, and $CD144^-$ [82,94,100]. Whereby ASCs are only $CD34^+$ at the first passage of the culture, which then decreases more and more after passaging [94,101]. However, what distinguishes them from BM-MSCs is that they are additionally positive for $CD36^+$ and negative for $CD106^-$ [91]. The localization of ASCs within AT has not been fully clarified so far. Some studies have suggested that the niche of ASCs is in the vasculature of AT [102]. Other studies have reported that ASCs are also found in the AT at a perivascular site, which has been demonstrated by histological investigations [103,104]. Traktuev et al. [104] have moreover noticed that ASCs are located at the interface between endothelium and adipocytes, whereby ASCs can both support vascular structure and generate adipocytes. This finding reinforces the assumption that processes like adipogenesis and angiogenesis are closely related [64,105].

Apart from the easy accessibility of ASCs and their availability in high numbers in AT, they have prominent implications in tissue regeneration due to their capacity for self-renewal, their ability to undergo multi-lineage differentiation [86,106,107], including osteogenic, chondrogenic, adipogenic, cardiomyocytic, hepatic and neurogenic differentiation and to secrete various cytokines and immunomodulatory factors [108]. These properties contribute to ASCs being promising candidates for cell-based therapeutic applications in the field of regenerative medicine.

1.3 Regenerative properties of ASCs

Regenerative medicine is a multidisciplinary field of research that aims to develop methods to regrow, repair or replace damaged or diseased cells, organs, or tissues by using biomaterials, growth factors and especially stem cells [109–112]. ASCs are ideal candidates in the field of regenerative medicine as they possess the ability to self-renew, to differentiate along multiple cell lineages, and have above all paracrine and immunomodulatory properties [73,106,113,114]. Moreover, because ASCs are available in large quantities, can be harvested through minimally invasive procedures and transplanted safely and effectively, they have acquired increased importance for therapeutic uses than other stem cell sources [93,115,116]. The postulated regeneration mechanisms of ASCs are very multifaceted in the different areas of tissue injury, transplantation, or tissue repair. These range from direct differentiation

[82,117,118], increased perfusion [119,120], antioxidant effects [117,121], tissue remodeling [122–124], immunomodulatory and anti-inflammatory actions [125–127], among others.

1.3.1 Differentiation potential of ASCs

The regenerative effects of ASCs can be attributed to various mechanisms. ASCs are of mesodermal origin and considered to be a promising tool for multiple clinical applications due to the differentiation capability into cells of ectodermal, endodermal, and mesodermal origin, both *in vitro* and *in vivo* [107,128]. The induction of ASC differentiation *in vitro* is thereby achieved using appropriate culture media containing lineage-specific induction factors [107,129].

One of the most intuitive uses of ASCs in tissue repair and regeneration is the replacement of adipose tissue itself [130–132]. Therefore, the generation of *de novo* adipose tissue by supplemented ASCs is of great importance for various therapeutic purposes. Numerous studies have reported on the adipogenic differentiation capability of ASCs with the underlying mechanisms [37,115,133]. The adipogenic differentiation, termed adipogenesis, is initiated and regulated by a multitude of integrated stimulatory and inhibitory signals from soluble factors, cell-cell, and cell-matrix interactions. The pH as well as the local oxygen tension are also reported to be crucial factors that are related to adipogenesis [38,134,135]. The transition of MSCs into preadipocytes and finally into lipid-laden adipocytes, is described to occur in two main phases, involving adipocyte determination and differentiation [39,136–138]. The determination phase includes the commitment of MSCs to preadipocytes. Preadipocytes are morphologically indistinguishable from their progenitor cell, but at this state they lose the potential to differentiate into other cell types, although the molecular mechanisms are not yet fully understood [38,137,139,140]. There is strong evidence that a network of signaling pathways targeting the promoters of lineage-specific transcription factors is involved in the determination process [139,141,142]. Terminal differentiation results in the development of functional and phenotypic mature adipocytes, characterized by insulin and hormone sensitivity, a unilocular lipid vacuole, tissue-specific ECM, and the ability to produce adipokines and to have secretory function [38,39,137,138] (Figure 1.2).

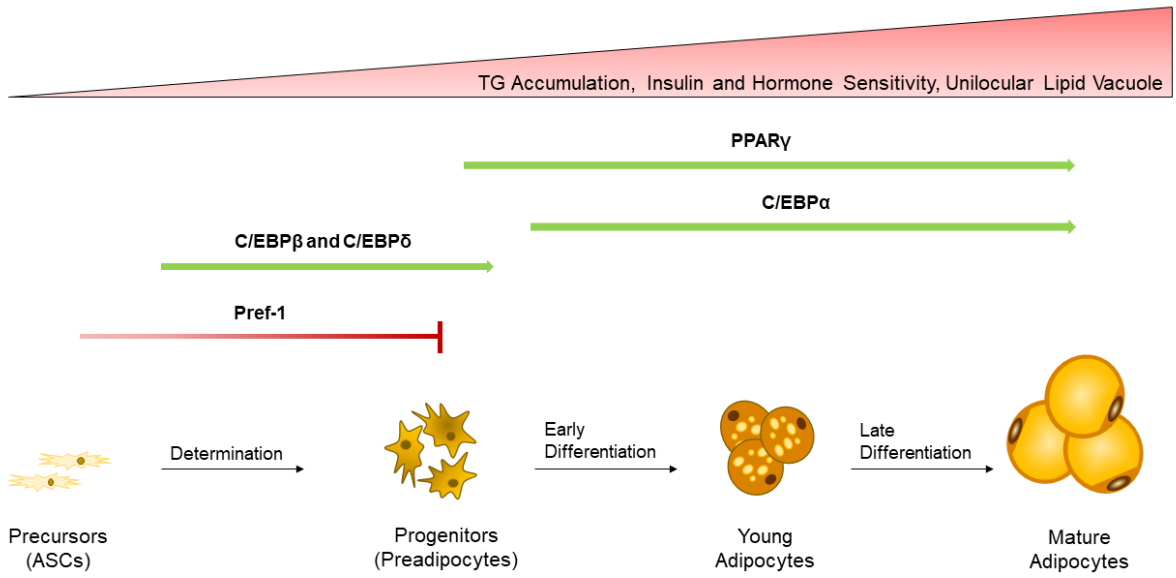

Figure 1.2: Schematic outline of adipogenic differentiation.
Adipogenic development from progenitor cells to lipid-laden mature adipocytes occurs in a two-phase process. During determination, ASCs are committed to preadipocytes and lose their potential to differentiate into other cell types. For further terminal differentiation into functional and phenotypic mature adipocytes, a sequential cascade of transcription factors is spatially and temporally activated. Abbreviations: TG: triglycerides; Pref-1: preadipocyte factor-1; C/EBPα/β/δ: CCAAT/enhancer binding proteins alpha/beta/delta; PPARγ: peroxisome proliferator-activated receptor gamma. Adapted in part from Bourgeois et al. 2019, and de Villiers et al. 2018 [143,144].

The differentiation is regulated by a number of transcription factors that coordinate the expression of hundreds of genes and has been examined intensively, mainly in mouse preadipocyte cell lines such as 3T3-L1, 3T3-F442A, C3H10T1/2, and NIH 3T3 [38,39,133,137]. In response to inductive stimuli, the transcriptional cascade of adipogenesis starts with the expression of CCAAT enhancer-binding protein (C/EBP) family members, including C/EBPβ and C/EBPδ, which are responsible for the subsequent expression of peroxisome proliferator-activated receptor gamma (PPARγ and C/EBP) and control the overall terminal differentiation process and regulation of adipogenesis [145–148]. After activation of PPARγ and C/EBPα, the expression of several adipocyte-specific genes, such as adipocyte protein 2 (aP2), glycerol-3-phosphate dehydrogenase (GPDH), leptin, adiponectin, lipoprotein lipase (LPL), and the glucose transporter GLUT-4 is promoted [39,133,135,142,149]. Apart from these central players involved in adipogenic differentiation, there are several other factors that promote adipogenesis, such as several Krueppel-like factors (KLFs), signal transducer and activator of transcription 5 (STAT5), sterol regulatory element-binding protein 1 (SREBP-1), whereas GATA binding protein 2/3 (GATA2/3), hairy and enhancer of split-1 (HES-1)

and T-cell factor/lymphoid enhancer factor (TCF/LEF), and KLF2 demonstrate inhibitory effects [150–153].

Adipogenic differentiation *in vivo* is triggered by progenitor cells receiving appropriate signals from their cellular environment. Instead, adipogenic differentiation *in vitro* depends on respective culture media containing specific induction factors to induce ASCs towards the adipogenic lineage [154]. The formulation of the culture media varies depending on the cell culture model, but the hormonal cocktail usually contains pharmacological inducers such as insulin or IGF-1, a glucocorticoid (e.g., dexamethasone), 3-isobutyl-1-methylxanthin (IBMX) and indomethacin [155,156]. After a one-week induction with media changes every three days, vacuoles filled with lipids form and the expression of adipogenic markers at mRNA level can be detected. After about two weeks of differentiation, the mature adipocytes express an adipocyte-specific ECM, which consists mainly of laminin , COL IV, and COL VI as components of the basal lamina [33,115].

Apart from the differentiation towards the adipogenic lineage, ASCs can also differentiate into other mesodermal (bone, cartilage, muscle types), ectodermal (neurons, endothelium, epidermis/skin), and endodermal (liver) lineages [118]. For chondrogenic differentiation, cells are cultivated in a pellet culture, which provides a 3D environment that increases cell-to-cell interaction and leads to the production of a cartilage-like matrix [157,158]. The differentiation medium should contain the chondrogenic inducing factors such as basic fibroblast growth factor (bFGF), dexamethasone, TGF-β1, insulin, ascorbate-2-phosphate (A2P) and bone morphogenetic protein (BMP-6) [159], leading to the secretion of extracellular matrix proteins of cartilage, including type II and VI collagen and aggrecan [160,161]. The osteogenic differentiation in the field of bone tissue regeneration can be induced in medium that typically contains dexamethasone, β-glycerol phosphate, TGF-β, vitamin, and amino acid [162–165]. During the differentiation along the osteogenic-lineage, several expressed genes are upregulated like BMP-2, BMP-4, COL I, osteoponin, alkaline phosphatase, and osteocalcin [149,166,167]. Apart from the above-mentioned lineages there are also studies reporting that ASCs cultured with media containing butylated acid, valproic acid and insulin, have the potential for ectodermal differentiation with morphological similarity to neurons and possess both neuronal associated (neuron-specific enolase, nestin, and NeuN) and glial lineage (S100, p75, nerve growth factor (NGF), receptorm, and NG2) markers [168]. Thereby, the applicability of ASCs in the regeneration of the central or peripheral nervous system following traumatic injury still needs to be much better investigated and characterized [169]. In addition, the differentiation of ASCs into cells of endodermal origin such as hepatocytes and beta-islet cells is induced *in vitro* by the differentiation factors activin-A, exendin-4, HGF as well as pentagastrin and leads to insulin-producing cells [170–173].

Besides the many advantages that ASCs provide, the differentiation of ASCs is more complex and challenging *in vivo* than *in vitro*. As the transplantation site is often characterized by a hypoxic or even ischemic microenvironment, which seems to be detrimental to cells, most injected or implanted cells die [174]. The great differentiation potential of ASCs is also exploited by the use of biomaterials combined with a variety of biodegradable 3D scaffolds *in vivo* that stimulate ASCs into the specific cell type for the desired clinical application via their chemical compositions and physical properties [175]. Moreover, the use of biomaterial scaffolds within clinical applications additionally supports attachment, proliferation, and differentiation of cells [176,177]. The multi-lineage differentiation potential of ASCs is not the only property that makes these cells particularly attractive and promising for therapeutic uses. Researchers assume that the regenerative effects of ASCs are caused by the secretion of various trophic factors, including cytokines and growth factors [178,179], rather than by direct differentiation into tissue-specific cell types, so that the paracrine properties of ASC are of great importance.

1.3.2 Secretory capacity of ASCs

ASCs are considered mediators of tissue regeneration, mainly through the secretion of specific soluble factors, which include cytokines, chemokines, growth factors and glycoproteins, as well as vesicular fractions containing exosomes and microvesicles (MVs) [73,180–184]. This secretome composition is capable of enhancing a variety of cellular mechanisms, particularly by promoting cell survival, cell proliferation, differentiation and migration, vascularization, and immunoregulation [158] and by the recruitment of endogenous stem cells, which reside in the adult tissue or organs [185,186] (Figure 1.3). The reported advantage of ASCs over BM-MSCs is the increased secretion of growth factors and bioactive factors such as NGF, IL-6 and IL-8, HGF, monocyte chemotactic protein 1 (MCP-1), granulocyte macrophage factor colony stimulator (GM-CSF) and others [180], leading to better differentiation, migration, proliferation, and autocrine activity, highlighting their promising regenerative capacity [187–189].

The therapeutic outcomes in tissue regeneration and repair are improved by the variety of secreted proteins with their different mechanisms of action [190]. In terms of angiogenesis and revascularization, the release of several angiogenic factors and growth factors can contribute to the induction and promotion of angiogenesis and to the support of existing vascular structures [108,191]. The pro-angiogenic factors in the ASC secretome mainly includes VEGF, HGF, TGF-β2, FGF-2, and GM-CSF, placental growth factor (PGF) and Ang-1 and Ang-2 [178,192–195]. VEGF, a mitogen-activated protein and one of the key players within vascularization also has a crucial role in relation to hypoxia [196]. Due to an initial lack of blood supply after transplantation leading to a hypoxic or ischemic state, transplanted tissues or cells

tend to undergo apoptosis [197,198]. The upregulation of VEGF under hypoxia with the resulting significant secretion levels leads to protective effects against ischemia [191,199–201]. Moreover, VEGF and HGF are also reported to greatly influence cell viability, proliferation, and migration, with HGF particularly stimulating EC proliferation and migration [195,202,203].

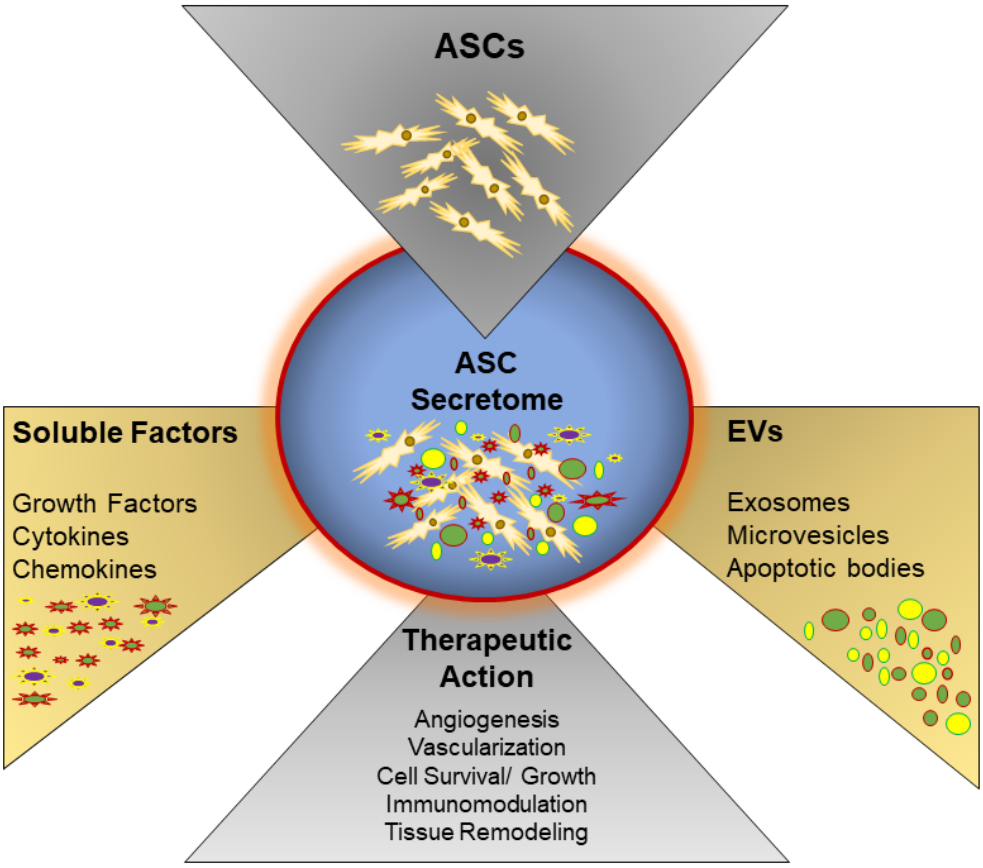

Figure 1.3: Composition and the main therapeutic effects of ASC secretome.
The ASC secretome is composed of soluble factors such as growth factors, cytokines, chemokines, and the EVs including exosomes, microvesicles and apoptotic bodies. The potential therapeutic mechanisms that can be harnessed through the use of the ASC secretome are diverse and include, in particular, angiogenesis, enhanced vascularization, cell proliferation, differentiation and migration, as well as anti-inflammatory and immunomodulatory actions. Abbreviation: EVs: Extracellular vesicles.

In tissue regeneration and repair, the regulation of inflammatory processes plays a crucial role, particularly in the control of local and systemic inflammatory responses [204]. The highly immunomodulatory properties of ASC, besides the reduction of dendritic cell surface markers (CD80, CD83, CD86), are mainly attributed to the secreted amounts of anti-inflammatory mediators such as IL-1 receptor antagonist (IL-1Ra), IL-4, IL-6, IL-10, IL-13 as well as TGF-β1, which attract immune cells [205–207]. Delarosa et al. [208] also report interferon gamma (IFN-γ), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO) and nitric oxide

as important effector molecules secreted by ASCs. The secretion of IL-6 and IL-8, which can act as chemo-attractants, support the recruitment of monocytes and macrophages to sites of tissue repair [209–211]. However, it is also reported that IL-6, likewise IL-2, can also have pro-inflammatory properties under different states [212]. A study by Ma et al. [213] examined the influence of the ASC secretome on macrophages. They pointed out decreased expression of pro-inflammatory cytokines, including IL-6, TNF-α and MCP-1, due to a reduction in pro-inflammatory M1 macrophages and an increase in anti-inflammatory M2 macrophages. Moreover, the ASC seretome can exert anti-inflammatory effects by reducing the proliferation of peripheral blood mononuclear cells, inhibiting NK cells, and suppressing the proliferation of cytotoxic T cells while increasing the number of regulatory T cells [214,215]. Inflammation is also in relation with wound healing. Thus, the paracrine capacity of ASCs also plays a crucial role in the therapeutic treatment of wound healing processes [216,217]. Ebrahimian et al. [218] have shown in their animal model that besides VEGF, keratinocyte growth factor (KGF) also has the potential to enhance the proliferation phase of the healing process and that these are key factors for the proliferation and migration of keratinocytes. Moreover, IL-6 and IL-8 are equally important for wound healing processes as they also trigger keratinocyte migration, fibroblast migration, leukocyte infiltration and collagen synthesis [219–221]. In addition, TGF-β1 and HGF are also considered to support the regenerative potential in wound healing [222]. However, studies have equally shown that the secretion of these factors promotes neurogenic responses, while tissue inhibitor of metalloproteinase-1 (TIMP-1) and progranulin (PGRN) have neuroprotective potential [223]. The effects of the secretome of ASCs in regulating breast cancer is highly controversial. O'Halloran et al. [224] have highlighted that secreted adipokines such as TNF-α, IL-6, IL-8 and MCP-1 are responsible for promoting tumor growth. Another study by de Miranda et al. [225] has also demonstrated that the ASC secretome increases tumor-initiating cells and the migration capacity of MCF-7 and MDA-MB-231 cells. Contrary to these studies, it has been shown that tumor growth in MCF-7 breast cancer cells can be suppressed via STAT1 activation resulting from IFN-β secretion [226]. Although the function of the secretome on breast cancer cells shows very different results, it is assumed that the negative effect of the secretome only affects active breast cancer cells [227]. To clarify the effects of ASCs and its secretome on remaining breast cancer cells in the context of breast reconstruction after tumor resection, further studies would be needed to specifically assess the resulting effects.

Besides the secretion of growth factors and cytokines of the soluble fraction, extracellular vesicles (EVs), including the subtypes exosomes, MVs, and apoptotic bodies, also promote cell survival, angiogenesis, and immunomodulation based on their release pathways, content, and function [228–230]. EVs, which consist of lipids, nucleic acids (e.g., mRNA, rRNA, miRNA) and small proteins, are able to modulate their actions through membrane fusion by interacting

directly with cell membranes or releasing their bioactive cargo into the cell cytoplasm [231,232]. Exosomes, defined as nano-scaled vesicles between 30-150 nm in diameter [233,234], have gained increased popularity for therapeutic uses due to their specific regenerative properties and their stability in the human body [182]. Researchers identified several proteins in ASCs-derived exosomes and demonstrated their role within metabolic and cellular processes as well as the link to proliferation and regeneration pathways [235,236]. Li et al. [237] demonstrated in their model the improved wound healing by ASCs exosomes. In addition to exosomes, Kang et al. [238] found that MVs released by ASCs positively influence migration and vascular formation of ECs. In addition, Togliatto et al. [239] demonstrated the potential of MVs regarding proangiogenesis within diabetic states by activating the extracellular signal-regulated protein kinase 1/2/mitogen-activated protein kinase (Erk1/2/MAPK) pathway. The ASC secretome represents a promising tool in the field of regenerative medicine due to the possible interaction between different molecules present in the secretome. However, whether the paracrine actions are mainly attributed to the secreted cytokines and growth factors or to the EVs remains under investigation and is of great relevance to find appropriate strategies for clinical settings. ASCs have shown that they can alter their secretome properties and composition depending on their microenvironment or the disease status [179,239,240]. For instance, modulation of ASCs paracrine activity by oxygen deprivation is well documented with an increased production of chemokines (e.g., IL-8, MCP-1), angiogenic mediators (ANG, VEGF) and an increase in inflammatory modulators (IL-6, IL-1Ra) [241–243]. Knowledge about the paracrine capacity of ASCs in nutrient deprivation, which have been reported in the context of ischemic diseases or at transplantation sites, where cell-loaded constructs are initially not adequately supplied with nutrients [244,245], is still pending and is therefore the main topic of this work.

ASCs and their secretome represent a valuable tool in regenerative medicine due to the composition of numerous factors that mediate beneficial contributions to tissue repair, regeneration, and immunomodulation [246]. Moreover, ASCs respond to various stimuli from their dynamic environment by increasing their expression of trophic factors. Over the last years, there has been a growing interest on strategies that stimulate ASC paracrine secretion [179]. Various strategies including genetic, pharmacological, physiological, physical, and cytokine and preconditioning are used to trigger the expression of the desired multiple trophic factors and vesicles [178,179,247]. Nevertheless, further research is needed to understand the mechanisms and components underlying the beneficial secretory effects of ASC under adverse *in vivo* conditions, such as at transplant sites or in diseased tissue, in order to potentially improve their efficacy in clinical settings.

1.4 Clinical application of ASCs

The ability of ASCs to differentiate into multiple lineages and their secretion of various trophic factors make them an attractive tool for clinical stem cell therapies. Over the last years, there has been a significant increase in studies and preclinical studies on the effectiveness of ASCs in tissue reconstruction and regeneration. Their clinical application has also been investigated in numerous clinical trials, but only a small number of phase III trials are reported today [169,192,248]. The use of ASCs is multifaceted and is applied in a wide variety of therapies [249]. ASCs have been shown to be useful for the treatment in wound healing [250]. Through their secretion of growth factors and cytokines such as FGF, EGF (epidermal growth factor), TGF-β, VEGF, HGF, SDF-1 (stromal cell-derived factor 1), they are able to activate immune cells, fibroblasts and MSCs, promote cell proliferation, support skin remodeling, and contribute to improved vascularization [218,251–253]. In a study by Rigotti et al. [254], the use of ASCs demonstrated improved ultrastructural tissue characteristics with new vessel formation in a group of patients who had received radiotherapy with resulting severe radiation wounds. ASCs have also been shown to be effective in treatment of wounds that are complicated by ischemia such as occurring in diabetes as well as by severe symptoms such as atrophy, fibrosis, and retraction [255–257]. Moreover, the treatment with ASCs in pathologic wounds as for instance with aberrant scar formation is also well reported. Here, the injection of ASCs in animal models showed a reduced area of wound with improved colour and pliability [258–260]. Due to the immunosuppressive and anti-inflammatory effects of ASCs and their secretome they can counteract inflammatory processes associated with scar formation [261,262]. The reported immunomodulatory effects of ASCs also play a crucial role with regard to immune tolerance for transplant patients, where ASCs suppress inflammatory and stimulate anti-inflammatory cytokines and are able to induce antigen-specific regulatory T cells [263]. These properties make ASCs important regulators of immune tolerance and promising candidates for immunologic disorders [264–266].

Additionally, the use of ACSs has shown promising results in bone tissue repair. The ability of ASCs to differentiate into osteoblasts and chondrocytes is well known and therefore makes them attractive for bone tissue reconstruction [267,268]. Lendeckel et al. [269] reported for the first time on new bone formation with ASCs three months after reconstruction in a patient with extensive calvarial defects. Other studies also reported the formation of new bone as well as the healing of defects in the maxilla and mandible using ASCs [270,271]. Promising results were further shown in the treatment of cartilage defects, but so far only in animal studies where ASCs fully repaired hyaline cartilage defects [272,273]. Even in the treatment of osteoarthritis, a disease that affects millions of people worldwide, the use of ASCs could improve pain and mobilization. In addition, imaging techniques showed bone matrix formation at the site of osteonecrosis and an increase in the thickness and height of the meniscal cartilage [274].

However, there is no distinction whether these promising results can be attributed to the direct differentiation of the ASCs into target cells or to their secretory capacity. In the field of tissue engineering, the use of ASCs seeded onto natural or synthetic biomaterial scaffolds is used to support cell attachment, proliferation, migration, interaction, and differentiation [177,275], whereby the material is degraded or absorbed into the patient so that the newly formed tissue restores the defective site [276,277]. This application is used in reconstructive procedures such as tissue regeneration after tumor removal, in burn treatment and cleft lip/palate repair [23,278]. Besides, ASC therapy is also promising in neural related diseases, like Alzheimer disease, Parkinson disease, multiple system atrophy and further neurodegenerative diseases [279–282]. In the treatment of Parkinson, in a mouse model it could be demonstrated that the injection of ASCs recovered the mitochondrial functions, improved motor abilities, and had neuroprotective effects [282]. Again, it should be mentioned that most of the studies were conducted in animal models [283]. However, in humans, is has been shown, that the application of ASCs had a positive influence on cell viability and cell proliferation of nucleus pulposus cells in the intervertebral disc [284,285]. ASCs also showed angiogenic effects through their secretion of angiogenic factors in the treatment of cardiovascular diseases and diseases associated with ischemia, such as ischemic stroke, ischemic heart disease or critical limb ischemia [286–288].

In recent years, the clinical use of ASCs has also increased in the field of plastic and aesthetic surgery, in particular, through the use of cell-assisted lipotransfer (CAL) technology. ASCs are an attractive, readily available cell source, especially in Korea and Japan, for aesthetic treatments such as breast augmentation, augmentation in facelift and facial contouring surgeries, or for facial lipoatrophy, where ASCs have shown promising clinical results due to their regenerative potential [289].

1.5 ASCs in cell-assisted lipotransfer (CAL)

For several decades, the importance and use of stem cell therapies has been gaining more and more attention. The therapeutic procedures thereby aim to exploit the properties of the stem cells. The clinical application of ASCs in the field of regenerative medicine is very multifaceted, as described above, and more and more research is focusing on the use of ASCs. ASCs have been shown to be particularly ideal candidates for the treatment in conjunction with the transplantation of AT using CAL.

In plastic surgery, autologous fat transfer or lipofilling is used for soft-tissue correction, reconstruction, and augmentation and has become a well-established method for cosmetic and reconstructive indications [290–292]. Thereby, the adipose tissue is removed by liposuction or excision, followed by purification steps, and immediately reimplanted into the

same patient [293]. Autologous fat is an ideal and promising filler because it is host-compatible, non-toxic, easy and repetitive to harvest and indicates a low complication rate [294–297]. The main indications for the use of autologous fat grafting are the correction of tissue defects after cancer removal or radiotherapy, soft tissue augmentations (mainly breast augmentations), facial rejuvenation through filling and correcting post-traumatic defects [298–301]. However, autologous fat grafting is associated with several complications that significantly reduce the efficacy of this technique. These include fat necrosis, calcification, the formation of oil cysts, and above all, fat resorption with a long-term volume loss ranging from 25 to 80% [302–308]. To overcome these limitations of this technique, researchers have sought to increase the survival rate and persistency of the transplanted fat by a concurrent transplantation of fat with ASCs, called CAL. In 2006, Matsumoto et al. [309] introduced this novel fat grafting technology in animal models, whereby the lipoaspirate was enriched with the SVF, which contains mainly ASCs in large quantities, in addition to other cells such as endothelial cells and stromal cells. The application of ASCs in CAL has yielded findings suggesting a supportive effect in autologous lipotransfer, improving distinctly the outcome of transplantation through various mechanisms. First, ASCs could differentiate into mature adipocytes and thus serve to maintain volume [310], or differentiate into vascular endothelial-like cells, which could promote neoangiogenesis and vascularization of the graft and simultaneously prevent fat necrosis [68,191,311,312]. Second, ASCs can be stimulated by the ischemic microenvironment prevalent at transplantation site, and thus release a wide variety of paracrine factors that support the surrounding tissue and promote vascularization through secreted factors such as VEGF, HGF, and SDF-1, providing major benefits over conventional autologous lipotransfer [191,202,313–315]. In 2008, the CAL technique was first applied in humans by Yoshimura et al. [316] for cosmetic breast augmentation to boost the efficacy of autologous lipoinjection. Since this first use of CAL in humans, several studies have evaluated the efficacy of the CAL technique in comparison to conventional autologous fat grafting, with divergent results depending on the indication [317–322]. For the realization of CAL, a portion of a volume of fat removed by conventional liposuction or extraction is used, washed (phosphate-buffered saline) and digested using a mixture of proteolytic enzymes. After filtration, the liquid SVF fraction is obtained and washed again several times. This process takes about 90 minutes in total. The remaining AT and the SVF obtained are mixed gently for about 10 to 15 minutes and filled into the injection syringe [323], the injection being performed mainly according to Coleman's technique [294]. The SVF isolation procedure can be done manually or using automatic commercial systems that enable the rapid, automated generation of a cell-enriched lipoaspirate [324,325]. The outcomes after the application of different SVF isolation techniques are contradictory. While Gentile et al. [326] found that the optimal SVF extraction method was the automatic one, Doi et al. [327] showed no differences in terms of

resulting cell number and cell viability. In order to better compare the results of clinical trials, a standardized method of SVF isolation with a constant percentage of cells in the graft is therefore required. However, the number of cells needed for the best augmenting effect of CAL is not known, but the reported dose for fat enhancement ranges from 5 x 10^5 to 2 x 10^7 cells/ml lipograft [318]. ASCs can be obtained clinically from liposuction aspirates in sufficient numbers without the need for cell expansion in culture [309]. The advantage is that the harvesting and injection can be done in a single surgical session, which is time and cost efficient and saves the patient an additional surgical procedure with higher risks. In addition to the use of SVF for cell enrichment, there are also studies that have investigated the efficacy of CAL with cultured ASCs. Kølle et al. [328] have conducted a blinded, randomized clinical trial comparing the use of autologous ASCs in CAL with conventional lipotransfer. This study included 10 patients with a bolus injection of fat into the upper arm with a fat graft enriched with 2.2 x 10^7 ASC/ml fat on one side compared to the other side with the fat graft alone. After 121 days, the grafts were explanted and analyzed histologically. The fat survival rate was 80.9% (76.6-85.2) in ASC-enriched grafts compared to control grafts with 16.3% (11.1-21.4) of the initial volume, with higher quality of the remaining graft in CAL. Similarly, Koh et al. [329] demonstrated reduced resorption in Parry-Romberg disease therapy, resulting in improved survival of ASC-enriched grafts compared to microfat-only grafts. A study by Moseley et al. [312] aimed to highlight the comparison between the use of ASCs and SVF, with the SVF group showing a 2.5-fold improvement in survival of transplanted fat in a mouse model compared to the control group, while cultured ASCs showed a trend towards improvement without statistical significance. However, due to the limited data available on studies up to now, no conclusion can be reached as to whether the fresh cell population, the SVF, or cultured ASCs result in better clinical outcomes. Interestingly, there are also studies reporting oncological risk associated with CAL, both in human studies showing an increase in breast cancer [330,331] and in *in vitro* cultures or animal studies indicating promotion of ASCs in breast tumor growth and invasion [332–334].

Overall, the promising effect of ASCs in CAL has been demonstrated in a variety of clinical settings. However, further randomized controlled clinical trials are needed to clarify the pivotal role and mechanisms of action of ASCs in CAL and also to assess the impact on long-term oncological safety. It has been widely discussed that the positive effects of ASCs are mainly related to their secretory capacity. For this reason, it is of great importance to understand the conditions that prevail in the setting of CAL *in vivo* as best as possible in order to find out which specific stimuli promotes the secretory activity of ASCs in this context. Therefore, in basic and translational science, 3D culture *in vitro*-models are becoming increasingly interesting to mimic the 3D environment of the native adipose tissue and the complexity of the interactions between

AT and ASCs. Therefore, studies with a physiologically relevant model of AT *in vitro* would be an ideal tool for more detailed studies on the function of ASCs in CAL.

1.6 Goals of the thesis

The potential use of human ASCs has been reported in a variety of clinical approaches, especially for repair and regeneration of various tissues and organs. Their regenerative properties from self-renewal and multipotential differentiation are not the only characteristics that make these cells attractive for therapeutic purposes. The therapeutic effects are mainly attributed to the trophic actions of their secreted growth factors, cytokines, chemokines, extracellular MVs and exosomes, that contribute to promotion of angiogenesis, reduction of inflammation, and functional recovery [178,335–339]. The use of ASCs in cell-based approaches, such as CAL, has been shown to promote higher survival rates and persistence of the transplanted cell-loaden construct [316,340]. However, the survival rate of the ASCs upon transplantation is very low due to the ischemic conditions to which cells are initially exposed. As the transplantation site is characterized by an initial lack of blood supply [244,245], cells undergo severe oxidative and nutritional stress, which is considered to be detrimental to cells [341]. The response of ASCs to the individual factors of ischemia remains poorly understood. In order to optimally exploit their use in cell-based approaches and tissue regeneration, a comprehensive characterization of ASCs in terms of ischemic conditions is necessary. This includes a broad knowledge of their viability, differentiation capacity and, in particular, of their secretory capabilities. In regenerative approaches, angiogenesis and wound healing are key processes whereby secreted growth factors and cytokines of ASCs can contribute to beneficial effects [342,343]. Thus, the underlying effect of the secretome of ischemia-challenged ASCs on ECs and fibroblasts also remain to be elucidated. The function of added ASCs in CAL could be examined in more detail apart from cell cultures on 2D surfaces by using a 3D culture that mimics functions and responses of cells and tissue in a more physiological manner. Thus, the establishment of a 3D culture to study ASC function in CAL would be of great relevance. Therefore, this thesis includes the following objectives to get a better understanding of the mechanisms responsible for the beneficial role of ASCs under ischemia and to further improve their therapeutic efficacy in CAL and in regenerative approaches.

1. The characterization of ASCs under ischemia-like stress conditions and the comprehensive analysis of their secretory properties and regenerative effects in terms of ischemia.

2. The evaluation of the effect of stanniocalcin-1 (STC-1) on ASC secretion and endothelial cell tube formation by the addition of exogenous recombinant human STC-1 (rhSTC-1) and by specific inhibition of STC-1.

3. The development of a 3D adipose tissue-ASC co-culture *in vitro* and the investigation on the secretory capacity of ASCs in this set-up.

1.6.1 Characterization of ischemia-challenged ASCs and analysis of secretory properties and regenerative effects

Despite the multitude of regenerative properties of ASCs, a low survival of ASCs upon transplantation has been documented [197,198]. This may be due to an ischemic environment to which the cells are exposed due to the initial lack of blood supply and the associated deprivation of nutrients, oxygen, and growth factors [344,345]. In order to understand and to further improve their therapeutic efficacy in regenerative medicine, a comprehensive characterization of ASCs under ischemia-like stress conditions is essential. The secretory response of ASCs to ischemic stress and the different components, however, has been rarely investigated so far. Therefore, first in this study, two different ischemic conditions, moderate and harsh, were examined with regard to the viability and secretory response of ASCs (see Chapter 4.1). Since studies regarding the modulation of the paracrine activity of ASCs have so far only referred to low oxygen concentrations, in a next step, investigations focused on the effect of glucose deprivation in conjunction with severe hypoxia were conducted. Therefore, a cytokine antibody array for screening of markedly expressed anti-apoptotic, angiogenic and immunomodulatory factors in glucose/oxygen-deprived ASCs was used to further investigate secretion profiles. The assumption that regenerative effects are mainly attributed to the secreted factors of ischemia-challenged ASCs was further evaluated by investigating the effect of the secretome of ischemia-challenged ASCs on endothelial cell viability, metabolic activity, and tube formation as well as on fibroblast migration (see Chapter 4.2).

1.6.2 Effect of stanniocalcin-1 (STC-1) on endothelial cell tube formation and on the secretion of vascular endothelial growth factor (VEGF) by ASCs

The beneficial effects of ASCs are mainly attributed to the large number of various secreted growth factors and cytokines [134,346]. Some of these trophic factors play an important role, for example in promoting neo-vascularization [197,347]. For STC-1, one major

anti-apoptotic factor [348,349] involved in the response to oxidative stress, survival, proliferation and especially in the control of angiogenesis [350,351], a significant expression in ischemia-challenged ASCs was observed (see Chapter 4.2). Therefore, the possible effect by STC-1 on endothelial cell tube formation and on the secretion of VEGF by ASCs was examined further. Different exogeneous rhSTC-1 concentrations were used and the VEGF expression downstream of STC-1 was investigated. Furthermore, a STC-1-targeted blocking approach, by applying different antibody concentrations, was conducted to examine the possible influence of this newly detected factor in ASCs on EC tube formation (see Chapter 4.3).

1.6.3 Development of an *in vitro* 3D autologous lipograft-ASC co-culture system

The use of ASCs in CAL is of great interest due to their high abundance, easy harvesting [106,352], and especially their regenerative properties, due to the variety of secreted factors that promote higher survival rates and persistence of the transplanted cell-laden construct [328,340]. In order to gain deeper insights into the functional role and effects of added ASCs on lipografts, an adipose tissue *in vitro* model, mimicking the *in vivo* adipose environment and reflecting the complexity of the native tissue, would be highly desirable. Therefore, in this study, a 3D, physiologically relevant, long-term volume stable *in vitro* lipograft-ASC co-culture system was established and evaluated with regard to its usability for studying ASC function in CAL and tissue regeneration. For this purpose, lipografts and ASCs with a clinically relevant concentration were co-cultured in a transwell-based culture system under ischemia-mimicking conditions. After establishing the co-culture of lipografts and ASCs to closely mimic *in vivo* cellular environments, the secretory response of ASCs within this co-culture was examined by measurement of the secreted amounts of selected angiogenic, anti-apoptotic, and immunomodulatory factors by ELISA (see Chapter 4.4). This served as a first approach to investigate the function of ASCs in CAL in a physiologically relevant manner and should be the basis for further experiments to get closer to an identification of factors that are able to foster the long-term stability of lipografts *in vivo.*

2 Materials

2.1 Instruments

Table 2.1: Overview of instruments.

Instrument	Supplier	Central office
Analytical balance	Ohaus	Zürich, Switzerland
Centrifuge Rotina 420 R	Hettich	Tuttlingen, Germany
Centrifuge SIGMA 1-14	SIGMA Laborzentrifugen GmbH	Osterode, Germany
ChemiDoc™ Touch	Bio-Rad	Munich, Germany
COBAS INTEGRA 800	Roche	Basel, Swiss
CO_2 incubator	IBS Integra Biosciences	Fernwald, Germany
CO_2 incubator (hypoxia)	Binder GmbH	Tuttlingen, Germany
CytoFlex	Beckman Coulter GmbH	Brea, USA
Fluorescence spectrometer Tecan Infinite M200	Tecan	Crailsheim, Germany
Freezers	Liebherr	Biberach, Germany
Fridges	Liebherr	Biberach, Germany
Haemacytometer Neubauer	Paul Marienfeld GmbH	Lauda, Germany
Laminar flow box Typ-HS18	Heraeus	Hanau, Germany
Orbital shaker Unimax 1010	Heidolph	Schwabach, Germany
Mastercycler® Gradient	Eppendorf	Hamburg, Germany
Magnetic stirrer	VWR	Darmstadt, Germany
Microscope BX51/ DP71 camera	Olympus	Hamburg, Germany
Microscope IX51/ XC30 camera	Olympus	Hamburg, Germany
Microtome Histoslide 2000 R	Leica Biosystems	Wetzlar, Germany
NanoDrop 2000c	Thermo Scientific	Waltham, USA
pH-meter HI2210	Hanna Instruments	Kehl am Rhein, Germany
Pipettes Research® Plus	Eppendorf	Hamburg, Germany
Pipette controller accu-jet® pro	Brand	Wertheim, Germany
Pipette repetitive	Brand	Wertheim, Germany

Instrument	Supplier	Central office
proOx-C chamber system C-274	BioSpherix	Parish, USA
Real-Time PCR Detection System CFX96T™	Bio-Rad	Munich, Germany
Thermomixer comfort MTP	Eppendorf	Hamburg, Germany
Ultrasonic homogenizer SonoPlus	Bandelin	Berlin, Germany
Vortexer, IKAR MS3 basic	IKAR	Staufen, Germany
Waterbath	Memmert	Schwabach, Germany

2.2 Consumables

Table 2.2: Overview of used consumables.

Consumable	Supplier	Central office
Bottle top-filter Nalgene®	Thermo Scientific	Waltham, USA
Cannulas sterican	B. Braun	Melsungen, Germany
Coverslip 24 x 60 mm	Menzel	Braunschweig, Germany
Cultur-Insert 2 well	Ibidi GmbH	Gräfelfing, Germany
Combitips® Plus 2.5 mL	Eppendorf	Hamburg, Germany
Disposable forceps ratiomed®	Megro GmbH	Wesel, Germany
Falcon cell strainers 100 µm	BD Biosciences	Heidelberg, Germany
Glass pasteur pipettes	Brand	Wertheim, Germany
Hardshell PCR plates, 96-well, thin wall	Bio-Rad	Munich, Germany
Microseal® ´C´ Film	Bio-Rad	Munich, Germany
Microtome blades	Feather	Osaka, Japan
6, 12, 24 & 96- well plates	Greiner Bio-One	Frickenhausen, Germany
24-well TC-plates	Sarstedt	Nümbrecht, Germany
24-well TC-inserts	Sarstedt	Nümbrecht, Germany
96-well plates black	Thermo Scientific	Waltham, USA
Parafilm®	Pechiney	Chicago, USA

Consumable	Supplier	Central office
PAP pen liquid blocker	Sigma-Aldrich	Munich, Germany
PCR-strips 8-tubes 0.2 mL	Carl Roth GmbH	Karlsruhe, Germany
Pipette filter tips	Sarstedt	Nümbrecht, Germany
Pipette tips	Starlab	Hamburg, Germany
Pipettes serological	Greiner Bio-One	Frickenhausen, Germany
Polypropylene Tubes 15 mL/50 mL	Greiner Bio-One	Frickenhausen, Germany
Reagent Reservoirs	VWR	Darmstadt, Germany
SafeSeal micro tubes 1.5 mL/2.0 mL	Sarstedt	Nümbrecht, Germany
Sample cup PE 2.5 mL	Hartenstein	Würzburg, Germany
Scalpels	Feather	Osaka, Japan
Scissors surgical	B. Braun	Melsungen, Germany
Syringe Filter Minisart® 0.2 µm	Sartorius AG	Göttingen, Germany
Tissue culture flasks T25 / T75 / T175	Greiner Bio-One	Frickenhausen, Germany
µ-Slide Angiogenesis	Ibidi GmbH	Gräfelfing, Germany

2.3 Chemicals

If not otherwise stated in Table 2.3 or the Methods section (Chapter 3), all chemicals and reagents utilized for the preparation of buffers and solutions were obtained from Sigma-Aldrich (Munich, Germany) and Carl Roth GmbH (Karlsruhe, Germany).

Table 2.3: Overview of used chemicals.

Chemicals	Supplier	Central office
Accutase	Sigma-Aldrich	Munich, Germany
Antibody diluent, Dako REAL™	Dako	Hamburg, Germany
Aqua ad iniectabilia	B. Braun	Melsungen, Germany
β-Estradiol	Sigma-Aldrich	Munich, Germany
bFGF (basic fibroblast growth factor)	BioLegend	London, UK
BODIPY 493/503	Invitrogen	Karlsruhe, Germany

Chemicals	Supplier	Central office
COBAS INTEGRA reagents	Roche	Basel, Swiss
Collagenase NB4	Serva Electrophoresis	Heidelberg, Germany
Corning® Matrigel® Basement Membrane Matrix, Growth Factor Reduced,Phenol Red Free	BD Biosciences	Heidelberg, Germany
CytoFLEX Daily QC Fluorospheres	Beckman Coulter GmbH	Brea, USA
CytoFLEX Sheath Fluid	Beckman Coulter GmbH	Brea, USA
D-(+) Glucose solution	Sigma-Aldrich	Munich, Germany
DAPI mounting medium Immunoselect®	Dako	Hamburg, Germany
Dulbecco´s phosphate-buffered saline (DPBS), no calcium, no magnesium	Thermo Scientific	Karlsruhe, Germany
Dexamethasone	Sigma-Aldrich	Munich, Germany
Distilled water (DNase/RNase free)	Life Technologies	Karlsruhe, Germany
Hoechst 33258 dye	Polysciences	Warrington, USA
Human insulin	PromoCell	Heidelberg, Germany
IBMX (3-Isobutyl-1-methylxanthine)	Serva Electrophoresis	Heidelberg, Germany
Indomethacin	Sigma-Aldrich	Munich, Germany
MESA GREEN qPCR™ Mastermix Plus for SYBR® Assay	Eurogentec	Cologne, Germany
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma-Aldrich	Munich, Germany
Phalloidin-iFluor 488 Reagent - CytoPainter	Abcam	Cambridge, UK
Phenol Red	Sigma-Aldrich	Munich, Germany
Phosphate-buffered saline (Dulbecco A) tablets	Thermo Scientific	Waltham, USA
Proteinase K (Digest-All 4)	Life Technologies	Karlsruhe, Germany
Recombinant human STC-1	R&D Systems	Minneapolis, USA
Resazurin sodium salt	Sigma-Aldrich	Munich, Germany
Terralin Liquid® disinfectant	Schülke	Norderstedt, Germany
Thesit®	Gepepharm	Hennef, Germany

Chemicals	Supplier	Central office
TRIzol® reagent	Life Technologies	Karlsruhe, Germany
Trypsin-EDTA 0.25%	Thermo Scientific	Karlsruhe, Germany

2.4 Antibodies

Table 2.4: Overview of used antibodies.

Antibody	Type/Source	Application/Dilution	Supplier
Anti-HIF-1α	Monoclonal IgG mouse	IHC: 1:200	Abcam (ab16006)
Anti-CD31	Monoclonal IgG mouse	IHC: 1:200	Dako Clone JC70A (M0823)
Anti-Perilipin 1	Polyclonal IgG rabbit	IHC: 1:200	Thermo Scientific (PA5-72921)
Anti-STC-1	Polyclonal IgG goat	Blocking: 5 µg/mL 20 µg/mL	R&D Systems (AF2958)
IgG 1 isotype control	Polyclonal IgG goat	Blocking: 5 µg/mL 20 µg/mL	R&D Systems (AB-108-C)
IgG1 negative control	Polyclonal IgG mouse	IHC: according to primary antibody concentration	Dako (X0931)
IgG1 isotype control	Polyclonal IgG rabbit	IHC: according to primary antibody concentration	Dianova Clone pAK (DLN-13121)
Cy3-conjugated donkey anti-mouse secondary antibody	Polyclonal IgG donkey	IHC: 1:400	Dianova (715-165-150)
Cy3-conjugated goat anti-rabbit secondary antibody	Polyclonal IgG goat	IHC: 1:400	Dianova (111-165-003)

2.5 Primers

Table 2.5: Overview of used primers.

Gene	Gene Symbol	Unige Assay ID/ Sequence	Supplier
EF1α	*EF1A1*	F: 5´-CCCCGACACAGTAGCATTTG-3´ R: 5´-TGACTTTCCATCCCTTGAACC-3´	Biomers
IL-6	*IL6*	qHsaCID0020314	Bio-Rad
STC-1	*STC1*	qHsaCID0006115	Bio-Rad
VEGF	*VEGFA*	qHsaCED0043454	Bio-Rad

2.6 Cells

Table 2.6: Overview of used cells.

Cell Type	Source	Ref./Lot Number	Supplier
Adipose-derived stem cells	Human abdominal adipose tissue (female)	PT5006 Lot 0000560032	Lonza, Walkersville, USA
Human umbilical vein endothelial cells	Human umbilical vein	C-12200 Lot 445Z011	PromoCell, Heidelberg, Germany
NIH/3T3 Fibroblasts	Mouse, *mus musculus*, embryo	CRL-1658™	ATCC, Manassas, USA
MCF7 Breast cancer cells	Human breast, mammary gland	HTB-22™	ATCC, Manassas, USA
MDA-MB-231 Breast cancer cells	Human breast, mammary gland	HTB-26™	ATCC, Manassas, USA

2.7 Cell culture media

Table 2.7: ASC medium.

Medium	Composition
ASC basal medium	Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12, Thermo Scientific, Waltham, MA, USA) supplemented with 1% penicillin/streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin), 10% fetal bovine serum (FBS; Thermo Scientific), and 3 ng/mL basic fibroblast growth factor (bFGF; BioLegend, London, UK).

Medium	Composition
ASC growth medium	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 (both from Thermo Scientific).
ASC differentiation medium	Growth medium with 1.7 µM insulin (PromoCell, Heidelberg, Germany), 1 µM dexamethasone, 200 µM indomethacin (both from Sigma-Aldrich, Munich, Germany) and 500 µM 3-isobutyl-1-methylxanthin (IBMX; Serva Electrophoresis, Heidelberg, Germany).
ASC cryopreservation medium	Basal medium, supplemented with 5% Dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Table 2.8: HUVEC medium.

Medium	Composition
HUVEC growth medium	Endothelial Cell Growth Medium 2 (449M460) with SupplementMix (449M236, PromoCell, Heidelberg, Germany), supplemented with 1% penicillin/streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin; Thermo Scientific).
HUVEC cryopreservation medium	Cryo-SFM (PromoCell).

Table 2.9: NIH/3T3 fibroblast medium.

Medium	Composition
NIH/3T3 growth medium	Dulbecco´s Modified Eagle´s Medium (4.5 g/L D-glucose, 4 mM L-glutamine, 1 mM sodium pyruvate; Thermo Scientific), supplemented with 1% penicillin/streptomycin (Thermo Scientific), and 10% bovine calf serum (BCS; Sigma-Aldrich).
NIH/3T3 cryopreservation medium	Growth medium, supplemented with 5% Dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Table 2.10: MCF7 and MDA-MB-231 medium.

Medium	Composition
MCF-7 and MDA-MB-231 basal medium	Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12, Thermo Scientific, Waltham, MA, USA) supplemented with 1% penicillin/streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 10% fetal bovine serum (FBS; Thermo Scientific).
MCF-7 and MDA-MB-231 cryopreservation medium	Basal medium, supplemented with 5% Dimethyl sulfoxide (DMSO, Sigma-Aldrich).

2.8 Buffers and solutions

Table 2.11: Overview of used buffers and solutions.

Medium	Composition
Adipogenic inducer stock solutions	Insulin (1.7 mM): 10 mg/mL dissolved in 30 mM HCl. IBMX (25 mM): 5.55 mg/mL dissolved in ddH_2O, Na_2CO_3. Dexamethasone (10 mM): 3.925 mg/mL dissolved in abs. ethanol. Indomethacin (50 mM): 17.89 mg/mL dissolved in abs. ethanol.
Basic FGF stock solution	10 mg/mL are dissolved in PBS with 1% BSA.
Blocking buffer (WMS)	1.5% BSA dissolved in PBS with 0.3% Triton X-100.
Blocking solution (IHC)	1.5% BSA dissolved in PBS
BODIPY stock solution	1 mg/mL is dissolved in DMSO.
Buffered formalin	3.7% formalin (37% stock solution) diluted in PBS.
Hoechst 33258 stock solution	2 mg/mL dissolved in ddH_2O.
ORO staining solution	0.5 g ORO dissolved in 100 mL isopropanol. Addition of 66.6 mL ddH_2O. Filtered twice before use.
PBS	10 PBS (Dulbecco A) tablets are dissolved in 1 L ddH_2O.
PBS/Triton X-100	0.3% Triton X-100 diluted in PBS.
Phosphate saline buffer	50 mM ($Na_2HPO_4*2H_2O$ (178 g/mol), $NaH_2PO_4*H_2O$ (138 g/mol)), 2 mM $Na_2EDTA*2H_2O$, and 2 M NaCl dissolved in ddH_2O and pH was adjusted to 7.4 with NaOH or HCl.
Resazurin solution	0.15 mg/mL resazurin sodium salt dissolved in PBS.
TEN buffer	0.1 M NaCl, 1 mM EDTA, 10 mM Tris dissolved in ddH_2O; pH adjusted to 7.4.

2.9 Assay kits and multicomponent systems

Table 2.12: Overview of used assay kits.

Assay kit	Supplier	Central office
DetachKit	PromoCell	Heidelberg, Germany
DuoSet Ancillary Reagent Kit 2	R&D Systems	Minneapolis, USA
Human Cytokine Antibody Array C5	RayBiotech	Norcross, USA
Human ANG DuoSet ELISA	R&D Systems	Minneapolis, USA

Assay kit	Supplier	Central office
Human IL-6 DuoSet ELISA	R&D Systems	Minneapolis, USA
Human IL-8 DuoSet ELISA	R&D Systems	Minneapolis, USA
Human MCP-1 DuoSet ELISA	R&D Systems	Minneapolis, USA
Human STC-1 DuoSet ELISA	R&D Systems	Minneapolis, USA
Human TIMP-1 DuoSet ELISA	R&D Systems	Minneapolis, USA
Human VEGF DuoSet ELISA	R&D Systems	Minneapolis, USA
ImProm-II™ Reverse Transcription System	Promega	Mannheim, Germany
Live/Dead Cell Staining Kit II	PromoKine	Heidelberg, Germany
Serum Triglyceride Determination Kit	Sigma-Aldrich	Munich, Germany

2.10 Software

Table 2.13: Overview of used software.

Software/Version	Supplier	Central office
ACAS Image analyzer, Tube formation module	Ibidi GmbH	Gräfelfing, Germany
CellSense™ 1.16	Olympus	Hamburg, Germany
GraphPad Prism Version 8.3	GraphPad Software	San Diego, USA
Image Lab™ Software	Bio-Rad	Munich, Germany
Inkscape 0.92.4	Open Source	-
Microsoft Office 365	Microsoft	Redmond, USA

3 Methods

3.1 Isolation of ASCs

Adipose tissue (AT) was obtained from healthy female patients, between 30 and 40 years of age, following abdominoplasty that were conducted in the plastic surgery department, University Hospital of Wuerzburg. AT samples were obtained as subcutaneous excised adipose tissues. The procedure was approved by the local Ethics Committee of the University of Wuerzburg (141/14), and informed consent was obtained from all patients. The obtained AT was minced and extensively washed with equal volumes of phosphate-buffered saline (DPBS; Thermo Scientific, Waltham, MA, USA), and then digested at 37 °C for 2 h in fresh collagenase buffer (Hepes 0.1 M, NaCl 0.12 M, KCl 0.05 M, $CaCl_2$ 0.001 M, glucose 0.005 M, 1.5% bovine serum albumin (BSA) and 0.1% Collagenase NB4 from *Clostridium histolyticum*) under dynamic conditions (50 rpm) on an orbital shaker, to degrade the tight junctions and the components of the extracellular matrix. When enzymatically digested, the AT was centrifuged at 300 rcf for 10 min. Afterwards, the floating top fat layer was decanted, and the remaining suspension was filtered through a 100 µm nylon mesh to remove undigested tissue fragments. The cell suspension was centrifuged once again (300 rcf for 10 min) and the remaining cell pellet was washed with DPBS. The obtained stromal vascular fraction (SVF) was resuspended in basal medium, consisting of Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12; Thermo Scientific) containing 1% penicillin/streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 10% fetal bovine serum (FBS), seeded and used for the subsequent co-culture experiments (see Chapter 3.14).

3.2 Cell culture of ASCs, HUVECs, NIH/3T3 fibroblasts and breast cancer cell lines

3.2.1 Expansion culture

Both purchased human ASCs, obtained from Lonza (Walkersville, MD, USA), and isolated ASCs (described in Chapter 3.1), which were used for lipograft co-culture experiments, were routinely expanded and cultured in 175 cm^2 cell culture-treated plastic flasks in basal medium consisting of Dulbecco´s Modified Eagle´s Medium/Ham´s F-12 (DMEM/F-12; Thermo Scientific, Waltham, MA, USA), supplemented with 1% penicillin/streptomycin (Thermo Scientific), 10% fetal bovine serum (FBS; Thermo Scientific), and 3 ng/mL basic fibroblast growth factor (bFGF; BioLegend, London, UK) dissolved in phosphate-buffered saline (PBS) containing 1% BSA (bovine serum albumin). Cultures were maintained under a sterile humidified 37 °C, 5% CO_2, and 95% air environment. The culture medium was replaced every

other day. At 80–85% confluence, the ASCs were detached using a 0.25% trypsin-EDTA solution (Thermo Scientific) and passaged. ASCs were used at passage 4 for the subsequent experiments. Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (Heidelberg, Germany). HUVECs were plated in 25 cm^2 flasks and cultured in endothelial cell growth medium 2 (PromoCell). When the cells reached 70–80% confluence, they were detached with DetachKit (PromoCell) and expanded to passages 2–3. NIH/3T3 fibroblasts were obtained from ATCC (Manassas, VA, USA). Fibroblasts were cultured in fibroblast growth medium (DMEM; 4.5 g/L D-glucose, 4 mM L-glutamine, 1 mM sodium pyruvate; Thermo Scientific), supplemented with 1% penicillin/streptomycin (Thermo Scientific), and 10% bovine calf serum (BCS; Sigma-Aldrich, Munich, Germany). Cultures were maintained under a sterile humidified 37 °C, 5% CO_2, and 95% air environment. The culture medium was replaced every other day. At 70–80% confluence, the fibroblasts were detached using a 0.25% trypsin-EDTA solution (Thermo Scientific) and passaged. NIH/3T3 fibroblasts were used at passage 5-6 for the subsequent experiments. MCF-7 and MDA-MB-231 breast cancer cells (ATCC) were cultured in Dulbecco´s Modified Eagle´s Medium/Ham´s F-12 (DMEM/F-12; Thermo Scientific), supplemented with 1% penicillin/streptomycin (Thermo Scientific), and 10% fetal bovine serum (FBS; Thermo Scientific). When the cells reached 80-85% confluence, they were detached with 0.25% trypsin-EDTA solution (Thermo Scientific) and expanded for 4–5 passages.

3.2.2 Ischemic culture conditions of ASCs

ASCs were exposed to glucose and oxygen deprivation separately and in combination. Cells were seeded (25,000 cells/cm^2) in tissue culture-treated well plates (6-well and 24-well plates) in basal medium (DMEM/F-12; Thermo Scientific), and after incubation overnight with 21% O_2 for attachment of cells, ASCs were washed twice with DPBS (Thermo Scientific) and then cultured under the respective ischemic conditions as described in the according chapters. Hypoxic conditions of 2% O_2 were achieved using the CO_2 incubator (Binder, Tuttlingen, Germany) and of 0.2% using the well-established and finely controlled proOx-C chamber system (C-Chamber, C-274; BioSpherix, New York, NY, USA). The oxygen concentrations of 2% or 0.2% were maintained with the residual gas mixture composed of 5% CO_2 and balance nitrogen. In order to ensure sustained hypoxic conditions, cell cultures were left undisturbed without medium changes over the course of experiments.

3.2.3 Preparation of conditioned medium of ASCs

ASCs were seeded at 25,000 cells per cm^2 in basal medium (DMEM/F-12; Thermo Scientific) and allowed to adhere overnight at 21% O_2. ASCs were then washed twice with DPBS (Thermo Scientific), and the medium was replaced with basal medium (D-glucose-, L-glutamine-, phenol red-, and sodium pyruvate-free DMEM; Thermo Scientific) containing no serum and supplemented with 0.1 g/L glucose (Sigma-Aldrich). Cells were incubated under 0.2% O_2, to generate a conditioned medium (CM) of ASCs exposed to glucose/oxygen deprivation. After four days, the medium was harvested as ASC-$CM_{ischemic}$.

3.2.4 Adipogenic differentiation of ischemia-treated ASCs

To assess the adipogenic differentiation capability of ischemia-treated ASCs, cells were cultured under combined glucose/oxygen deprivation for four days. The cells were then trypsinized, re-seeded, and cultured in Preadipocyte basal medium-2 (PBM-2; Lonza) containing 10% FCS and 1% penicillin/streptomycin. After two days, adipogenic differentiation was induced by changing to differentiation medium (PBM-2 with 1.7 µM insulin; PromoCell, Heidelberg, Germany), 1 µM dexamethasone, 200 µM indomethacin (both from Sigma-Aldrich), and 500 µM 3-isobutyl-1-methylxanthin (IBMX; Serva Electrophoresis, Heidelberg, Germany) for 14 days. Non-induced cells were cultured in PBM-2 during the differentiation period. In parallel, control ASCs were cultured in basal medium (DMEM/F-12; Thermo Scientific) for four days and then treated in the same way for adipogenic differentiation. The respective medium of each group was changed every other day until the end of the experiment. After 14 days of culture in adipogenic medium, cells were fixed in 3.7% buffered formalin and the visualization of intracellular lipid accumulation was histologically assessed by staining with Oil Red O solution (3 mg/mL Oil Red O in 60% isopropanol; Sigma-Aldrich). The cell nuclei were counterstained with hematoxylin (Bio Optica, Milan, Italy). Samples were imaged using an Olympus IX51 inverted light microscope and analyzed with the Olympus CellSens™ Software 1.16 (Olympus, Hamburg, Germany).

The quantitative determination of intracellular lipid accumulation was performed using the Serum Triglyceride Determination Kit from Sigma-Aldrich (Munich, Germany). Cells were harvested in Thesit solution (0.5% Thesit in H_2O; Gepepharm, Hennef, Germany) and sonicated with an ultrasonic homogenizer (SonoPlus, Bandelin electronic, Berlin, Germany). The triglyceride quantification was performed according to the manufacturer's instructions and measured with a microplate reader (Infinite M200; Tecan) at a wavelength of 570 nm. Triglyceride contents were normalized to the DNA content of the respective samples.

3.2.5 Proliferation and metabolic activity of fibroblasts and breast cancer cell lines

Conditioned medium from glucose/oxygen-deprived ASCs (ASC-$CM_{ischemic}$) was prepared as described above. NIH/3T3 fibroblasts and breast cancer cells (MCF-7 and MDA-MB-231) were both treated with nutrient deprived basal medium (D-glucose-, L-glutamine-, phenol red, and sodium pyruvate-free DMEM, w/o FBS) or ASC-$CM_{ischemic}$, each supplemented with 1 g/L glucose (Sigma-Aldrich), under normoxic conditions. Proliferation and metabolic activity of the cells were analyzed at the indicated time points using a DNA and MTT assay as described below.

3.2.6 Addition of recombinant human STC-1 (rhSTC-1)

The lyophilized rhSTC-1 purchased von R&D Systems (Minneapolis, MN, USA) was reconstituted with sterile DPBS at 500 µg/mL, according to the manufacturer's instructions. This stock solution was further diluted in sterile DPBS before use to yield different concentrations of 40, 400, 4.000 and 40.000 pg/mL. The recombinant human STC-1 was added to the respective medium, depending on the according experiment, and pre-incubated for 1 h at 37 °C under gentle shaking prior treatment with cells.

3.2.7 Inhibition of STC-1

For a specific inhibition of STC-1, an affinity purified polyclonal anti-STC-1 antibody (R&D Systems, Minneapolis, MN, USA) was used for blocking. The lyophilized antibody (100 µg) was reconstituted with sterile DPBS at 0.2 mg/mL, according to the manufacturer's instructions. This stock solution was further diluted in sterile DPBS before use to yield a final concentration of 2, 5, 10 and 20 µg/mL. The antibody was added to ASC-$CM_{ischemic}$, with the respective concentration depending on the analysis, and pre-incubated for 1 h at 37 °C under gentle shaking prior treatment with cells. The respective isotype IgG1 control (R&D Systems) added to the respective medium served as control.

3.3 Investigations on cell and adipose tissue viability

3.3.1 Live/dead staining

ASCs, HUVECs and adipose tissue were cultured in their respective basal or growth medium and after attachment of cells or short regeneration of tissue, they were cultured according to the respective condition. The viability was determined using live/dead cell staining

kit (PromoKine, Heidelberg, Germany) according to the manufacturer´s instructions. Cells or adipose tissue were washed three times with PBS and stained by applying 0.5 mL (for cells) or 1 mL (for tissue) of live/dead staining solution containing 4 µM ethidium bromide homodimer III (EthD-III) and 2 µM calcein acetoxymethyl ester (Calcein-AM) in DPBS per well. After 45 min of incubation in the dark, the staining solution was removed, and a washing process with DPBS was made 3 times. Living cells or tissue were stained with calcein (green) and dead were stained with ethidium bromide (red). Images were taken using an Olympus IX51 fluorescence microscope and analyzed with the Olympus CellSens™ Software 1.16 (Olympus, Hamburg, Germany).

3.3.2 Flow cytometry of calcein-stained cells

ASCs were seeded in basal medium (DMEM/F-12; Thermo Scientific) in 6-well plates and after attachment of cells overnight with 21% O_2, there were washed twice with DPBS (Thermo Scientific) and cultured according to the respective condition. At the indicated time points, cells were treated with Accutase (Sigma-Aldrich, Munich, Germany) for 5-10 min at 37 °C and the detached cells were transferred into the FACS-tube. Cells were centrifuged at 400 rcf for 10 min at 4 °C and the supernatant was discarded. After repeated washing steps with DPBS, the cell pellet was incubated in a staining solution of 2 µM Calcein-AM (PromoKine, Heidelberg) for 45 min at room temperature. After repeated washing, the cell pellet was resuspended in FACS buffer (DPBS + 1% BSA) for flow cytometry analysis. The calcein fluorescence was analyzed in the fluorescein channel using the CytoFlex (Beckman Coulter, Brea, USA).

3.3.3 MTT assay

ASCs, HUVECs, NIH/3T3 fibroblasts, and MCF-7 / MDA-MB-231 breast cancer cells were seeded in their respective growth medium and after attachment of cells, they were cultured according to the respective condition. At the indicated time points, cells were treated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at a final concentration of 0.5 mg/mL and incubated for 3 h at 37 °C. Cells were then washed with phosphate-buffered saline (DPBS), incubated with dimethyl sulfoxide (DMSO) for 5 min with gentle shaking, and mixed to ensure complete solubilization of the dye formed. The respective light absorbance of this solution was recorded using a microplate reader (Infinite M200; Tecan) at a wavelength of 570 nm. The mean value of day 0 samples was taken as reference and set as 100%.

3.3.4 WST-8 assay

ASCs were seeded in basal medium in 96-well plates and after attachment of cells, they were cultured according to the respective condition. At the indicated time points, 10 µl Cell Counting Kit-8 (Sigma-Aldrich) containing the water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was directly added into 100 µl culture medium. Cells were then incubated at 37 °C for 4 h. The respective light absorbance of the samples was recorded using a microplate reader (Infinite M200; Tecan) at a wavelength of 450 nm. The mean value of day 0 samples was taken as reference and set as 100%.

3.3.5 Resazurin assay

Resazurin assay was used to measure the viability of lipografts and injection material. Resazurin as a cell permeable redox indicator, used for cell metabolism analysis, converts resazurin, a purple and non-fluorescent product to its reduced form resorufin, a red color fluorescent product. For this purpose, 40 µl of resazurin stock solution (0.15 mg/mL resazurin sodium salt (Sigma-Aldrich) dissolved in DPBS was added directly into each well of the 48-well plate containing lipografts or injection material in 400 µl medium. The tissues were further incubated with the supplemented resazurin for 3 h on an orbital shaker at 37 °C and 5% CO_2. Afterwards, 150 µl of each well was transferred into a black 96-well plate for fluorescence measurement using a 560 nm excitation and 590 nm emission filter set with a fluorescence spectrometer (Infinite M200; Tecan).

3.4 Immunofluorescence staining

For histological evaluation, 2D monolayers and adipose tissue were fixed in 3.7% buffered formalin and stored until immunohistological investigations in DPBS. Immunohistochemical analyses of 2D monolayers were performed directly in the 24-well plates. Adipose tissue were cut into 2 µm thick sections using a Leica Histoslide 2000 R microtome (Leica Biosystems, Wetzlar, Germany).

3.4.1 HIF-1 alpha staining in 2D monolayers

The presence of the hypoxia-inducible factor 1 alpha (HIF-1 alpha), as a mediator of cellular response to hypoxia, was examined by immunohistochemical staining. Cells were stained with primary antibody against HIF-1 alpha (ab16006; monoclonal mouse anti-HIF-1 alpha), which was purchased from Abcam (Cambridge, United Kingdom).

Antigen retrieval was performed by incubation with Proteinase K (Life Technologies, Karlsruhe, Germany) for 10 min at 37 °C. After a permeabilization step (0.3% Triton X-100 diluted in DPBS) for 45 min at room temperature, cells were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, Munich Germany) for 30 min. Afterwards, cells were incubated with the primary antibody overnight in appropriate dilution (1:200). After several washing steps with DPBS, the secondary antibody (Cy3-conjugated AffiniPure donkey anti-mouse; 1:400; Dianova) in addition with the CytoPainter Phalloidin-iFluor 488 Reagent (Abcam) were applied for 1 h in the dark under gentle shaking. Finally, the cells were counterstained with IS DAPI mounting medium (Dako, Hamburg, Germany) and imaged with an Olympus IX51 inverted light microscope equipped with a XC30 digital camera.

3.4.2 Perilipin staining on adipose tissue sections

Lipid vacuoles of lipograft and injection material sections were visualized by immunostaining against perilipin. The paraffin cross-sections were deparaffinized and antigen retrieval was performed using Pepsin (Digest All™-3; Invitrogen, Karlsruhe, Germany). After antigen retrieval, unspecific binding was blocked with blocking solution (1% BSA in DPBS) prior to overnight incubation with a polyclonal rabbit anti-perilipin (PA5-72921; Thermo Scientific) in appropriate dilution (1:200). Slides were thoroughly washed with DPBS and an Cy3-conjugated goat anti-rabbit secondary antibody (1:400; Dianova) was added for 60 min at room temperature in the dark. Nuclei were counterstained with IS Mounting Medium DAPI (Dako) and analyzed under the Olympus BX51 fluorescence microscope.

3.5 Whole mount staining

For the visualization of tissue structure of lipografts they were whole mount-stained adapted to Wittmann et al. [353]. The prepared lipografts (see Chapter 3.13) were fixed in 3.7% buffered formalin overnight. Next, the lipografts were incubated in 100% ice-cold methanol for 30 min and washed with DPBS for 1 h under gentle shaking. Following, lipografts were incubated overnight in blocking buffer (1.5% BSA in PBST; PBS with 0.3% Triton X-100). After removal of the blocking buffer solution, the tissue samples were washed with PBST for 2 h. The primary antibody anti-human CD31 (Clone JC70A, monoclonal mouse anti-human CD31 (1:200); Dako) was diluted in antibody diluent (Dako) and added overnight to the lipograft samples. Next, samples were washed with PBST for 1.5 h to remove unbound antibody, and further incubated for 1.5 h in blocking buffer. Afterwards, whole mounts were incubated with the respective secondary antibody (Cy™3-conjugated AffiniPure donkey anti-mouse (1:400; Dianova) for another 2 h, followed by washing with DPBS overnight. For staining of the lipid vacuoles, samples were incubated with a 1:100 dilution of BODIPY in DPBS (stock 1 mg/mL)

for 30 min. The BODIPY solution was removed, and the samples were rinsed with DPBS for 2 h. All incubation steps were performed at room temperature on an orbital shaker (40 rpm). Before imaging, they were treated with DAPI (IS DAPI Mounting Medium; Dako) and examined using the Olympus BX51 fluorescent microscope.

3.6 Quantification of DNA content

For determination of total DNA content, the intercalating dye Hoechst 33258 was used (Polysciences, Warrington, PA, USA). For this purpose, cells were harvested in phosphate-saline-buffer (50 mM phosphate buffer, 2 mM Na_2 EDTA*2 H_2O, 2 M NaCl, pH 7.4; all obtained from Carl Roth, Karlsruhe, Germany) and subsequently sonicated with an ultrasonic homogenizer (SonoPlus, Bandelin electronic, Berlin, Germany) for breaking cell membranes. Quantification of DNA content was carried out by measuring fluorescence intensities at an excitation wavelength of 365 nm and an emission wavelength of 458 nm with a fluorescence spectrometer (Infinite M200; Tecan).

3.7 Glucose and lactate determination

Exogenous glucose and lactate levels in the cell culture supernatants of ASCs were measured with GLUC3 and LACT2 COBAS INTEGRA substrate reagents using the related COBAS INTEGRA 800 robot (Roche, Basel, Swiss). A total number of 3 biological replicates per sample were used for these analysis. The measurements were kindly provided by the central laboratory, University Hospital Wuerzburg, Germany.

3.8 RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA from cultured cells was isolated using TRIzol® reagent (Invitrogen, Karlsruhe, Germany). First-strand cDNA was synthesized from total RNA using the ImProm-II Reverse Transcription System (Promega, Mannheim, Germany). Quantitative PCR analyses were performed using the MESA GREEN qPCR MasterMix Plus with MeteorTaq polymerase (Eurogentec, Seraing, Belgium). The cDNA for genes of interest was amplified with the PrimePCR™ SYBR® Green Assay using the following cycle conditions: 95 °C for 15 min initial denaturation followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 70 °C for 30 s using the following primers: IL-6 (qHsaCID0020314, IL6, human), VEGF (qHsaCED0043454, VEGFA, human), and STC-1 (qHsaCID0006115, STC1, human), all from Bio-Rad (Hercules, CA, USA). The mRNA expression levels were normalized to the eukaryotic translation elongation factor 1 alpha (EF1α) (forward, 5′-ccccgacacagtagcatttg-3′; reverse, 5′-tgactttccatcccttgaacc-3′) (Biomers, Ulm, Germany). The relative expression levels were

determined using the $2^{-\Delta\Delta CT}$ method and were further normalized to the respective day 0 sample.

3.9 Human cytokine antibody array

To identify factors secreted by glucose/oxygen-deprived ASCs, a profiling of human cytokines was performed using an antibody array covering 80 cytokines (Human Cytokine Antibody Array C5; RayBiotech, Norcross, GA, USA). Two biological replicates of cell culture supernatants from cells cultured under the following conditions were examined: (1) 1 g/L glucose and normoxia (21% O_2) (control); (2) 1 g/L glucose and hypoxia (0.2% O_2); (3) 0.1 g/L glucose and normoxia (21% O_2); (4) 0.1 g/L glucose and hypoxia (0.2% O_2). The cell culture supernatants were centrifuged at 1000 rcf for 5 min to remove cell debris. Array analyses was performed according to the manufacturer's instructions. Briefly, the array membranes were blocked with a blocking buffer and incubated with 1 mL of each supernatant overnight at 4 °C. Subsequently, the membranes were assayed for chemiluminescence signals (ChemiDocTM Touch; Bio-Rad, Munich, Germany). The Bio-Rad Image Lab™ Software was used to process and evaluate the array membranes.

3.10 Enzyme-linked immunosorbent assays (ELISAs)

The concentrations of individual cytokines in the cell culture supernatants from 2D monolayers and of lipograft co-culture under deprivation conditions and control condition after four days of culture were determined using ELISA kits for vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-8, angiogenin (ANG), TIMP metallopeptidase inhibitor (TIMP)-1, monocyte chemoattractant protein (MCP)-1, and stanniocalcin (STC)-1 from R&D Systems (DuoSet ELISA; Minneapolis, MN, USA). For 2D monolayers and co-culture, 1n consisted of the pooled medium of 2 wells of a 24-well plate (total volume of 2 mL). The ELISAs were performed according to the manufacturer´s instructions and measured with a microplate reader (Infinite M200; Tecan) at a wavelength of 450 nm, with a wavelength correction of 540 nm. For 2D monolayers, the concentration levels were normalized to the DNA content of the respective samples (pg/µg DNA).

3.11 Tube formation assay

Angiogenesis µ-Slides (Ibidi, Gräfelfing, Germany) were coated with 10 µL of growth factor- reduced matrigel (BD Biosciences, Heidelberg, Germany). HUVECs were suspended in nutrient deprived basal medium (D-glucose-, L-glutamine-, phenol red-, and sodium pyruvate-free DMEM, w/o FBS), ASC-$CM_{ischemic}$ or endothelial growth medium (PromoCell) and

plated with 1 × 10^4 cells per well on top of the matrigel. After 4, 6, and 10 h of incubation at 37 °C under hypoxic conditions (0.2% O_2), the formation of tube-like structures was examined microscopically. The tube length and branch count were quantified using the automated image analyzer ACAS from Ibidi (Tube formation ACAS image analysis module) at the indicated time points.

3.12 Fibroblast migration assay

The migratory activity of NIH/3T3 fibroblasts was assessed using a migration assay. Ibidi Culture-Inserts 2 well (Ibidi, Gräfelfing, Germany) were transferred into 6-well plates and 70 µL cell suspension containing 3 × 10^5 cells/mL was applied to each well. After an appropriate duration for cell attachment (24 h) the Ibidi Culture-Inserts were removed to create a cell-free gap of 500 µm. Cells were then washed with phosphate-buffered saline (DPBS) and incubated with basal medium (D-glucose-, L-glutamine-, phenol red-, and sodium pyruvate-free DMEM, w/o FBS) or ASC-$CM_{ischemic}$, each supplemented with 1 g/L glucose (Sigma-Aldrich), under normoxic conditions for 24 h. The fibroblast growth medium (DMEM; 4.5 g/L D-glucose, 4 mM L-glutamine, 1 mM sodium pyruvate; Thermo Scientific) was used as positive control. To monitor the progress of gap closure, micrographs were taken at different time points with an Olympus IX51 inverted light microscope equipped with a XC30 digital camera.

3.13 Preparation and cultivation of lipografts

All adipose tissue (AT) from healthy patients, following abdominoplasty, was kindly provided by the plastic surgery department, University Hospital of Wuerzburg.

AT was obtained as subcutaneous excised adipose tissue from female donors between 20 and 40 years of age with a body mass index ranging from 28 to 33. Written informed consent was obtained from all patients. The AT was cut into 2x2 mm sized tissue fragments (lipografts) and cultured in agarose-coated 48-well plates with the respective culture medium. For mimicking ischemic conditions, lipografts were cultured with nutrient deprived basal medium (D-glucose- and sodium pyruvate-free, w/o FBS) under 0.2% O_2. The cultivation of lipografts with basal medium DMEM/F-12 supplemented with 10% FBS under normoxia was used as control condition in this set-up.

3.14 Co-culture of lipografts with ASCs

Excised fat from particular donors was used to generate lipografts (described above) and in parallel to isolate ASCs (see Chapter 3.1) from the same donor. Lipografts were maintained in DMEM/F-12 basal medium until the ASCs were propagated to sufficient numbers, which usually took 3-4 days. Finally, lipografts and ASCs were co-cultured in a 24-transwell system. For this approach, 5 lipografts with an approx. total volume of 50 µl were incubated in one insert. To achieve a cell concentration of 10^6 ASCs/mL lipografts, ASCs were seeded at the bottom with a seeding density of 25,000 cells per cm^2.

3.15 Statistical analysis

Quantitative results are presented as means ± SD. Statistical analyses of variance comparisons between groups were performed using the ANOVA-test in conjunction with Bonferroni post-hoc adjustment. For statistical analyses of endothelial tube formation an unpaired Student's t-test was applied. For all analyses, differences at $p < 0.05$ were considered as statistically significant. All statistical analyses were performed using the GraphPad Prism Software 8.3 (GraphPad Software, San Diego, CA, USA)

4 Results and Discussion

4.1 Function of ASCs under ischemia-mimicking conditions

In recent years, ASCs have been most commonly used in tissue engineering and regenerative medicine [86,354]. ASCs were shown to contribute to tissue repair and regeneration in various clinical applications [355]. Apart from their potential to differentiate towards a specific cell type or stimulating the recruitment of endogenous stem cells to the damaged area and to commit in the proper lineage [356,357], ASCs may also promote tissue regeneration by modulating the immune response [358,359], and by secreting cytokines and growth factors that stimulate restoration of normal tissue function or reduce its damage [360–363]. However, despite these beneficial effects, previous studies have shown low survival of ASCs post transplantation [197,364,365]. One explanation for the massive cell death of transplanted cells is the ischemic environment, to which cells are exposed upon transplantation [344,345,366]. The ischemic environment is a consequence of insufficient blood supply at the transplantation site, which translates into a reduction of nutrients, grow factors and cytokines as well as reduced oxygen concentrations [367]. Therefore, it is essential to analyse the response of ASCs to *in vitro* models of nutrient deprivation to understand their function under such conditions and to improve their therapeutic efficacy. To date, little is known about how ASCs respond to ischemic stress. Only a few studies so far have investigated the effect of combined hypoxia and nutrient deprivation on the viability and metabolic response of ASCs [368,369]. Moreover, the factors that are secreted by ASCs under ischemic conditions have not been studied up to now. Thus, investigations on the effect of ischemic conditions on ASC secreted factors with trophic activity would help to better understand the beneficial effects of transplanted ASCs in regenerative approaches [367].

Therefore, the work presented in this first chapter aimed at the establishment of an *in vitro* model of the ischemic conditions occurring at the transplantation site. This model was then used to assess the impact of ischemia on ASCs. To models ischemic conditions, we exposed ASCs *in vitro* to sustained moderate ischemic conditions simulating the periphery and to harsh ischemic conditions, that might best reflect the core of tissue engineering constructs. First, their viability and especially their secretory potential in this set-up was assessed. To further modify the harsh ischemic conditions, the role of glucose deprivation under severe hypoxia on a set of factors that are secreted by ASCs has also been studied. Altogether, these investigations should contribute to a first characterization of ASCs under ischemic conditions and to further understand the role of ASCs in tissue regeneration.

4.1.1 Ischemia-mimicking conditions - Experimental set-up

In order to mimic ischemic conditions during the early post-transplantation phase, that is characterized by an initial lack of blood supply, ASCs were cultured under different deprivation conditions (Figure 4.1). There is literature describing harsh conditions inside tissue engineered constructs. A study by Moya et al. [370] describes that 0.13% oxygen best reflected 24 hours post-implantation due to the non-existent blood supply within transplanted constructs. For glucose concentration *in vivo*, such data do not exist in the literature so far. Therefore, it can be assumed that if no oxygen arrives at the construct *in vivo*, that no glucose and other nutrients will arrive either. Since these conditions are certainly not consistent over the whole implant, as better conditions will exist at the periphery than in the core of the construct, the approach resulted in different deprivation conditions. Thus, in this set-up, ASCs were subjected to harsh deprivation conditions with complete withdrawal of glucose and serum and exposure to severe hypoxia (0.2% O_2) and moderate ischemic conditions, which were set to 0.5 g/L glucose, 0.5% serum and 2% oxygen. These conditions were compared to standard cell culture conditions with 3.1 g/L glucose, 10% serum and 21% oxygen. With this experimental set-up, ASCs were cultured for 7 days undisturbed and without medium change over the course of the experiments to prevent reoxygenation and to ensure cell-driven nutrient depletion.

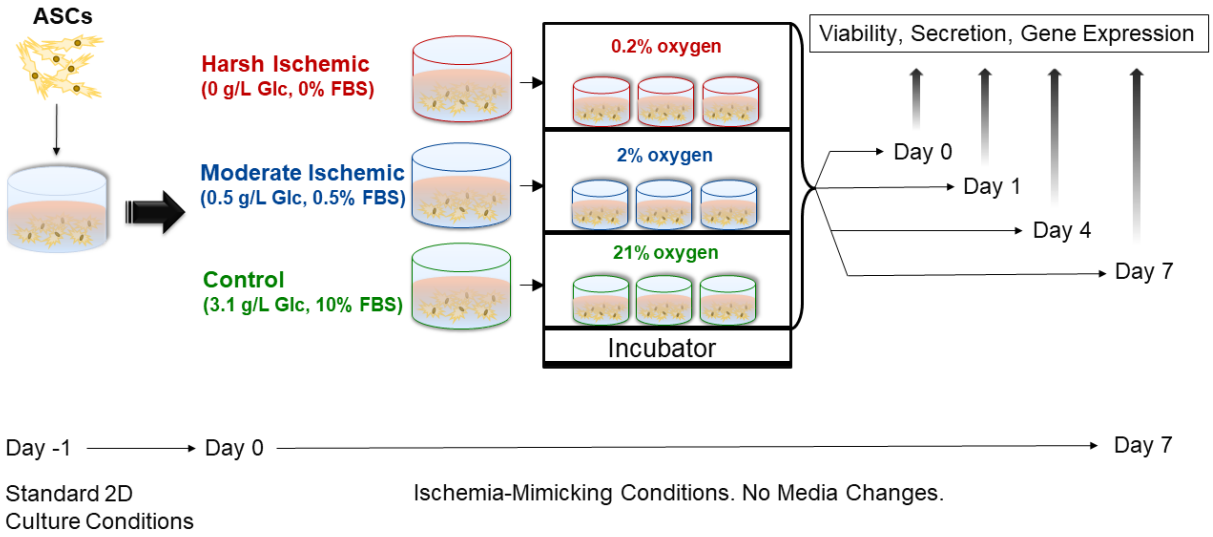

Figure 4.1: Outline of the experimental set-up to mimic ischemic conditions.
ASCs were seeded at a density of 25,000 cells per cm^2 under standard culture conditions (day -1) and were then subjected to harsh ischemic conditions (0 g/L Glc, 0% serum, 0.2% O_2) or moderate ischemic conditions (0.5 g/L Glc, 0.5% serum, 2% O_2), respectively. Standard culture conditions (3.1 g/L Glc, 10% serum, 21% O_2) served as control. ASCs were cultured for 7 days without medium change. The viability, secretion and gene expression were analyzed at the indicated time points. Abbreviation: Glc: Glucose. Modified from Deschepper et al. 2013 [371].

4.1.2 Characterization of ASCs exposed to ischemia

The hypoxia-inducible factor 1 alpha (HIF-1 alpha), as mediator of cellular response to hypoxia, activates and regulates a large panel of genes, including those especially important for cell survival [372–374]. To examine whether the selected external oxygen conditions are also adopted by the cells in this manner, immunohistochemistry of HIF-1 alpha expression on ASCs cultured for 7 days under moderate (2% O_2) and harsh ischemic conditions (0.2% O_2) was performed (Figure 4.2). Under control conditions (normoxia), no expression of HIF-1 alpha was detected, whereas HIF-1 alpha was found under moderate as well as under harsh ischemic conditions as early as 24 hours after cell adhesion under the respective condition. Thus, these results demonstrated that ASCs were exposed to sustained hypoxia during the period of the experiment. Moreover, the additional F-Actin staining demonstrated very clearly the typical stellate morphology of ASCs under moderate ischemic conditions over 7 days compared to harsh ischemic conditions, with only a few shrunken and altered cells.

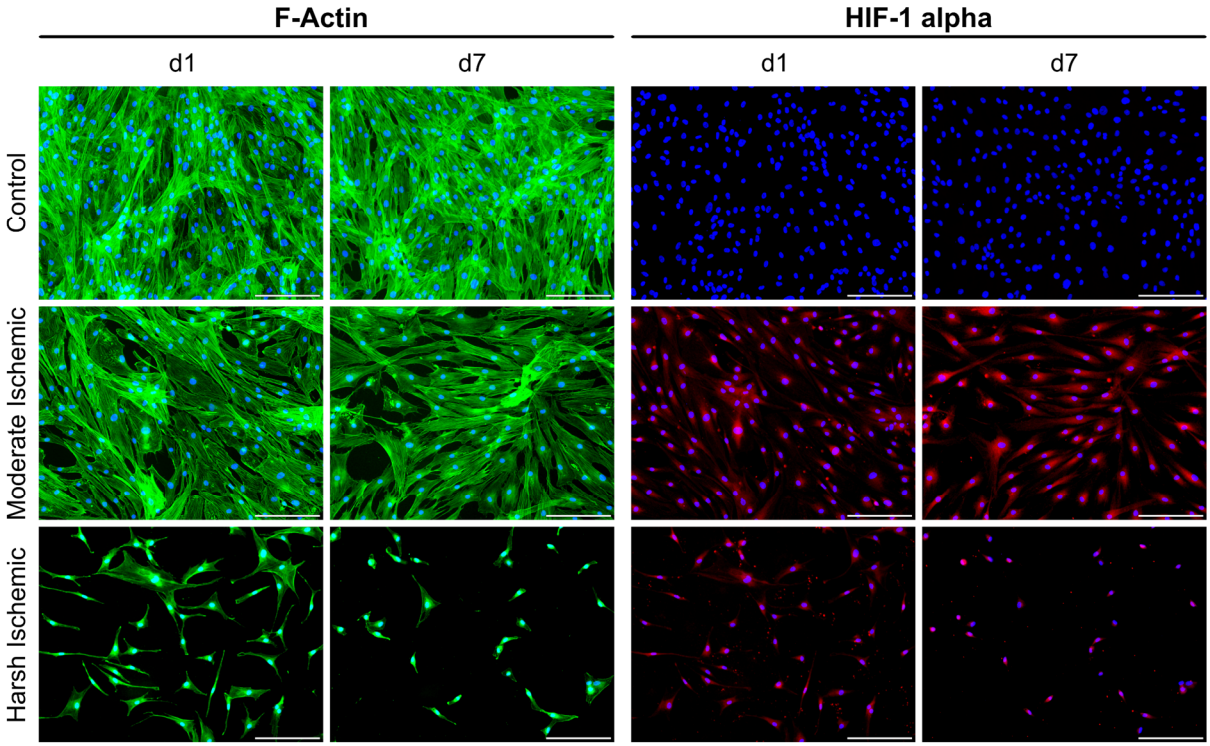

Figure 4.2: Immunohistochemical validation of hypoxia under ischemia-mimicking conditions.
The cell nuclei were labelled with DAPI (blue). For visualization of the cytoskeleton the cells were stained for F-Actin (green, left). To determine the presence of hypoxia under moderate and harsh ischemic conditions, the cells were stained for HIF-1 alpha expression (red, right). Scale bar represents 200 µm.

To determine the viability of ischemia-challenged ASCs over a period of 7 days, a live/dead assay was performed (Figure 4.3 A). Under moderate ischemic conditions, a similar viability was observed as in the control group. ASCs retained their typical stellate morphology up to 7 days of culture. In contrast, under harsh ischemic conditions only few viable cells were present after 4 days. Dead (red) cells were hardly detectable in this set-up, as they rapidly detached from the cell-culture plates and consequently were washed out during the staining process. Moreover, ASCs under harsh ischemic conditions lost their typical stellate morphology and shrank progressively. Quantitation of viable cells by FACS confirmed a sharp decrease in the number of viable cells, already at day 1 to 20% with less than 10% of the cells remaining at day 7 (Figure 4.3. B). The strong reduction in cell number under harsh ischemic conditions was also reflected by the DNA content (Figure 4.3. C), which decreased to approx. 7% at day 4 and remained stable until day 7. Under moderate ischemic conditions, the DNA content decreased only to approx. 80% at day 4, with 60% of the cells remaining at day 7. The metabolic activity of the cells (determined using the MTT assay) was also significantly reduced under harsh ischemic conditions, already at day 1 with less than 30% compared to the metabolic activity from the start of the culture. Under moderate conditions, the viable cell number remained mostly unaffected, but the cellular metabolic activity significantly decreased over time (Figure 4.3. D).

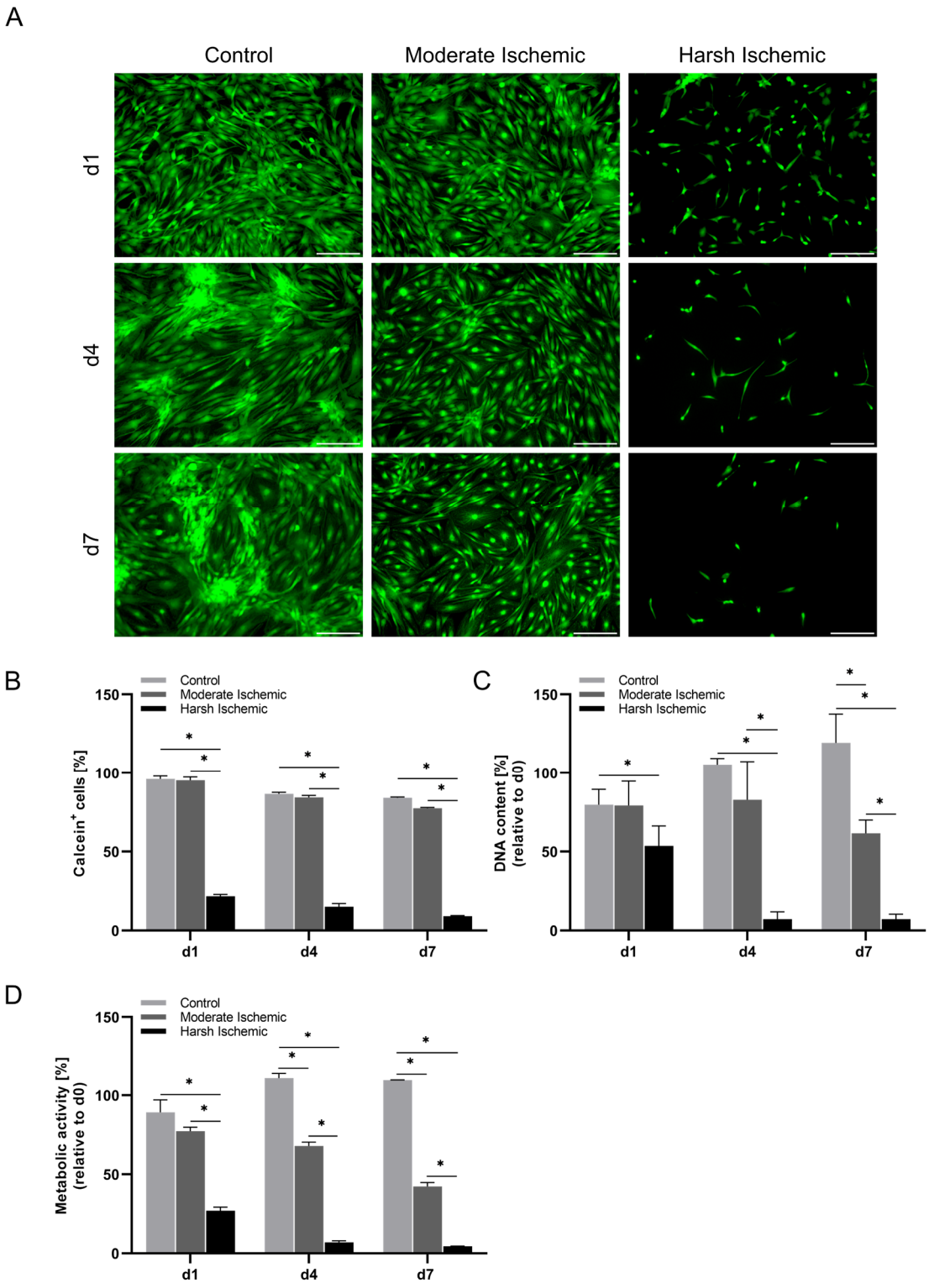

Figure 4.3: Viability of ASCs under ischemia-mimicking conditions.

(**A**) Microscopic images of live/dead staining. Viable cells appear green, dead cells red. Scale bar represents 200 µm. (**B**) FACS analysis of viable ASCs in relation to the total cell number stained with calcein. (**C**) Quantitative determination of total DNA content relative to the mean DNA content at day 0. (**D**) Metabolic activity as determined by a MTT assay. % Metabolic activity was calculated relative to the mean activity at day 0. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviation: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

4.1.3 Angiogenic and anti-apoptotic marker expression of ASCs under ischemia-like stress conditions

In order to begin to characterize the secretory response of ASCs under moderate and harsh ischemic conditions, the concentration of a set of factors with known anti-apoptotic, angiogenic or immune-modulatory functions (IL-6, VEGF, STC-1), in the culture supernatants after 4 days of culture was quantified (Figure 4.4 A). Significantly increased levels for IL-6, VEGF, and STC-1 were measured in response to culture under harsh ischemic conditions, compared to moderate ischemia. The concentrations of the selected factors in the culture supernatant under moderate ischemic conditions were similar to the concentrations in the supernatants of cells cultured under the control condition, thus the combined moderate deprivation of glucose and oxygen had no influence on the secretion of these factors. STC-1 was only detectable in supernatants of cells cultured under ischemia-mimicking conditions while it was not detected in supernatants of cells cultured under control conditions.

The changes in the levels of these factors measured in the supernatants of ASCs were in good agreement with the mRNA expression levels determined by real time PCR. A significant upregulation of IL-6, VEGF and STC-1 expression was observed until day 4 in ASCs that were cultured under harsh ischemic conditions, with an additional small increase for IL-6 and STC-1 up to day 4, whereas VEGF expression stayed at a constant high level from beginning (Figure 4.4 B). Due to the low number of surviving cells at day 7 of culture under harsh conditions it was not possible to obtain mRNA expression data from this time point.

Taken together, these results demonstrated that under moderate ischemic conditions the gene expression and secretion of anti-apoptotic, angiogenic and immune-modulatory factors was only marginally elevated, whereas under harsh ischemic conditions the gene expression and secretion of the investigated factors was significantly upregulated.

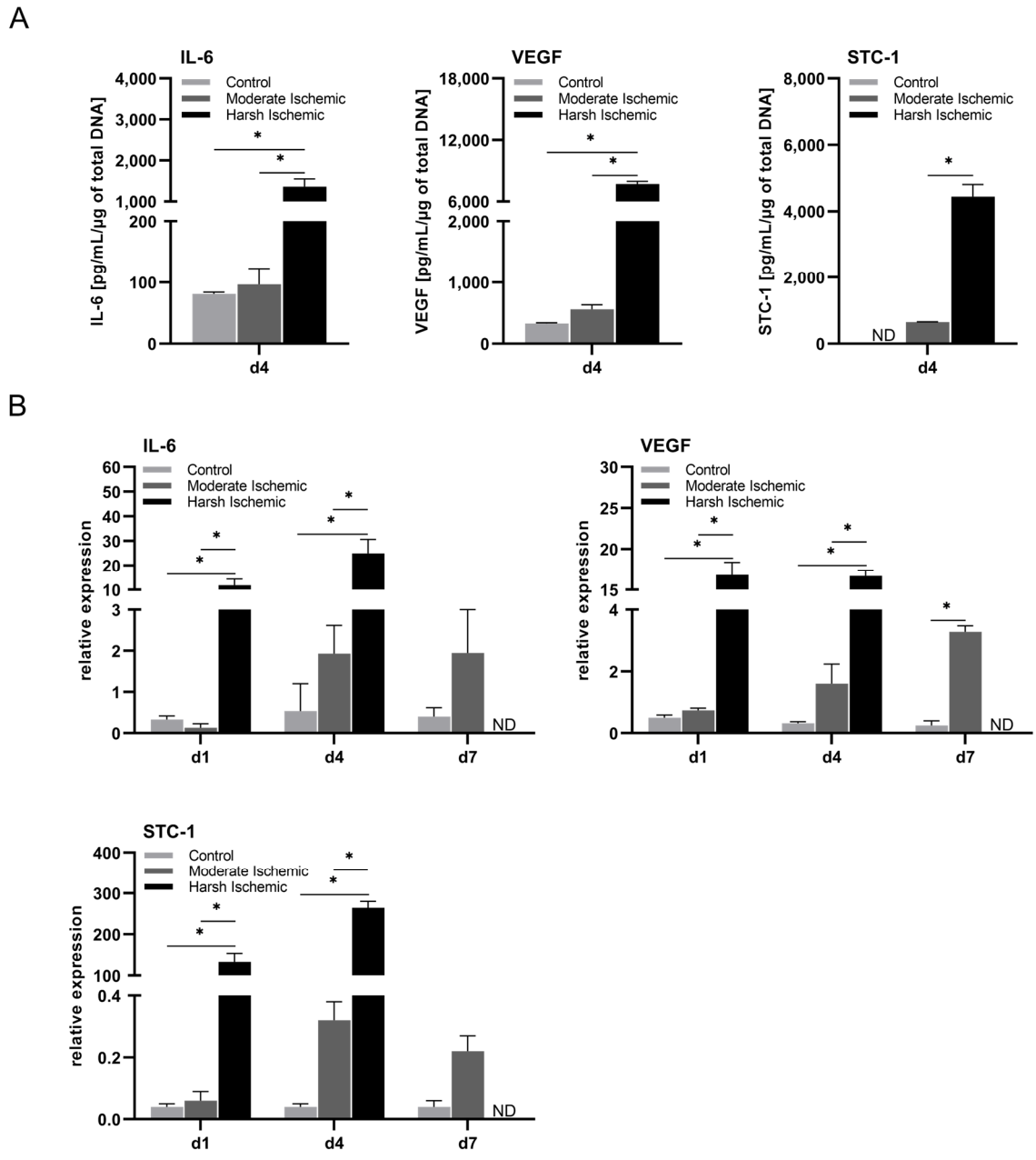

Figure 4.4: Expression of cytokines in ASCs under combined deprivation conditions.

(**A**) Enzyme-linked immunosorbent assays for IL-6, VEGF, and STC-1 in the medium at day 4 of culture; the values were normalized to the DNA content of the respective samples. (**B**) Corresponding relative gene expression of IL-6, VEGF, and STC-1 at day (d) 1, d4 and d7 by qRT-PCR. Gene expression was normalized to EF1α; the values were further normalized to day 0. (Note: d0 values are the same for all groups.) Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviations: IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; STC-1: Stanniocalcin-1; ND: Not detectable.

4.1.4 Effect of phenol red and 17-β Estradiol on the viability of ASCs under conditions mimicking harsh ischemia

A preliminary experiment, where different media compositions with regard to the contained nutrients including with and without phenol red (PSP) were examined, led to hypothesize that PSP, which is used ubiquitously as a pH-indicator in tissue culture media, had an effect on the viability of ASCs, when they were cultured under harsh ischemic conditions for 7 days (data not shown). To avoid interference between the effects of PSP and ischemic culture conditions, the viability and metabolic activity of the cells under harsh ischemic culture conditions with PSP-free basal medium (D-glucose-, L-glutamine-, and sodium pyruvate-free DMEM; containing no serum) and basal medium containing $4x10^{-5}$ M of PSP was further analyzed. Due to previously published studies that report about an estrogenic activity of PSP at concentrations, which are found in tissue culture media [375,376], the effect of different concentrations of the sex hormone 17-β Estradiol (E2) in the ASC culture medium was additionally examined. Viability of ASCs under harsh ischemic culture conditions was determined by live/dead staining in the presence or absence of PSP or E2, respectively. Dead (red) cells were hardly detectable in this set-up, as they rapidly detached from the cell-culture plates and consequently were washed out during the staining process. The treatment with PSP at a concentration of $4x10^{-5}$ M markedly enhanced cell viability after 1 to 7 days, whereas in medium lacking PSP (w/o) a strong decrease of viable cells especially at day 1 was evident (Figure 4.5 A), confirming that PSP had a positive effect on the viability of ASCs exposed to harsh ischemic culture conditions. In basal medium, which contained different concentrations of E2, cell viability was generally enhanced, compared to basal medium to which PSP was added. E2 most effectively improved cell viability at a concentration of 10^{-4} M, especially at day 7 (Figure 4.5 B). In contrast, the metabolic activity of the cells was not affected by the addition of PSP or E2 (Figure 4.5 C). Based on these results, PSP was omitted in all further experiments to rule out this effect on the viability of the cells.

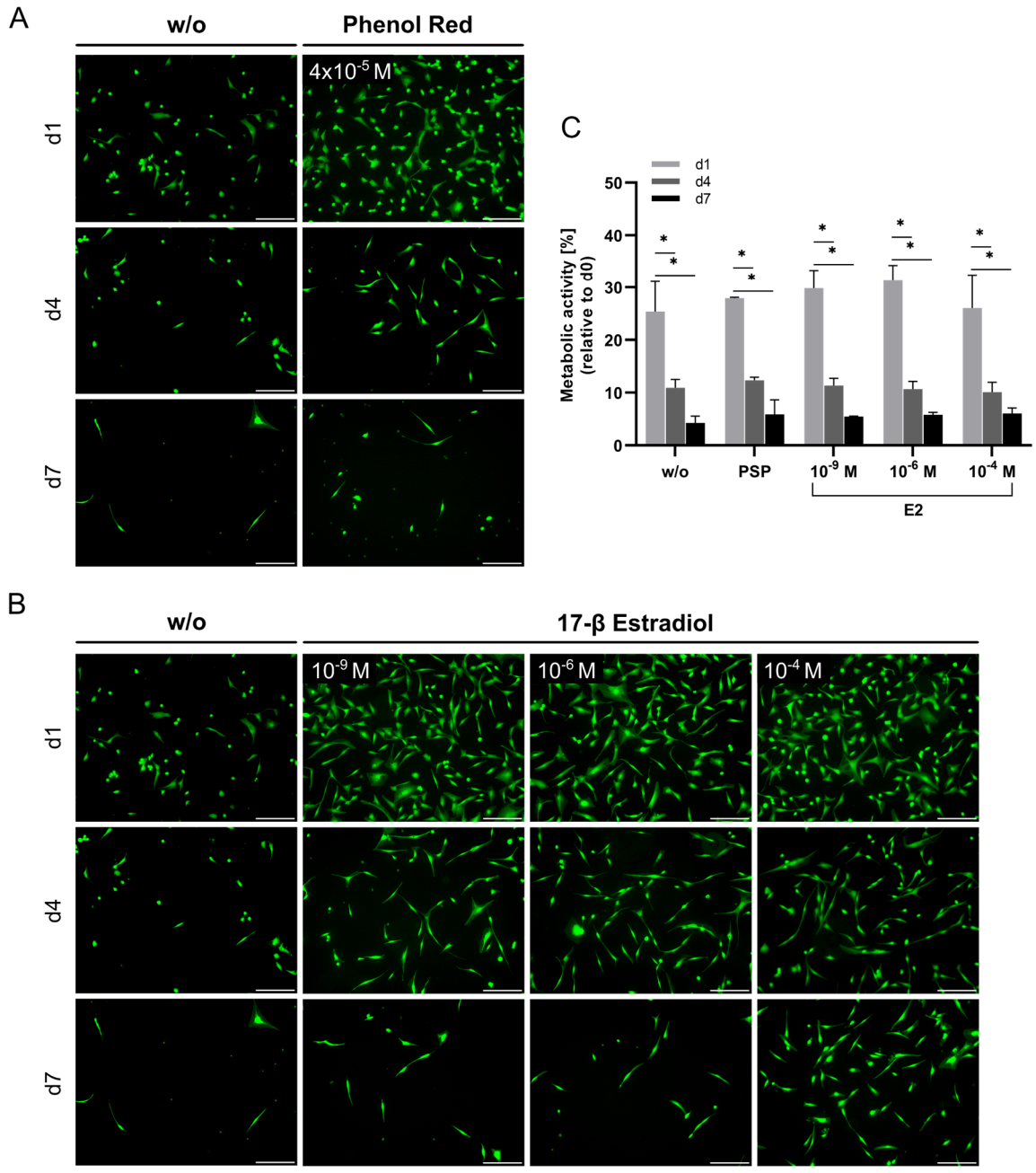

Figure 4.5: Effect of PSP and E2 on viability and metabolic activity of ischemia-challenged ASCs.

(**A**) Qualitative microscopic analysis of live/dead stained ASCs in the absence or presence of PSP and (**B**) in the presence of different E2 concentrations over 7 days under severe hypoxia (0.2% O_2) and complete glucose deprivation determined by live/dead assay. Living cells were stained with calcein (green) and dead cells were stained with ethidium bromide (red). Scale bar represents 200 µm. (**C**) Metabolic activity as determined by a MTT assay. MTT accumulated in ASCs was solubilized and the optical density was measured at 570 nm; % metabolic activity was calculated in relation to the mean value of day 0. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviations: MTT: 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide; E2: 17-β Estradiol; PSP: Phenol Red; w/o: without.

4.1.5 Role of glucose under severe hypoxia

To assess the impact of glucose concentration in the harsh ischemia-mimicking model, the viability and secretory potential of ASCs in the presence of 0.5 and 0.1 g/L glucose during severe hypoxia (0.2% O_2) over 7 days of culture was investigated. Glucose is an essential component for cell survival in ischemia [367,371]. As it was shown that PSP positively influences the viability of ASCs in case of complete glucose deprivation, PSP was omitted in this set-up. As the complete omission of glucose in a medium without PSP led to a massive cell death already at day 1, we aimed to adjust the glucose concentration for the harsh ischemic condition in order to prevent immediate cell death. Therefore, the influence of very low glucose concentrations (0.1 and 0.5 g/L) on cell survival over 7 days was assessed by live/dead staining. Similar cell numbers were observed independent of the glucose concentration for all time points (Figure 4.6 A). Even after 7 days with only 0.1 g/L glucose markedly more viable cells were present compared to the culture in the absence of glucose. This observation was well reflected by the assessment of the cell number as well as by the DNA content (Figure 4.6 B). Comparable effects on cell numbers without significant differences between the different glucose concentrations were found. However, the metabolic activity was significantly impaired in the presence of 0.1 g/L glucose in the culture medium compared to 0.5 g/L glucose. Especially at day 7, the metabolic activity of cells cultured in the presence of 0.1 g/L glucose decreased to approx. 10% of the activity measured in control-cultured cells, whereas cells that were cultured in the presence of 0.5 g/L glucose showed more than 40% metabolic activity (Figure 4.6 C). To gain further insight into the available residual exogenous glucose levels in the cultures under the different glucose concentrations, the glucose levels in the culture media were analyzed at different time points of culture. The presence of 0.1 g/L glucose under severe hypoxia led to a rapid decline in the residual available glucose within the first 3 days of culture followed by complete exhaustion by day 4. In contrast, the cultures supplemented with 0.5 g/L glucose demonstrated slightly decreased exogenous glucose levels that were not depleted over the course of 7 days. The corresponding lactate levels were significantly increased in 0.5 g/L compared to 0.1 g/L glucose cultures at all time points. Moreover, these data revealed a shift to anaerobic glycolysis under sustained oxygen deprivation (Figure 4.6 D). A gradual increase of lactate was observed until day 4 corresponding to the complete exhaustion of glucose.

Taken together, these results demonstrated the key role of glucose for cell survival under ischemic conditions and showed, that even very low glucose concentrations (0.1 g/L) support the viability of cells under severe hypoxia.

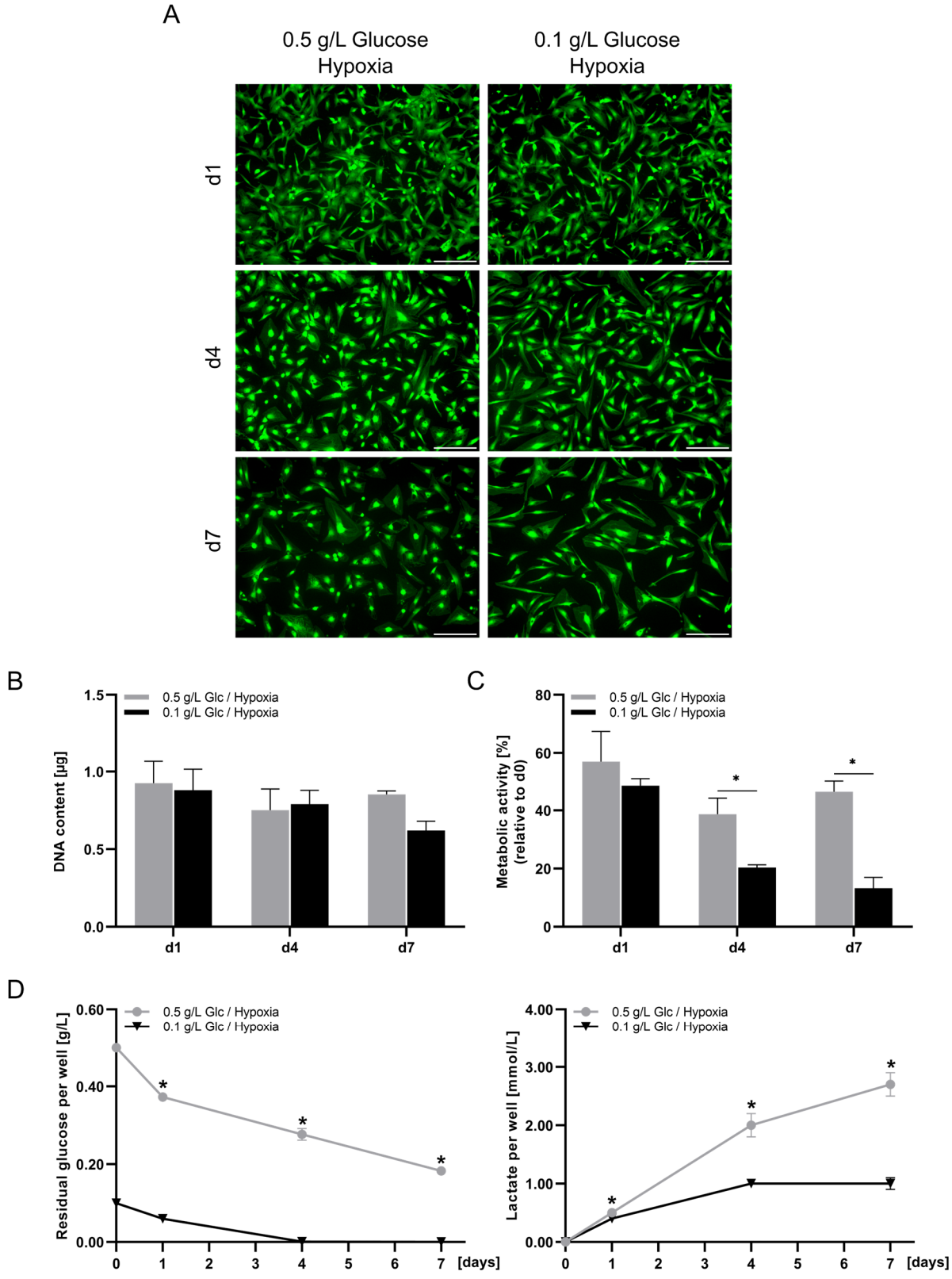

Figure 4.6: Effect of different glucose concentration under severe hypoxia.

(**A**) Viability of ASCs at different time points. Analysis of viable cells stained green (calcein) and dead cells, stained red (ethidium bromide). Scale bar represents 200 µm. (**B**) Quantitative determination of total DNA content. (**C**) Metabolic activity as determined by a MTT assay. MTT accumulated in ASCs was solubilized and the optical density was measured at 570 nm;

% metabolic activity was calculated in relation to the mean value of day 0. (**D**) Time course of exogenous glucose levels per well in the supernatant media (left) and of the respective lactate production per well (right). Data are presented as means ± SD of n = 3; * p < 0.05. Abbreviation: MTT: 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide.

To further investigate the influence of glucose on the secretory function of ASCs, the concentration of selected cytokines (IL-6, VEGF, and STC-1) in the culture supernatants using ELISA assays at different time points was quantified (Figure 4.7). Day 7 was included in the analysis to monitor the secretory activity of cells that were completely deprived of glucose. Results showed that the glucose concentration significantly influenced the secretion of the factors. In the presence of 0.1 g/L glucose the concentration of IL-6, VEGF, and STC-1 in ASC cultures was significantly increased compared to 0.5 g/L glucose. Moreover, the cultivation of cells with initially 0.1 g/L glucose, yielded markedly increased secretion levels of all factors at day 4 and day 7, when glucose was exhausted in the culture medium. These data demonstrate that glucose represents an important factor influencing the secretion of IL-6, VEGF, and STC-1 by ASCs under hypoxic conditions.

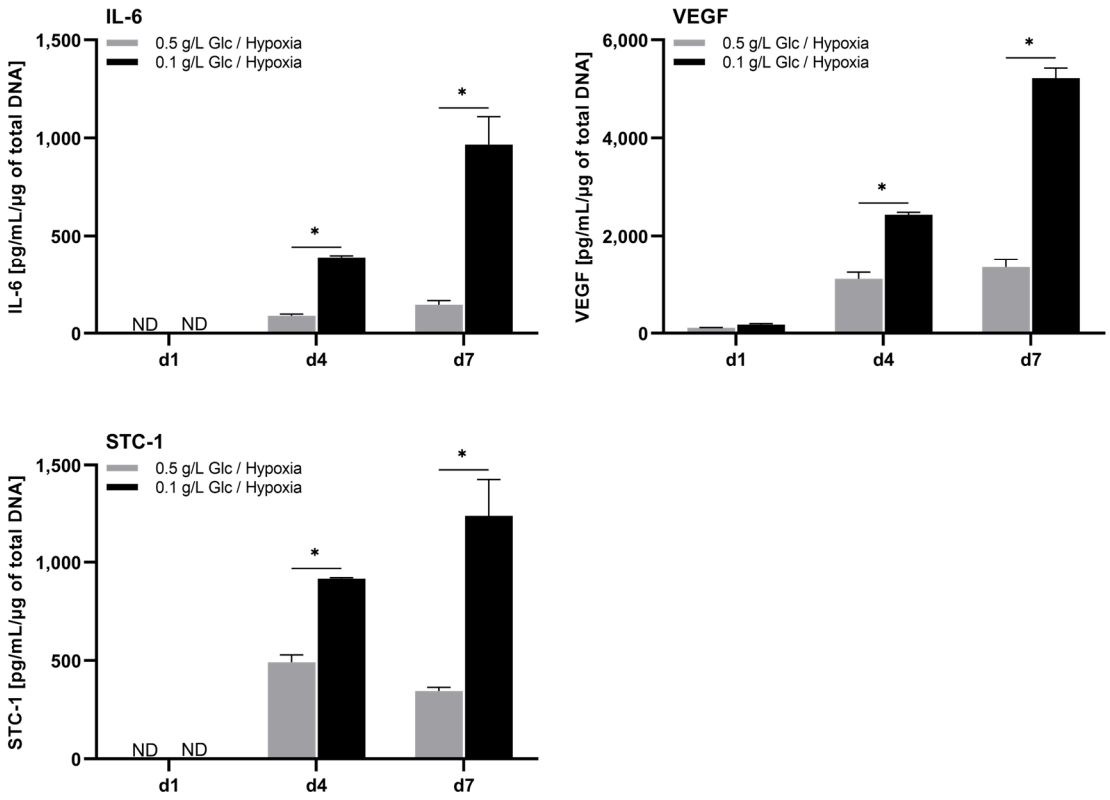

Figure 4.7: Effect of glucose on secretion of anti-apoptotic, angiogenic and immune-modulatory factors.
Enzyme-linked immunosorbent assays for IL-6, VEGF, and STC-1 in the medium of ASCs cultured under severe hypoxia (0.2% O_2) with either 0.5 or 0.1 g/L glucose at different time points of culture; the values were normalized to the DNA content of the respective samples. Data are presented as means ± SD of n = 3; * p < 0.05. Abbreviations: Glc: Glucose; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; STC-1: Stanniocalcin-1; ND: Not detectable.

4.1.6 Discussion

ASCs represent a promising source for cell-based therapies because of their widely acknowledged capacity to support regenerative functions in damaged tissues [134,175,377]. Due to their multi-potency, ASCs can differentiate into various cell types e.g. adipocytes or endothelial cells and in this way contribute to *de novo* tissue repair [93,378,379]. However, in transplantation settings these functions may not play a significant role, as it has been shown, that transplanted ASCs survive for only a short period *in vivo.* The transplantation site is characterized by an initial lack of blood supply, leading to an ischemic microenvironment, which is considered to be detrimental to cells [380]. The primary aim of this chapter was to establish an *in vitro* model that mimics the hypoxic environment as well as nutrient deprivation experienced during the early phase of engraftment. In order to study the response of ASCs to different starvation conditions, harsh ischemic conditions, with complete deprivation of glucose and serum and exposure to 0.2% oxygen and moderate ischemic conditions, which were set to 0.5 g/L glucose, 0.5% serum and 2% oxygen, were therefore chosen. Although it is challenging to determine the accurate oxygen tension that a cell might experience occurring at the transplantation site, we decided in the reported set-up for an oxygen tension of 0.2% (harsh ischemic set-up) as Moya et al. [370] reported that 0.13% oxygen best reflected 24 hours post-implantation due to the non-existent blood supply within transplanted constructs. In the moderate ischemic set-up, 2% oxygen were selected to simulate the periphery of tissue engineering constructs.

The data presented in this chapter demonstrate that in the absence of glucose under severe hypoxia, the viability of ASCs was markedly reduced. Within 7 days of culture under harsh ischemic conditions, the number of viable cells, as well as the metabolic activity decreased drastically compared to moderate ischemic conditions. This observation is consistent with studies that reported a rapid cell death in the absence of glucose and serum [367,371,381], whereas the viability of ASCs was shown not to be affected in the presence of minimal concentrations of glucose and serum (moderate ischemic conditions) [341,368]. With regard to oxygen deprivation, HIF-1 alpha plays an important role as hypoxia-regulated factor and represents a central controller of oxygen-related gene expression [372,382,383]. For this reason, the expression of HIF-1 alpha was analyzed and it could be shown that it was expressed in ASCs under hypoxic conditions, thereby confirming the hypoxic status of the cells for both ischemia-mimicking conditions for 0.2% as well as 2% oxygen during the whole culture period.

Next, the secretion of a set of angiogenic, anti-apoptotic, and immune-modulatory factors by ASCs in response to harsh or moderate ischemic conditions was examined. The focus was on IL-6 and VEGF, as known major immunomodulatory and angiogenic factors [195,384–387],

and on STC-1, which is a peptide hormone reported to be involved in reduction of apoptosis, enhanced resistance to hypoxia and metabolic stress [349–351,388,389].To date nothing is known about the function of STC-1 in ASCs. Our results demonstrated distinct response patterns for the factors to the different ischemia-mimicking conditions. The concentration of IL-6, VEGF, and STC-1 in the ASC-culture medium was markedly increased in the absence of glucose and serum combined with severe hypoxia over a period of 4 days. In contrast, the concentration of the secreted factors in the culture medium under moderate ischemic conditions was almost identical to the control condition except STC-1, which was not detectable under control conditions. Accordingly, the gene expression analysis of all factors was immediately upregulated in response to harsh ischemic conditions. These findings are in line with the literature where a variety of studies describe in diseases with different ischemic pathologies a strong increase in the expression of several cytokines and chemokines [390–392]. Perera et al. [393] also revealed an increasing cytokine expression already after a few hours associated with the recruitment of different types of cells, including neutrophils, lymphocytes, monocytes, as well as activation of resident microglia, astrocytes, and endothelial cells into the ischemic area. Further approaches show that the cytokine and chemokine signaling is related to the inflammatory response. Thus, the intercommunicative actions of both results in a complex of feedback loops and cascades by interacting with specific membrane-associated receptors with an extracellular ligand-binding region and an intracellular region that is activated by binding of cytokines and chemokines and thus contribute to the mediation of signals from the cytoplasm to the nucleus [392,394]. In recent years, the multifaceted role of the complement system by regulation, orchestration, and amplification of the immune response by regulating tissue repair has become of increasing interest. The complement activation suggesting a release of a variety of endogenous ligands (DAMPs) to stimulate innate immune response [395]. The inducing inflammatory cell recruitment, including neutrophils and macrophages, and the release of various cytokines and chemokines is attributed to the cleavage of C3 and C5 and the subsequent release of anaphylatoxins (C3a and C5a) [396,397]. Recent research indicates that classically activated M1 macrophages may lead to the expression of MHC class II antigens and release of pro-inflammatory cytokines with further propagating inflammation, whereas M2 macrophages secrete several trophic factors promoting tissue regeneration and repair by cell proliferation, reduced apoptosis, and by stimulating angiogenesis [398]. It may be that due to the harsh ischemic conditions, ASCs initially directly secrete this panel of factors, the immune system is thereby activated, and macrophages additionally support tissue regeneration by the release of various cytokines and chemokines. Taken together this part, moderate ischemic conditions were well tolerated by ASCs. The viability under this condition was not affected and the gene expression and secretion of anti-apoptotic, angiogenic and immune-modulatory factors was

only marginally elevated. In contrast, harsh ischemic conditions markedly affected the viability of the cells, in parallel gene expression and secretion of factors was significantly upregulated in the absence of glucose and oxygen. Thus, harsh ischemic conditions severely affect the cells, and lead to an increased secretory activity of angiogenic, anti-apoptotic, and immune-modulatory factors.

By testing different medium compositions, we found out that PSP in the culture medium affected the viability of ASCs in our harsh ischemic culture set-up. Based on the estrogenic activity of PSP described in the literature at concentrations found in tissue culture medium [375,376], we investigated specifically the impact of PSP and E2 on ischemia-challenged ASCs viability. The results revealed that at the beginning of the culture markedly more viable cells could be detected with PSP as compared to the PSP-free medium. E2 has a multifunctional role influencing cell proliferation, differentiation, and metabolism in many tissues [399]. Previous studies reported about at least two estrogenic receptors, which are expressed in ASCs and mediated estrogenic actions, with different impacts dependent on the cellular context [400]. Zhang et al. [401] focused on determining the effects of both receptors ERα and β on male mouse ASCs. They demonstrated an enhanced ASC proliferation and migration with dose-dependent effects. Celojevic et al. [402] investigated the influence of physiologic concentrations of E2 ranging from 0.1–10 nM on cell viability of human lens epithelial cells. We further analyzed the viability of ASCs exposed to harsh ischemic conditions with different concentrations of E2, ranging from 10^{-9}-10^{-4} M, added to basal medium. Our concentrations were approx. in line with reported concentrations, however there also exists differences in the E2 receptors of ASCs in males and females [399]. The here presented results show that physiological concentrations of E2 (10^{-9} M) resulted in markedly better viability in the early phase of the harsh ischemic culture, comparable to PSP, whereas slightly higher concentrations of E2 (10^{-6} and 10^{-4} M) prevent cell death until day 7 under this condition, but the metabolic activity was not affected by the addition of PSP or E2 at all times. This observations may be due to the markedly reduced metabolism of the resting cells under complete withdrawal of glucose and serum exposed to severe hypoxia [403]. Altogether, the results give insights that PSP as well as to E2 restores viability of ASCs under ischemia. For this reason, it is important to be aware of this effect of PSP when culturing and analyzing cells under deprivation conditions. However, these results could indicate that estrogen-like compounds may modulate therapeutic effects and could be one possible approach for tissue engineering applications.

In the experiments it was found that cells (cultured without PSP) died very quickly without the availability of glucose. This is in line with other studies that indicate glucose as an essential factor in cell survival [341,371,404]. To study if very low concentrations of glucose can support cellular survival under severe hypoxia, ASCs were cultured with low glucose concentrations

and the viability and consumption of glucose was assessed. It could be demonstrated that ASCs viability remained nearly unaffected with only a low availability of glucose of 0.1 g/L, independent of the severe hypoxic environment. Thus, these results well reflect the observations according to that severe hypoxia (i.e. 0.2%) per se is not detrimental to MSCs [367,405] and that nutrient deprivation is the stronger factor that constitutes the main limitation for MSC survival after transplantation [345]. With 0.1 g/L glucose, a complete exhaustion from day 4, while 0.5 g/L glucose were not limiting over the period of 7 days, was observed. The significantly increased levels of the secreted factors in the medium with the lowest glucose concentration (0.1 g/L) indicated an effect of glucose deficiency on the secretion of ASCs.

Overall, the results of this chapter contribute to a first characterization of ASCs under ischemic conditions. By studying the viability and secretory potential of ASCs under different ischemia-mimicking conditions, it was shown that the cells were capable to maintain their viability and secretory function even under harsh ischemic conditions in the early culture phase. The focus was on the factors IL-6 and VEGF, which are widely described in the literature in association with tissue regeneration why these factors were examined exemplarily in this work. Thereby, STC-1 was additionally selected as a particular factor, which has not been described in literature so far in relation to ASCs. The established *in vitro* model of ischemia with ASCs facing both severe glucose and oxygen starvation, allows further investigations on the impact of glucose and oxygen deprivation on the secretory capacity of ASCs exposed to an ischemic environment by, for example, using a protein array, to figure out other key factors in this context. However, additional work on molecular mechanisms as well as on the role of complement in this context would also be of paramount importance to understand why particularly ASCs are able to exert beneficial effects and improve cell-based therapies in tissue regeneration or tissue repair.

4.2 Ischemia-like stress conditions stimulate trophic activities of adipose-derived stromal/stem cells

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Adipose tissue-derived stromal/stem cells (ASCs) represent a valuable tool for cell-based therapies because of their widely acknowledged capacity to exert beneficial functions in tissue regeneration or in tissue repair [86,134,175,354,377,406]. This has been demonstrated for example for cell-assisted lipotransfer, where autologous ASCs added to lipografts have been shown to enhance vascularity, to improve the survival rate of grafts, and to reduce postoperative atrophy [316,328,340]. The benefits of ASCs in this context were mainly attributed to secreted paracrine factors rather than to direct differentiation into tissue-specific cell types [178,407]. In recent years, numerous studies have shown that ASCs secrete a complex panel of trophic factors, including growth factors, cytokines, chemokines, extracellular microvesicles and exosomes, that contribute to angiogenesis, anti-apoptosis, immunomodulation, and the activation of resident and circulating stem cells [127,178,197,336,360,407–409]. However, in regenerative approaches such as tissue engineering or cell-assisted lipotransfer, the implanted cell-laden construct or lipograft is at least initially impaired by a lack of blood supply [244,245]. This leads to an ischemic environment that is characterized by the deprivation of nutrients, oxygen, and growth factors. It has been shown that depletion of oxygen and nutrients, in particular glucose, significantly affects cell survival and function, as they are both critically required for energy-related pathways [341,367,370].

To date, the response of ASCs to ischemic stress and the different components of ischemia remains poorly understood. Only a few studies so far examined the viability and metabolic response of ASCs to combined oxygen and glucose deprivation, but they did not focus on their secretory function under this condition [368,369]. The modulation of the paracrine activity of ASCs by low oxygen concentrations is well documented. However, little is known about how nutrient deprivation, a further major component of ischemic stress, can affect their secretory potential.

In this context, the present chapter aimed to investigate the effect of glucose and oxygen deprivation on the viability, metabolic activity, and secretory capacity of ASCs. Specifically, we focused on glucose starvation in concert with hypoxia as a potential modulator of the paracrine function of ASCs. In response to combined glucose and oxygen deprivation, ASCs demonstrated increased levels of secreted angiogenic and anti-apoptotic mediators including vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), IL-8, angiogenin (ANG), and stanniocalcin-1 (STC-1). We further investigated the impact of conditioned medium of ischemia-challenged ASCs on the viability and tube formation of endothelial cells, and the proliferation and migration of fibroblasts. The results of this study suggest that ASCs can maintain their secretory function and thus exert regenerative effects even under ischemia-like stress conditions.

4.2.1 Characterization of ASCs under glucose/oxygen deprivation

Viability and metabolic activity of ASCs might be strongly dependent on the availability of glucose and the presence of oxygen. The impact of glucose and oxygen deprivation, two conditions that are expected to occur in an ischemic situation, were investigated individually and in concert. For this purpose, the cells were exposed to culture conditions represented by glucose starvation (0.1 g/L glucose) and hypoxia (0.2% O_2) under serum-free conditions for a period of seven days.

Viability of the cells was examined by live/dead staining. Under glucose deprivation or hypoxia, a similar viability was observed as in the control group (1 g/L glucose, 21% O_2, without serum) with cells retaining their typical stellate morphology (Figure 4.8 A). Even under combined glucose and oxygen deprivation, a considerable number of viable cells were still present after seven days. Dead (red) cells were hardly detectable in this set-up, as they rapidly detached from the cell-culture plates and consequently were washed out during the staining process. This observation was corroborated by the assessment of the cell numbers as reflected by the DNA content (Figure 4.8 B). Deprivation conditions demonstrated comparable effects on cell numbers without significant differences between the investigated culture conditions. Across all tested conditions the DNA content decreased to approx. 60% by day 4, likely due to the lack of serum in the medium and remained stable until day 7. In contrast, the metabolic activity of the cells, determined using the MTT assay, was significantly impaired by the limitation of glucose (0.1 g/L) already at day 1 and also at day 4, independent of the oxygen concentration (Figure 4.8 C). As the MTT assay is only an indicator of mitochondrial dehydrogenase activities, WST-8 assay was additionally performed to evaluate the metabolic rate of the cells by other energy metabolism-related dehydrogenases in cytosols (Figures 4.8 D).

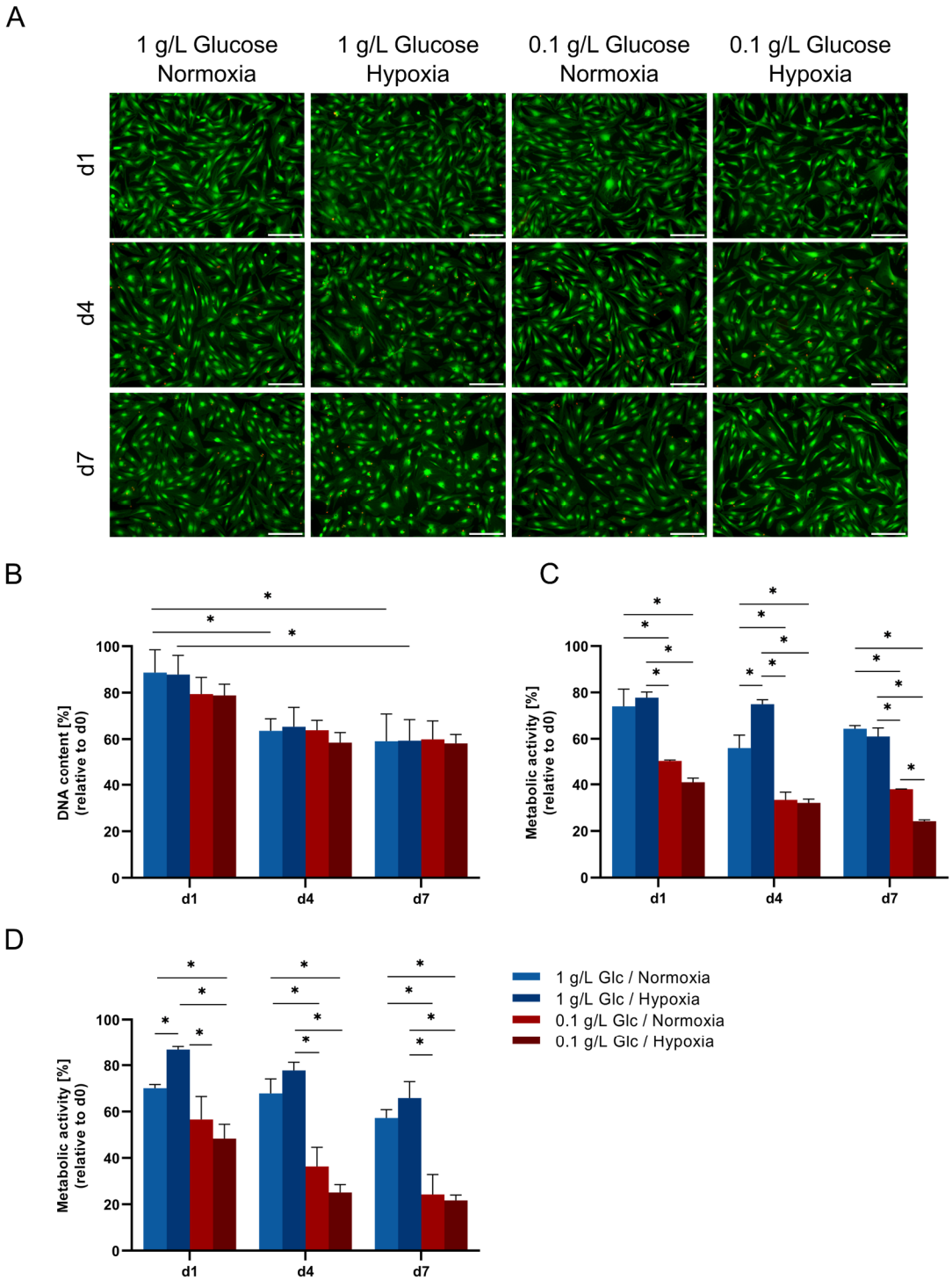


Figure 4.8: Viability and metabolic activity of human adipose-derived stromal/stem cells (ASCs) under glucose and oxygen deprivation.

(**A**) Microscopic images of live/dead staining. 25,000 cells per cm^2 were seeded in well plates and imaged at the indicated time points. Living cells were stained with calcein (green) and dead cells were stained with ethidium bromide (red). Scale bar represents 200 µm. (**B**) Quantitative determination of total DNA content in relation to the mean value of day 0. Metabolic activity as determined by a MTT assay (**C**); MTT accumulated in ASCs was solubilized and the optical density was measured at 570 nm and (**D**) by WST-8 assay; % metabolic activity was calculated in relation

to the mean value of day 0. Data are presented as means ± SD of n = 3; * p < 0.05. Abbreviation: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WST-8: 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.

In addition, we examined whether ASCs were still able to differentiate adipogenically after being subjected to ischemia-like stress conditions. For this purpose, ASCs were cultured under glucose/oxygen deprivation (0.1 g/L glucose, 0.2% O_2) for four days and subsequently differentiated in adipogenic medium. Control ASCs were cultured under standard conditions (growth medium, 21% O_2) and subsequently differentiated in the same way. Lipid accumulation was analyzed histologically and by quantification of intracellular lipid content. Similar amounts of stained lipid droplets were observed in ASCs previously treated with glucose/oxygen deprivation and in control ASCs (Figure 4.9 A). This finding was corroborated by the triglyceride assay, which showed comparable amounts of intracellular triglycerides in both groups (Figure 4.9 B). Thus, ASCs were not affected in their adipogenic differentiation capability after exposure to ischemia-mimicking conditions.

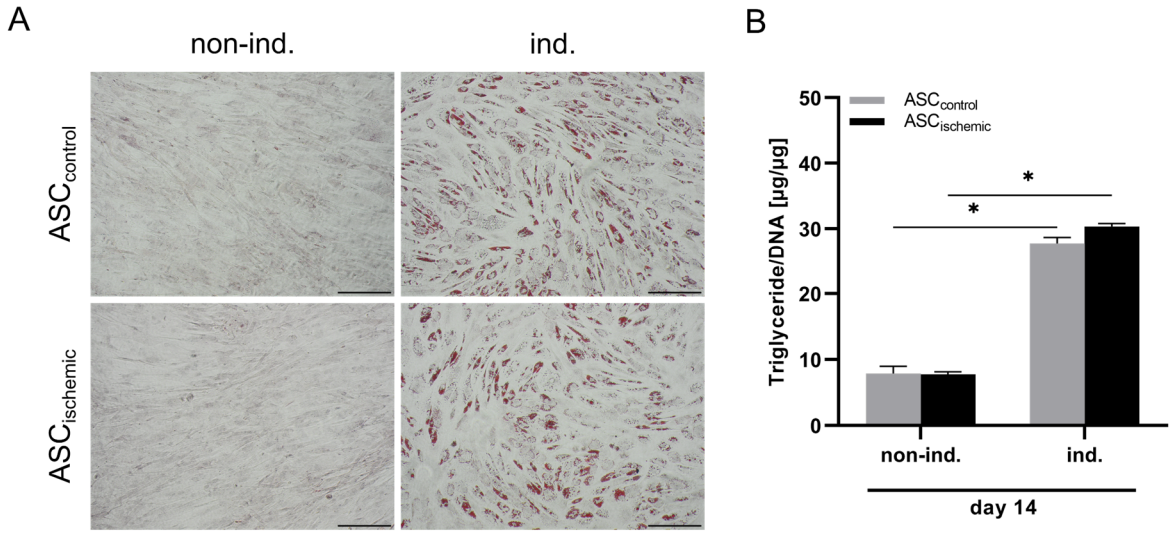


Figure 4.9: Adipogenic differentiation potential of ASCs after exposure to ischemia-like conditions.
$ASC_{ischemic}$ were cultured under glucose/oxygen deprivation (0.1 g/L glucose, 0.2% O_2) for four days before being adipogenically induced. $ASC_{control}$ were cultured in growth medium before induction. (**A**) Histological analysis of adipogenesis after 14 days of induction by staining with Oil Red O (ORO, red) and hematoxylin (blue). Non-induced cells served as controls. Representative images are shown. Scale bar represents 200 µm. (**B**) Quantitative analysis of triglyceride content. Analysis was performed at day 14. Triglyceride contents were normalized to the DNA content of the respective samples. Data are presented as means ± SD of n = 3; * p < 0.05. Abbreviation: Non-ind.: Non-induced; ind.: Induced; $ASC_{ischemic}$: ASCs cultured under ischemic conditions before induction; $ASC_{control}$: ASCs cultured in growth medium before induction.

To monitor available exogenous glucose levels in the cultures under the different conditions, the remaining glucose concentrations in the culture supernatants were analyzed at different time points. The culture of ASCs in the presence of 0.1 g/L glucose and hypoxia led to a rapid decline in the residual available glucose within the first three days of culture followed by complete exhaustion by day 4. In the cultures supplemented with 1 g/L glucose, the exogenous glucose concentration only slightly decreased in the hypoxic condition as compared to normoxia but was not depleted in either condition over the course of seven days (Figure 4.10 A). Corresponding lactate levels were significantly increased under hypoxic conditions irrespective of the glucose concentration in the media indicating a shift to anaerobic glycolysis under sustained oxygen deprivation (Figure 4.10 B). Under combined glucose/oxygen deprivation, a gradual increase of lactate was observed until day 4 corresponding to the complete exhaustion of glucose, followed by a further slight increase until day 7, which potentially indicates the use of endogenous glycolytic reserves of the cells after complete consumption of the glucose present in the medium.

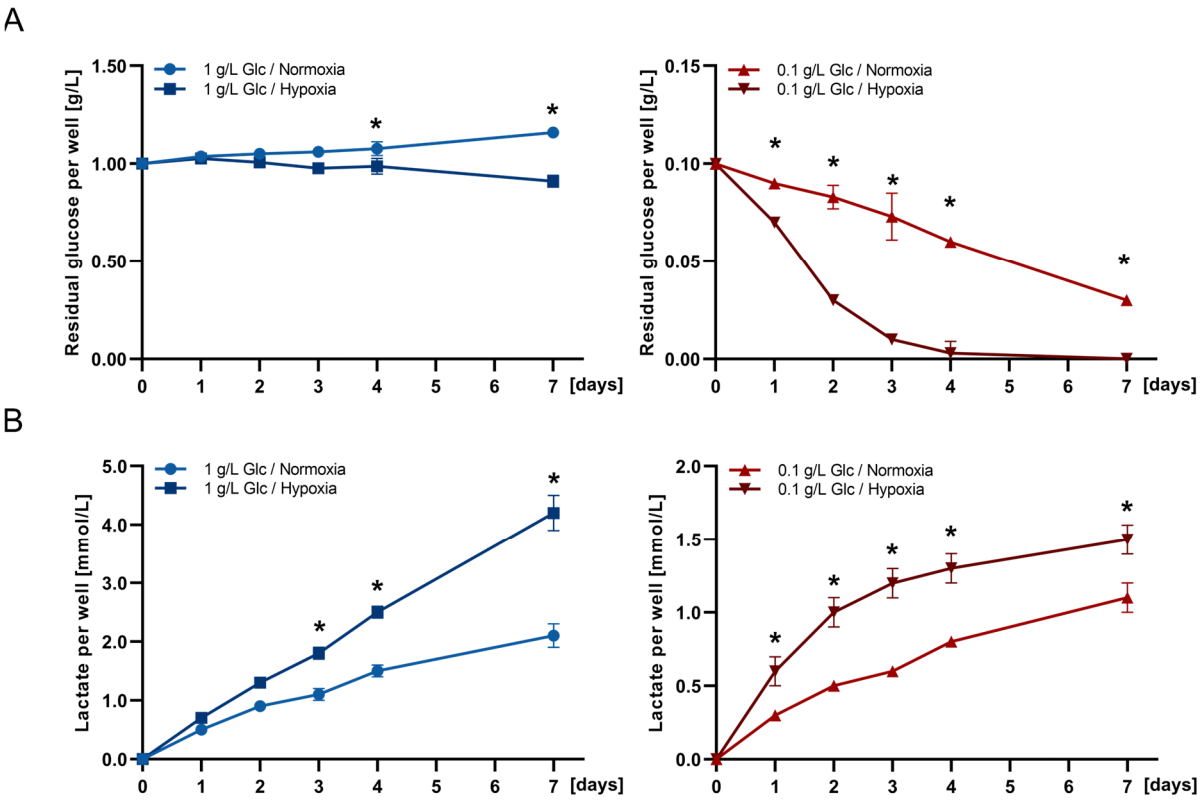


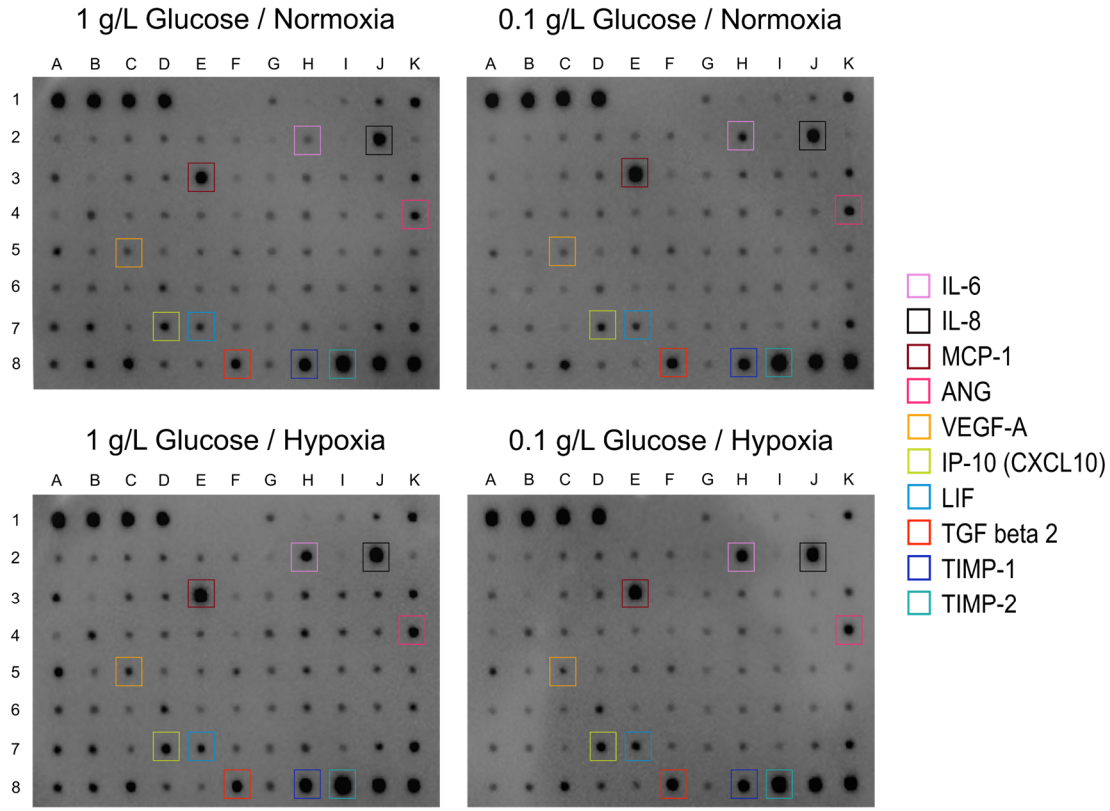
Figure 4.10: Glucose consumption and lactate production of ASCs cultured with 1 or 0.1 g/L glucose exposed to 21% or 0.2% oxygen over seven days.

(**A**) Time course of exogenous glucose levels in the supernatant media (per well). (**B**) Time course of lactate production (per well). Data are presented as means ± SD, n = 3; * $p < 0.05$.

4.2.2 Secretory potential of ASCs under glucose/oxygen deprivation

To gain insight into the secretory potential of ASCs exposed to an ischemic environment characterized by nutrient deprivation in combination with acute hypoxic stress, qualitative secretion profiles were established using a cytokine antibody array covering 80 cytokines. To this end, ASCs were cultured under (1) 1 g/L glucose and normoxia (21% O_2) (control); (2) 1 g/L glucose and hypoxia (0.2% O_2); (3) 0.1 g/L glucose and normoxia (21% O_2); (4) 0.1 g/L glucose and hypoxia (0.2% O_2). Cell culture supernatants were analyzed after four days of culture. It appeared that cells subjected to combined glucose and oxygen deprivation (0.1 g/L Glc, 0.2% O_2) were able to express a considerable number of growth factors and cytokines to a similar extent or higher as compared to control cells (1 g/L Glc, 21% O_2). These factors included angiogenic factors (VEGF, IL-6, IL-8, ANG), matrix-regulating proteins (TIMP-1, TIMP-2), chemokines (MCP-1/CCL2, IP-10/CXCL 10), and others (Figure 4.11 A). Factors that were less pronounced in the glucose/oxygen deprivation condition were also identified, e.g., GRO, FGF-4 and -6, IL-12, IGFBP-2, -3, and -4, NAP-2 (the array map is shown in Figure 4.11 B for identification of the respective spots).

A



B

	A	B	C	D	E	F	G	H	I	J	K
1	POS	POS	POS	POS	NEG	NEG	ENA-78 (CXCL5)	G-CSF	GM-CSF	GRO a/b/g	GRO alpha (CXCL1)
2	I-309 (CCL1)	IL-1 alpha (IL-1 F1)	IL-1 beta (IL-1 F2)	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8 (CXCL8)	IL-10
3	IL-12 p40/p70	IL-13	IL-15	IFN-gamma	MCP-1 (CCL2)	MCP-2 (CCL8)	MCP-3 (CCL7)	M-CSF	MDC (CCL22)	MIG (CXCL9)	MIP-1 beta (CCL4)
4	MIP-1 delta (CCL15)	RANTES (CCL5)	SCF	SDF-1 alpha	TARC (CCL17)	TGF beta 1	TNF alpha	TNF beta (TNFSF1B)	EGF	IGF-1	Angiogenin
5	OSM	TPO	VEGF-A	PDGF-BB	Leptin	BDNF	BLC (CXCL13)	Ck beta 8-1 (CCL23)	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	Eotaxin-3 (CCL26)
6	FGF-4	FGF-6	FGF-7 (KGF)	FGF-9	FLT-3 Ligand	Fractalkine (CX3CL1)	GCP-2 (CXCL6)	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10 (CXCL10)	LIF	LIGHT (TNFSF14)	MCP-4 (CCL13)	MIF	MIP-3 alpha (CCL20)	NAP-2 (CXCL7)	NT-3
8	NT-4	OPN (SPP1)	OPG (TNFRSF11B)	PARC	PLGF	TGF beta 2	TGF beta 3	TIMP-1	TIMP-2	POS	POS

Figure 4.11: Human cytokine antibody array.

(**A**) Array membranes covering 80 cytokines were probed with supernatants of ASCs collected under glucose and oxygen deprivation separately and combined (n = 2). Prominently appearing spots with similar or higher intensity in the deprivation conditions compared to the other were highlighted. (**B**) The map of the array (RayBio® human cytokine array C5, RayBiotech, Norcross, GA) showing the position of 80 human cytokines and positive and negative controls (as available on https://doc.raybiotech.com/pdf/Manual/AAH-CYT-5.pdf) for identification of the respective spots. Reprinted with permission from RayBiotech Inc.

We further quantified the concentration of selected, prominently appearing cytokines (VEGF, IL-6, IL-8, ANG, TIMP-1, MCP-1) from the above panel in the culture supernatants using ELISAs in order to reveal the impact of the individual stress condition (glucose deprivation, hypoxia) on their expression level. STC-1, a peptide hormone, which has been associated with angiogenesis, reduction of apoptosis, and enhanced resistance to metabolic stress [349,350,388], but has so far not been identified as a factor secreted by ASCs, was additionally included in the analysis.

As shown in Figure 4.12 A, increased levels for IL-6, VEGF, STC-1, ANG, IL-8, and TIMP-1 were secreted in response to culture under hypoxic conditions irrespective of the glucose concentration. STC-1 was expressed exclusively under hypoxic conditions. Under normoxic conditions, glucose concentration had no influence on the expression of the selected markers, with the exception of IL-6 and IL-8, which were upregulated under glucose deprivation. Under combined glucose and oxygen deprivation (0.1 g/L glucose, 0.2% O_2), IL-6, VEGF, and STC-1 levels each showed a striking increase that significantly exceeded the level expressed under hypoxia only (1 g/L glucose, 0.2% O_2). This effect was also observed for ANG and IL-8 albeit to a lesser extent. TIMP-1 secretion was not affected by the glucose concentration in the culture medium but was increased by hypoxia only. MCP-1 levels remained largely unaltered under all deprivation conditions.

The protein expression of the factors positively regulated by glucose deprivation in the presence of hypoxia was also mirrored on mRNA level. A significantly upregulated gene expression was shown for IL-6, VEGF, and STC-1 under this condition compared to all other conditions. Moreover, different time courses in the expression of these markers were discernible over a period of seven days. STC-1 showed an immediate upregulation upon culture under deprivation conditions with the highest expression level on day 1, whereas IL-6 expression reached its maximum on day 7 of culture. VEGF expression stayed at a constant level over the time course of seven days in this set-up (Figure 4.12 B).

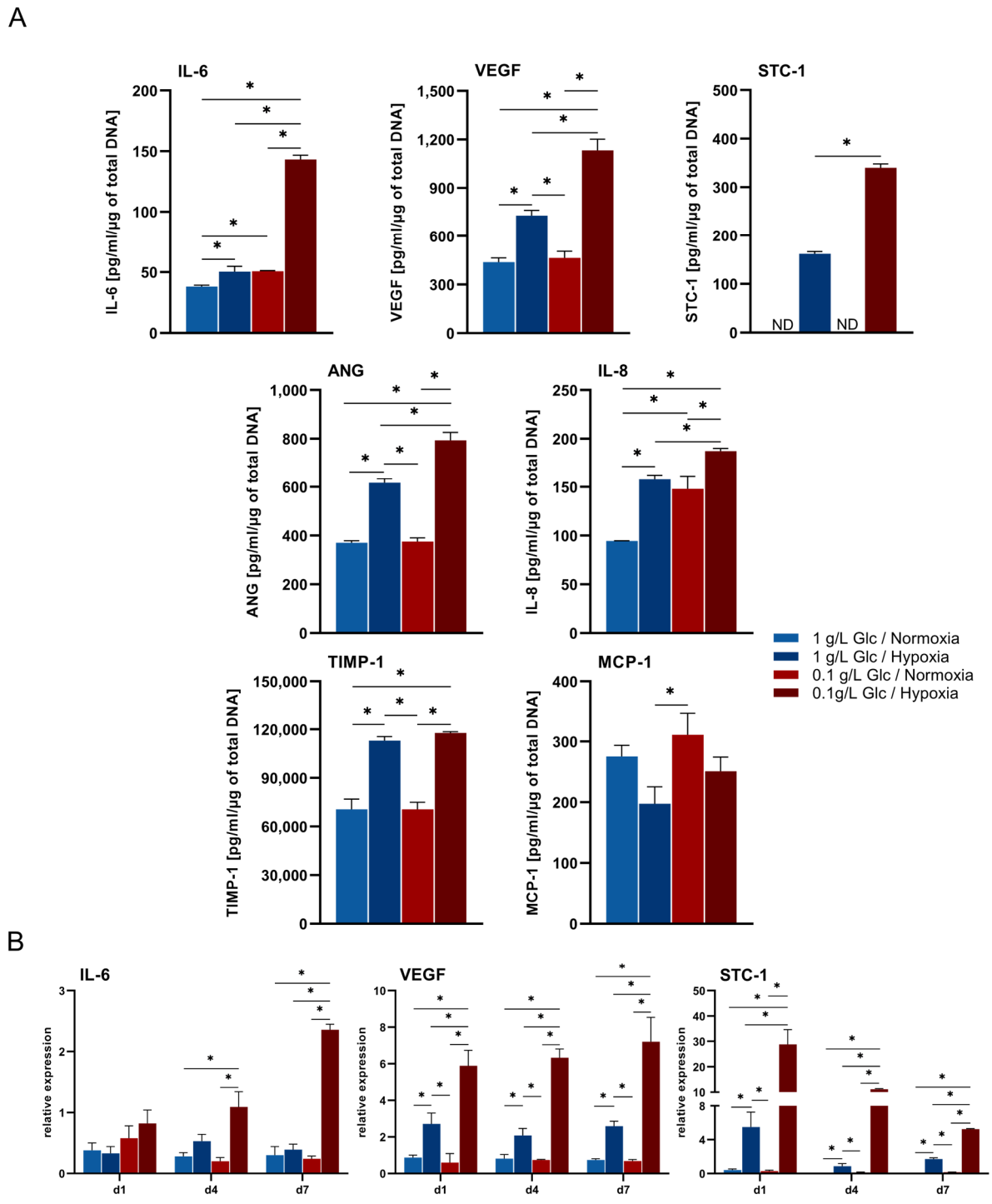


Figure 4.12: Expression of cytokines in ASCs under deprivation conditions.

(**A**) Enzyme-linked immunosorbent assays for IL-6, VEGF, STC-1, ANG, IL-8, TIMP-1, and MCP-1 in the supernatant medium of ASCs cultured under normoxia (21% O_2) or hypoxia (0.2% O_2) with either 1 or 0.1 g/L glucose at day 4 of culture; the obtained values were normalized to the DNA content of the respective samples.(**B**) Corresponding gene expression of IL-6, VEGF, and STC-1. Gene expression was normalized to EF1α; the obtained values were further normalized to day 0. (Note: Day 0 values are the same for all groups.) Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviations: IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; STC-1: Stanniocalcin-1; ANG: Angiogenin; IL-8: Interleukin-8; TIMP-1: TIMP metallopeptidase inhibitor 1; MCP-1: Monocyte chemoattractant protein-1; ND: Not detectable.

In order to gain a deeper insight into how the expression of cytokines depends on the availability of glucose under hypoxia, protein expression levels of IL-6, VEGF, and STC-1 were analyzed in the early phase (until day 4) of culture under deprivation conditions at shorter time intervals. In addition, a later time point (day 7) was included in the analysis to monitor the secretory activity of cells that were completely deprived of glucose. IL-6 showed markedly increased secretion levels under glucose/oxygen deprivation from day 4, when glucose was exhausted in the culture medium (Figure 4.13). Similarly, an enhanced secretion from day 4 was also evident for VEGF in cultures under combined glucose/oxygen deprivation. STC-1 expression was upregulated under glucose/oxygen deprivation on day 3 and demonstrated a further substantial increase until day 7. The expression of the respective factors remained at significantly lower levels under all other conditions.

These data revealed that ASCs were able to maintain their secretory capacity even under severe glucose/oxygen deprivation over a time course of seven days. Glucose deprivation associated with hypoxia was shown to be an important factor influencing the secretion of the pro-angiogenic and anti-apoptotic factors IL-6, VEGF, and STC-1.

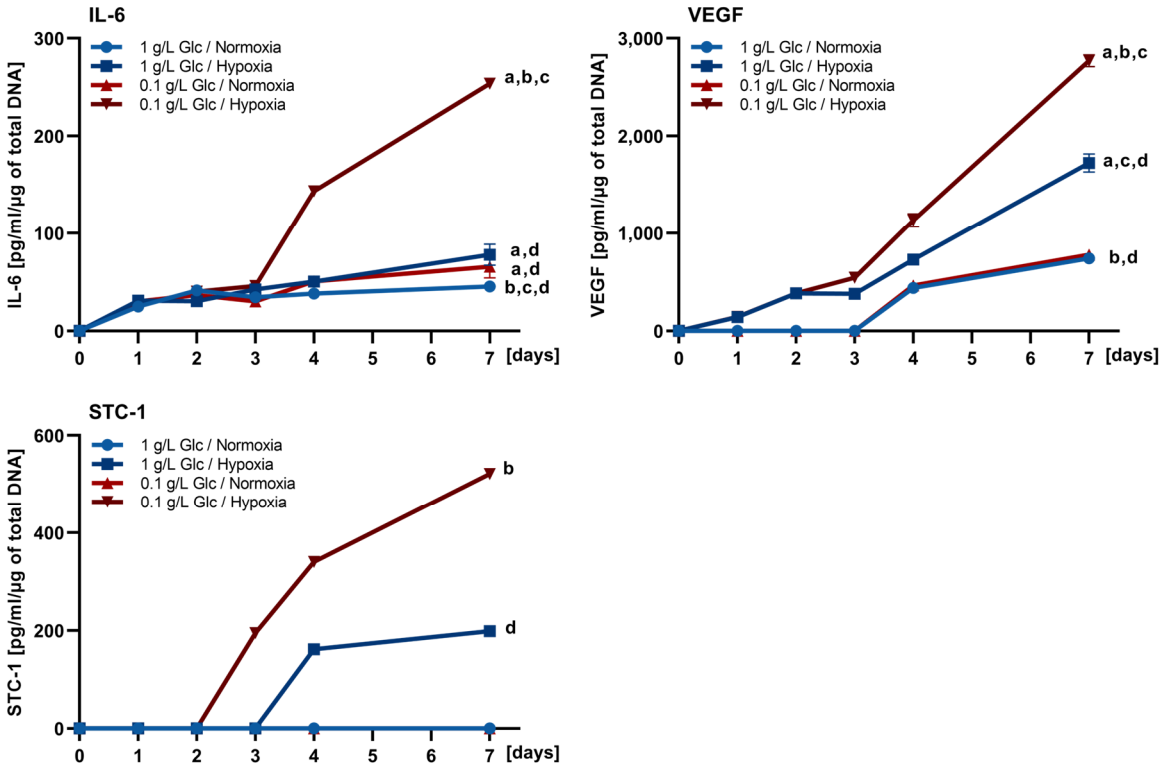


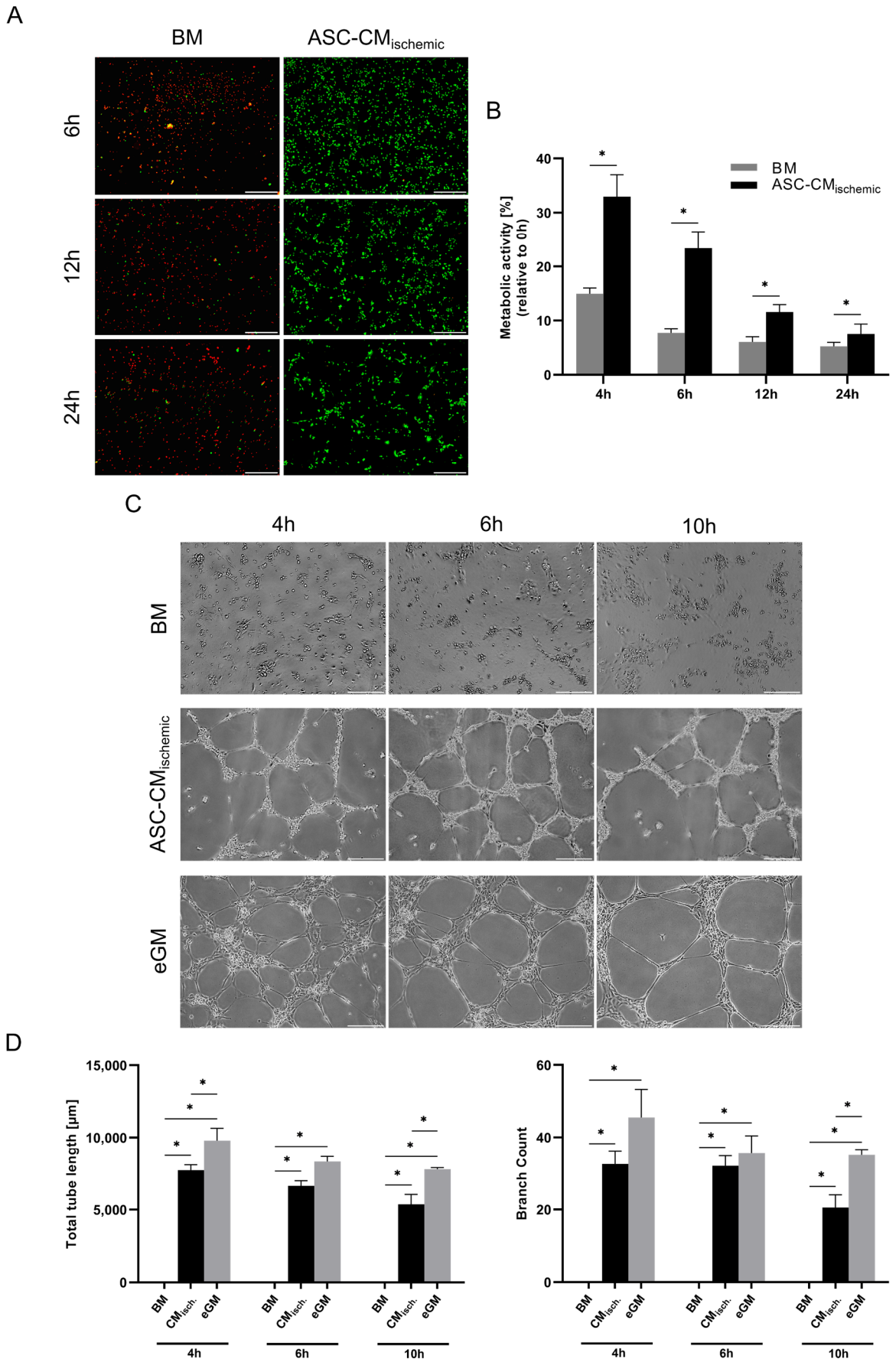
Figure 4.13: Time course of secretion of IL-6, VEGF, and STC-1 monitored over seven days under different deprivation conditions and quantified by ELISA.

The obtained values were normalized to the DNA content of the respective samples. Data are presented as means ± SD of n = 3; (a) significantly different to the value of group 1 g/L glucose and normoxia at day 7, (b) significantly different to the value of group 1 g/L glucose and hypoxia at day 7, (c) significantly different to the value of group 0.1 g/L glucose and normoxia at day 7,

(d) significantly different to the value of group 0.1 g/L glucose and hypoxia at day 7; * $p < 0.05$; for clarity, significance is only shown for day 7. Abbreviations: IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; STC-1: Stanniocalcin-1; ELISA: Enzyme-linked immunosorbent assay.

4.2.3 Regenerative effects of conditioned medium from glucose/oxygen-deprived ASCs

We next sought to study the angiogenic properties of conditioned medium from glucose/oxygen-deprived ASCs as this condition corresponds to the ischemic situation at the transplantation site. To this end, we cultured human umbilical vein endothelial cells (HUVECs) in basal medium conditioned by ASCs under glucose/oxygen deprivation and compared them to HUVECs cultured in basal medium. Viability, metabolic activity, and tube formation were assessed. Live/dead staining revealed a distinctly better viability of HUVECs in the presence of conditioned medium over a period of 24 h. In comparison, HUVECs in basal medium died rapidly, after 6 h no viable cells could be detected (Figure 4.14 A). The positive impact of the conditioned medium from glucose/oxygen-deprived ASCs was also reflected in the metabolic activity of the endothelial cells. The metabolic activity determined by a MTT assay was significantly higher in the ASC-conditioned medium than in the basal medium (Figure 4.14 B). The pro-angiogenic effect of secreted factors from glucose/oxygen-deprived ASCs was further analyzed by a tube formation assay on growth factor-reduced matrigel. Figure 4.14 C illustrates the distinct formation of tubular networks after incubation with conditioned medium at different time points (4, 6, and 10 h). In the unconditioned basal medium, HUVECs were not able to develop tube-like structures. The tube formation in endothelial growth medium is shown as a positive control. Accordingly, the tube length and the number of branch points were significantly higher at all time points in the conditioned medium group as compared to the basal medium-treated group (Figure 4.14 D). These results indicated that ASCs exposed to ischemia-mimicking conditions were able to restore endothelial cell viability, metabolic activity, and tube formation via their trophic function.



(Figure legend on the next page)

Figure 4.14: Effect of conditioned medium of glucose/oxygen-deprived ASCs ($CM_{ischemic}$) on viability and angiogenesis of human umbilical vein endothelial cells (HUVECs).

(**A**) Live/dead staining at different time points. Viable cells were stained with calcein (green), and dead cells were stained with ethidium bromide (red) at different time points. Scale bar represents 500 µm. (**B**) Determination of metabolic activity by a MTT assay; % metabolic activity was calculated in relation to the mean value of day 0.(**C**) Tube formation by HUVECs in basal medium, ASC-$CM_{ischemic}$ and endothelial growth medium on growth factor-reduced matrigel. Representative micrographs were shown at different time points (4, 6, and 10 h) for illustration of tube formation. Scale bar represents 200 µm. (**D**) Total tube length (left column) and the number of branch points (right column) determined by an automated image analyzer. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviations: BM: Basal medium; eGM: Endothelial growth medium; ASC-$CM_{ischemic}$: Adipose-derived stem cell-conditioned medium of glucose/oxygen-deprived ASCs.

In addition, the effect of the secreted factors from ischemia-challenged ASCs on the proliferation and migration of fibroblasts was investigated, since the activation of fibroblasts, in addition to angiogenesis, is considered as an important factor in wound healing processes. In the presence of conditioned medium from glucose/oxygen-deprived ASCs, the proliferation (reflected by the DNA content, Figure 4.15 A) and metabolic activity (as assessed by the MTT assay, Figure 4.15 B) of fibroblasts was markedly enhanced over a period of seven days. The migration assay further illustrates a clear stimulating effect of conditioned medium from glucose/oxygen-deprived ASCs on the migratory activity of fibroblasts compared to the basal medium. The migration of fibroblasts in fibroblast growth medium is shown as a positive control (Figure 4.15 C). Thus, ASCs in an ischemic environment might be able to exert positive effects on wound healing processes through the promotion of neo-vascularization and the activation of fibroblasts in terms of proliferation and migration. In contrast, the growth rate (Figure 4.16 A) and metabolic activity (Figure 4.16 B) of breast cancer cell lines (MCF-7 and MDA-MB-231) were not significantly increased by the trophic factors of ASCs under glucose/oxygen deprivation (except for the metabolic activity in MDA-MB-231 on day 1). This may be relevant in cell-assisted lipotransfer after mastectomy, a scenario in which ischemic conditions at the transplant site are likely to occur.

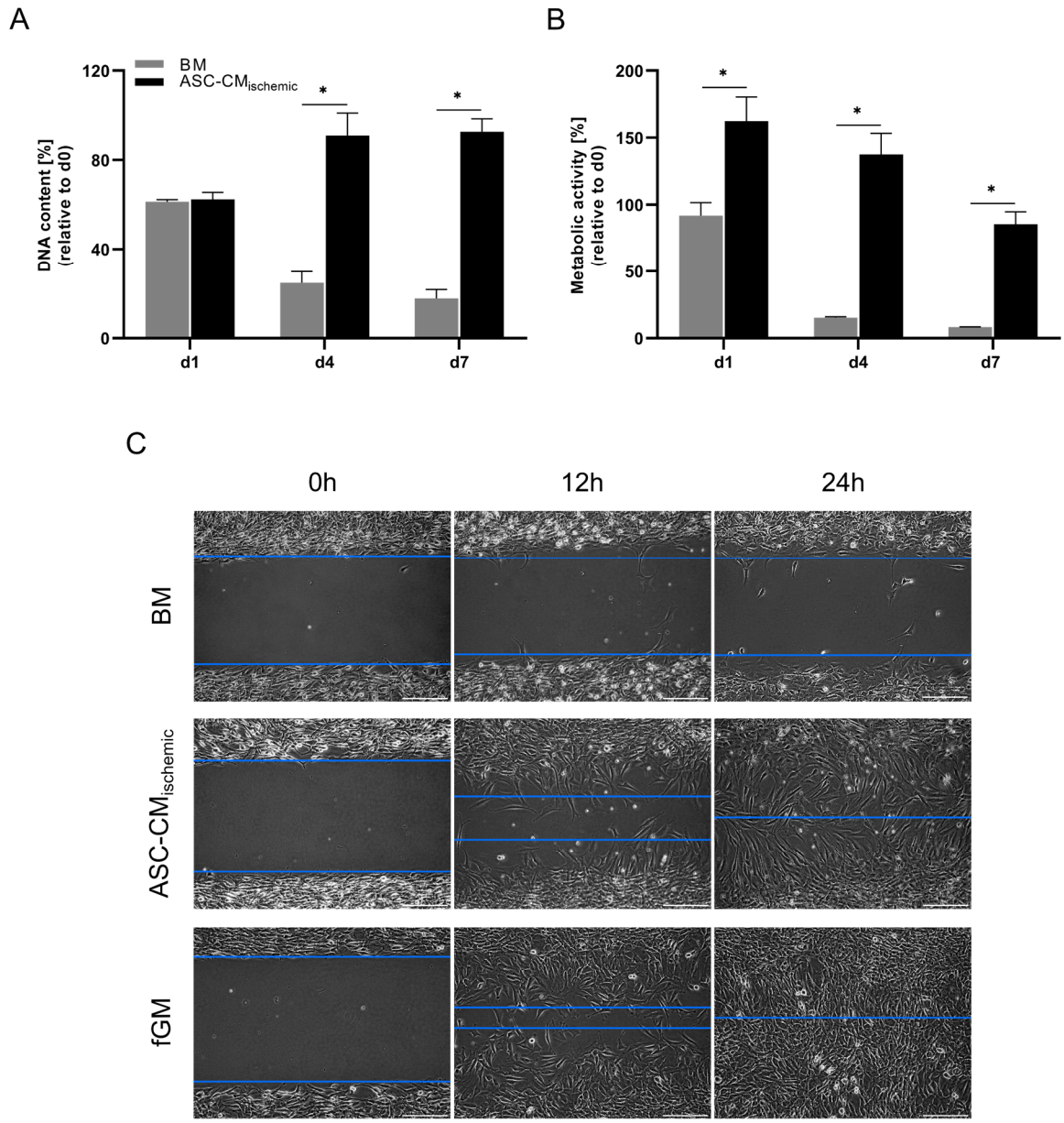


Figure 4.15: Effect of conditioned medium of glucose/oxygen-deprived ASCs ($CM_{ischemic}$) on proliferation, metabolic activity, and migration capacity of NIH/3T3 fibroblasts.

(**A**) Quantitative determination of total DNA content in relation to the mean value of day 0. (**B**) Metabolic activity as determined by a MTT assay. MTT accumulated in fibroblasts was solubilized and the optical density was measured at 570 nm; % metabolic activity was calculated in relation to the mean value of day 0. Data are presented as means ± SD of n = 3; * $p < 0.05$. (**C**) Migration of fibroblasts in conditioned medium from glucose/oxygen-deprived ACSs in comparison to the basal medium. Fibroblast growth medium served as a positive control. Representative micrographs at 0, 12, and 24 h were chosen for illustration, blue lines indicate the migration front. Scale bar represents 200 µm. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviation: MTT:3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide; BM: Basal medium, $ASC\text{-}CM_{ischemic}$: Adipose-derived stem cell-conditioned medium of glucose/oxygen-deprived ASCs; fGM: Fibroblast growth medium.

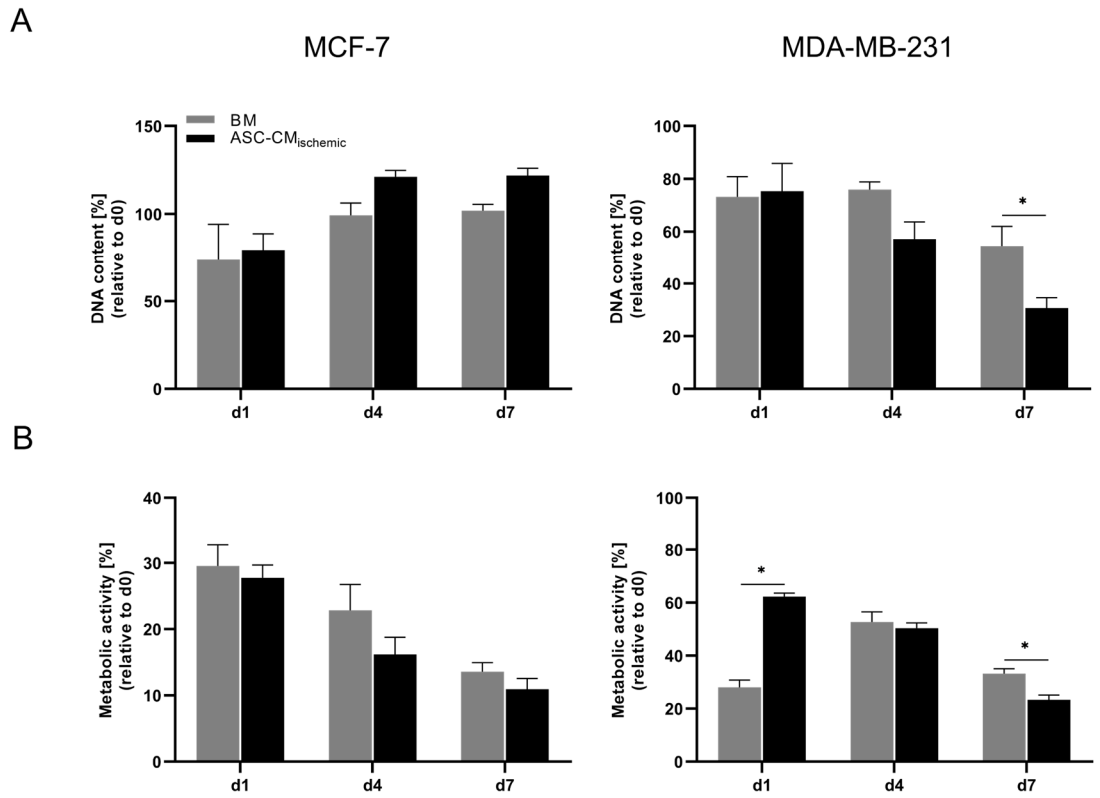


Figure 4.16: Effect of conditioned medium of glucose/oxygen-deprived ASCs ($CM_{ischemic}$) on MCF-7 and MDA-MB-231 cancer cells growth rate and metabolic activity.

(**A**) Quantitative determination of total DNA content in relation to the mean value of day 0. (**B**) Metabolic activity as determined by a MTT assay. MTT accumulated in MCF-7 and MDA-MB-231 was solubilized and the optical density was measured at 570 nm; % metabolic activity was calculated in relation to the mean value of day 0. Data are presented as means ± SD of n = 3; * $p < .05$. Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide; $ASC\text{-}CM_{ischemic}$, adipose-derived stem cell-conditioned medium of glucose/oxygen-deprived ASCs.

4.2.4 Discussion

The positive effects of ASC-based approaches in regenerative therapies have been demonstrated in preclinical and clinical studies, for example in cell-assisted lipotransfer or treatment of ischemic diseases [309,328,342,410]. However, a substantial loss of implanted cells has been documented during the early phase of engraftment [197,198]. Since ASCs, after transplantation into damaged tissues, are exposed to an ischemic environment characterized by the deprivation of nutrients and oxygen, a better understanding of the mechanisms underlying the beneficial effects in the early phase following transplantation is required. This would contribute to a more rational application of ASCs in cell-based approaches. The regenerative potential of ASCs nowadays is mainly attributed to their trophic activity through the secretion of angiogenic, anti-apoptotic, and immunomodulatory factors.

Hypoxia as one of the hallmarks of ischemia is well reported to enhance the secretion of such factors [191,411,412]. However, little is known about the ability of ASCs to maintain their secretory function under starvation conditions. Thus, we investigated the viability and secretory function of ASCs in response to glucose deprivation and severe hypoxia and the combination of both as major stress conditions in an ischemic environment. To our knowledge, no study has yet been conducted to investigate the effect of glucose deprivation as one of the components of ischemia on the secretion capacity of ASCs.

The hallmarks of ischemia were simulated in our *in vitro* set-up by culturing the ASCs under glucose deprivation (0.1 g/L glucose) and severe hypoxia (0.2% O_2) alone and in combination over a period of seven days in serum-free medium to mimic ischemic conditions during the early post-transplantation phase. Serum-free culture in combination with oxygen and also glucose deprivation is a widely used experimental model to mimic ischemic conditions *in vitro*. Furthermore, serum-free culture is also a common approach in studies analyzing the effects of secreted proteins by using a conditioned medium in order to avoid interference with serum proteins [413]. Viability and cell morphology were shown to be virtually not affected by the applied stress conditions over the culture period of seven days. Sustained viability of ASCs in response to adverse nutrient and oxygen levels was also reported by Mischen et al. [368] in a comparable set-up and time frame. In contrast to the limited cell death, the metabolic activity of the cells was significantly affected by glucose limitation from day 1, whereas severe hypoxia did not particularly influence the metabolic activity. Under the glucose-limiting condition (0.1 g/L), cells faced a complete exhaustion of glucose from day 4 under hypoxia, whereas the glucose level decreased more slowly in the normoxic condition. One g/L glucose was not limiting in this setup. In general, glucose levels in the medium demonstrated a steeper decline of the available glucose, when cells were exposed to hypoxia. The corresponding increase of lactate as an important by-product in glycolysis indicated that ASCs increasingly rely on anaerobic glycolysis for their metabolic demands when exposed to hypoxic conditions. Several studies exploring the metabolism of bone marrow-derived mesenchymal stem cells (MSCs) under glucose and oxygen deprivation demonstrated the metabolic flexibility of MSCs under such adverse conditions and their ability to rely on anaerobic glycolysis for energy supply in a hypoxic environment [341,367,404]. In this context, the crucial role of glucose for MSCs function in a hypoxic environment was emphasized. In the present study, the cells were able to maintain their viability for several days despite the complete exhaustion of glucose. This could possibly be due to an enhanced autophagic activity of the cells, as autophagy has been shown to be a survival mechanism for oxygen/glucose-deprived MSCs [404,414,415]. A further observation was that those ASCs that survived under the harsh ischemic condition (glucose/oxygen deprivation) were not affected in their adipogenic differentiation capability.

This finding additionally underlined the remarkable resilience of hASCs to an adverse environment.

The next step was to examine the secretion of the ASCs exposed to oxygen and/or glucose deprivation to determine whether cells under ischemic stress were able to maintain their secretory function. As displayed by a cytokine antibody array, ASCs were able to express a broad range of growth factors, cytokines, and chemokines, which in part appeared to be stimulated by hypoxia. When glucose deprivation was combined with hypoxia in order to mimic ischemia, ASCs were still able to maintain secretory function. Under this condition, the cells expressed growth factors and cytokines with angiogenic (VEGF, IL-6, IL-8, ANG) and matrix-remodeling (TIMP-1, TIMP-2) functions and chemokines (MCP-1/CCL2, IP-10/CXCL10) among others, while cytokines associated for example with the regulation of proliferation, cell division, and differentiation appeared to be expressed to a lesser extent (e.g., FGF-4 and -6, IGFBP-2, -3, and -4, NAP-2).

To reveal the impact of the individual stress condition on the expression level of selected factors (VEGF, IL-6, IL-8, ANG, TIMP-1, and MCP-1), their expression was investigated under glucose and oxygen deprivation separately and in combination. STC-1 was included in the analysis as a factor associated with the reduction of apoptosis, angiogenesis, and enhanced resistance of cells to metabolic stress [349,350,388]. Furthermore, STC-1 appears to be closely related to cellular metabolism, as a role of STC-1 in the activation of AMP-activated protein kinase (AMPK) has been postulated. AMPK in turn is a key regulator in the cellular adaptive response to ischemia [416]. STC-1 was shown to be expressed by different cell types including bone marrow-derived MSCs [349,389,417,418] but is a still unknown factor in ASCs. Thus, to the best of our knowledge, the secretion of STC-1 by ASCs was demonstrated for the first time in this study. We found different response patterns of the investigated factors to the individual stress conditions. It is generally accepted that hypoxia triggers the expression of a variety of growth factors and cytokines in ASCs [191,241,411]. Accordingly, we also found an increase in the expression of most of the factors investigated, when the cells were exposed to 0.2% O_2 (VEGF, IL-6, IL-8, ANG, TIMP-1, STC-1). MCP-1 showed no response to reduced oxygen levels. However, when hypoxia was combined with glucose deprivation in order to mimic ischemia, the secretion of VEGF, IL-6, IL-8, ANG, and STC-1, which are all factors with angiogenic and/or anti-apoptotic properties, increased markedly compared to the hypoxic condition alone. Thus, glucose deprivation (in conjunction with hypoxia) proved to be a factor that positively influenced the secretion of angiogenic and anti-apoptotic cytokines. The availability of glucose as a variable that influences the secretion performance of cells has hardly been investigated so far. Bakopoulou et al. [419] examined the secretion of human apical papilla mesenchymal stem cells subjected to glucose and oxygen deprivation and they also reported a stimulating effect of glucose deprivation on the secretion of angiogenic growth

factors in conjunction with hypoxia. In addition, VEGF has been described in early studies with glioma tumor cells as a “classical stress-induced gene”, whose secretion was enhanced by oxygen and glucose deficiency [200,201]. In contrast, Deschepper et al. [371] considered glucose essential for the response of hMSC to near-anoxic conditions. They reported a moderate increase in VEGF-C secretion with increasing glucose concentrations under severe hypoxia in MSCs. With regard to IL-6, the elevated expression under both glucose and oxygen deprivation determined in the present study is in accordance with reports that glucose deprivation triggers IL-6 expression by activation of ER stress signaling pathways [420,421]. The consideration of the time course of secretion additionally underlined the effect of glucose deficiency on the secretion of ASCs with a distinct increase in the levels of IL-6 and VEGF from the time point of complete glucose exhaustion in the culture (day 4). STC-1 secretion was detectable from day 3 under glucose/oxygen deprivation and was maintained until day 7 with a significant increase as compared to hypoxia alone. Gene expression of STC-1 was immediately upregulated in response to glucose/oxygen deprivation. This may indicate that STC-1 possibly elicits a stimulatory effect on VEGF expression. Several studies have shown that the expression of VEGF is associated with STC-1 and a positive feedback-loop between STC-1 and VEGF stimulation has been postulated [422,423].

Maintaining endothelial function and promoting angiogenesis in an ischemic environment to ensure adequate blood supply are considered key processes in regenerative approaches such as cell-assisted lipotransfer or treatment of ischemic diseases. The angiogenic function of secreted growth factors and cytokines of (co-)implanted ASCs could play an important role in this context [342,343]. For this reason, we investigated the impact of the secretome of ischemia-challenged ASCs on the viability, metabolic activity, and tube formation of endothelial cells (HUVECs). The results indicated that ASCs exposed to ischemia-mimicking conditions were able to restore endothelial cell viability, metabolic activity, and tube formation via their secretory function. The angiogenic response to VEGF, IL-6, IL-8, and ANG, which were shown to be major factors secreted under this condition, has been well documented [195,384–387]. Further studies are necessary to clarify to what extent the newly detected STC-1 in ASCs may contribute to this effect.

ASCs have further been shown to improve wound healing by promoting angiogenesis and fibroblast activation through their paracrine action [216,217,424]. Here, we demonstrated that factors secreted by ASCs in an ischemia-like environment, besides their angiogenic activity, stimulated the proliferation, metabolic activity, and migration of fibroblasts. IL-6 and IL-8 are known as factors that play an important role in wound healing by triggering fibroblast and keratinocyte migration, leukocyte infiltration, and collagen synthesis [219–221]. MCP-1 (CCL-2), which was also prominently expressed by oxygen/glucose-deprived ASCs, is a further important chemokine involved in wound healing processes, acting mainly through the

recruitment of macrophages [425]. Altogether, the findings support the regenerative potential of ischemia-challenged ASCs in wound healing, for example in chronic ischemic wounds. In contrast, the proliferation of breast cancer cell lines (MCF-7 and MDA-MB-231) was not increased in the presence of trophic factors from ASCs cultured under ischemia-like stress conditions. This may be relevant in the context of cell-assisted lipografting after mastectomy, where ischemic conditions at the transplantation site are likely to prevail. This observation may also be in line with reports from clinical studies that have suggested no increase in cancer recurrence rates in breast cancer patients treated with ASC-enriched lipografts, but further studies would be needed to more specifically assess the effects of the secretome of ischemia-challenged ASCs on breast cancer cells [426,427].

In conclusion, the present study demonstrated that ASCs showed sustained viability and metabolic flexibility under glucose deprivation and severe hypoxia as hallmarks of ischemia and were able to maintain their secretory function. The secretion of angiogenic and anti-apoptotic factors was influenced not only by hypoxia but was distinctly increased in conjunction with glucose deprivation. Under this condition, the ASCs also expressed STC-1, a factor with angiogenic and anti-apoptotic properties, but which is as yet unknown in ASCs. These results provide valuable insights into how ASCs, through their robustness and remarkable secretory function, may mediate regenerative effects even under adverse conditions such as those found at implantation sites or in damaged tissue.

4.3 Role of STC-1 in ASCs

The regenerative potential of ASCs in regenerative approaches is well documented [86,356,357,377], e.g. in the field of cell-assisted lipotransfer or treatment of ischemic diseases [328,426,428–430]. Beneficial effects were mainly attributed to the trophic factors that are secreted by ASCs [178,191,431,432]. Some of these factors play an important role in promoting angiogenesis during the early phase of engraftment. Angiogenesis is the predominant mechanism of blood vessel formation and it is activated by a variety of growth factors and cytokines [433,434]. The data presented in the previous chapter show that under ischemic conditions commensurate to the conditions that occur at transplantation sites or in damaged tissue, ASCs are able to secrete a range of angiogenic, anti-apoptotic, and immune-modulatory factors and can potentially also contribute to neo-vascularization [178,197]. STC-1, a peptide hormone discovered in the Corpuscles of Stannius (CS) of bony fish, was originally described as a hormone involved in calcium and phosphate homeostasis [388,435,436]. Moreover, mammalian STC-1 was shown to take part in numerous pathophysiological processes, associated with prevalent ischemic conditions [437]. Several studies reported STC-1 as an anti-apoptotic factor [348,389,438], and as a regulator of key pathways involved in the response to oxidative stress, cellular energy metabolism, survival, and proliferation and in the control of angiogenesis [350,351,439]. Some studies suggested that STC-1 could be a mediator for enhancing the expression of the vascular growth factor, VEGF. However, other studies reported that STC-1 expression is mediated by other cytokines [422,423,440–442]. To date, the role of STC-1 in the secretory response of ASCs to ischemic conditions is not known. In chapter 4.2. we showed, to the best of our knowledge, for the first time the secretion of STC-1 by ASCs. It was revealed that high levels of STC-1 are expressed by ischemia-challenged ASCs. Furthermore, we demonstrated that factors secreted into the cell culture media by ASCs under ischemia-like stress conditions promoted endothelial cell tube-formation and fibroblast migration.

In this context, it was hypothesized that STC-1 may contribute to these processes. Therefore, the aim was to investigate the effect of exogenous recombinant human STC-1 (rhSTC-1) as well as the effect of specific inhibition of STC-1 on human umbilical vein endothelial cell (HUVEC) tube-formation. As a positive feedback-loop between STC-1 and VEGF stimulation has been reported in the literature, a second set of experiments was performed to investigate VEGF expression downstream of STC-1.

4.3.1 Effect of rhSTC-1 on endothelial tube formation and VEGF secretion by ASCs

In the previous experiments, it was demonstrated that factors secreted by ASCs in an ischemic-like environment stimulated tube-formation by HUVECs (Figure 4.14 C). To assess a possible role of STC-1 in angiogenesis, we investigated whether rhSTC-1 could promote HUVEC tube-formation and whether rhSTC-1 also promoted ASCs to secrete VEGF. For this purpose, HUVECs were cultured in nutrient-deprived basal ASC culture medium (D-glucose-, L-glutamine-, and sodium pyruvate-free DMEM; containing no serum), which was supplemented with different concentrations (ranging from 400 to 40,000 pg/mL) of rhSTC-1. Analyses of tube-formation on growth factor-reduced Matrigel 6 hours post addition of rhSTC-1 containing medium revealed, that HUVECs did not form tube-like structures in the presence of rhSTC-1, whereas the formation of tubes was demonstrated for HUVECs cultured in endothelial growth medium (eGM), as positive control (Figure 4.17). As shown before, culture of HUVECs in ischemia-conditioned ASC medium also promoted tube formation. In conclusion rhSTC-1 alone does not promote HUVEC tube-formation.

Despite the lack of a direct effect of rhSTC-1 on HUVEC tube-formation, it was further investigated whether rhSTC-1 can significantly increases VEGF-secretion by ASCs. To this end, ASCs were treated with different concentrations of rhSTC-1 (40 to 40,000 pg/mL) added to nutrient-deprived basal medium (supplemented with 1 g/L glucose) and the cells were cultured under hypoxia. In agreement with the previous studies, only low STC-1 concentrations were present in the ASC medium under these culture conditions. The concentration of VEGF in the culture medium was quantified using ELISA on day 2 and day 3. On day 2, the VEGF level in the culture medium increased approx. 2-fold in the presence of 40 pg/mL rhSTC-1 as compared to the control-treated cells. The VEGF level did, however, not further increase in the media of cells that were exposed to higher STC-1 doses (Figure 4.18). On day 3, VEGF levels were slightly increased for all groups compared to day 2 VEGF levels.

In conclusion, these investigations showed that rhSTC-1 per se did not positively affect the tube formation of endothelial cells. However, the increased VEGF secretion levels of ASCs upon addition of rhSTC-1 indicated a possible correlation between VEGF and STC-1 secretion in ASCs.

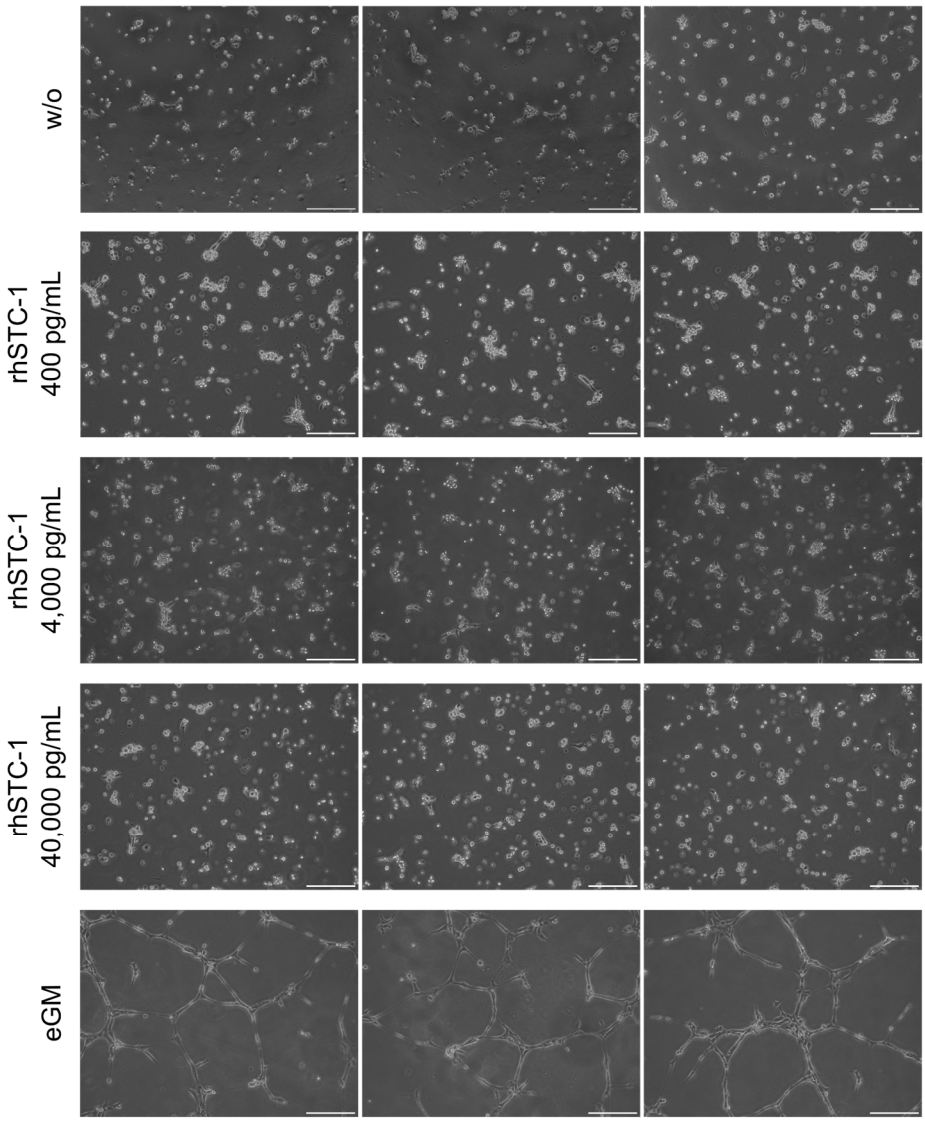


Figure 4.17: Effect of rhSTC-1 on tube formation of HUVECS.

For seeding, HUVECs were suspended in basal medium without supplements that contained rhSTC-1 at the indicated concentrations. Endothelial growth medium served as a positive control in this assay. Tube formation was assessed after 6 hours of culture on growth factor-reduced Matrigel. Representative micrographs are shown. Scale bar represents 200 µm. Abbreviations: w/o: without; rhSTC-1: recombinant human Stanniocalcin-1; eGM: endothelial growth medium.

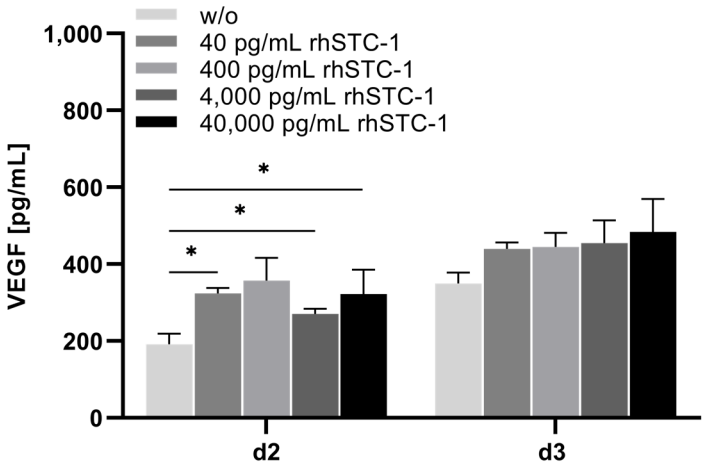


Figure 4.18: Influence of rhSTC-1 on VEGF secretion of ASCs

ASCs were cultured in the presence of different concentrations of rhSTC-1 (40 to 40.000 pg/mL) added to the culture medium and VEGF concentrations were determined in the culture supernatant after 2 and 3 days by enzyme-linked immunosorbent assays. ASCs without rhSTC-1 supplementation to the culture medium (w/o) served as control. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviations: VEGF: Vascular endothelial growth factor; rhSTC-1: recombinant human Stanniocalcin-1; w/o: without.

4.3.2 Specific inhibition of STC-1

To further investigate a potential function of STC-1 in angiogenesis and to confirm the putative positive feedback-loop between STC-1 and VEGF expression, we set out to determine whether specific inhibition of STC-1 in the supernatant of ASCs had an effect on tube formation or VEGF expression. For tube formation analysis, HUVECS were cultured in ischemia-challenged ASC-conditioned medium, which was preincubated with a polyclonal antibody against STC-1, as no STC-1 inhibitory antibody was commercially available. Two different antibody concentrations were used (5 and 20 µg/mL) along with the respective antibody isotype controls. The results show that tube formation was inhibited in the presence of SCT-1 antibodies in the conditioned medium. However, the same effect was observed in the presence of the isotype control, indicating that tube formation was inhibited by an unspecific effect, rather than a specific antibody-mediated effect on STC-1 in the conditioned medium (Figure 4.19). Furthermore, it was examined whether the specific inhibition of STC-1 affected the expression of VEGF in ASCs subjected to ischemia-like stress conditions. For this purpose, ASCs were cultured in nutrient-deprived basal medium with 0.1 g/L glucose under hypoxia, as it was already shown, that under this condition the secretion of STC-1 was significantly increased. The medium was supplemented with 2 µg/mL or 10 µg/mL anti-STC-1 antibody or the respective isotype control. ASCs were cultured for 7 days under hypoxia. Higher levels of VEGF within the groups supplemented with either anti-STC-1 or isotype antibody on day 2 as

well as on day 7 were demonstrated by ELISA (Figure 4.20), indicating again an unspecific effect.

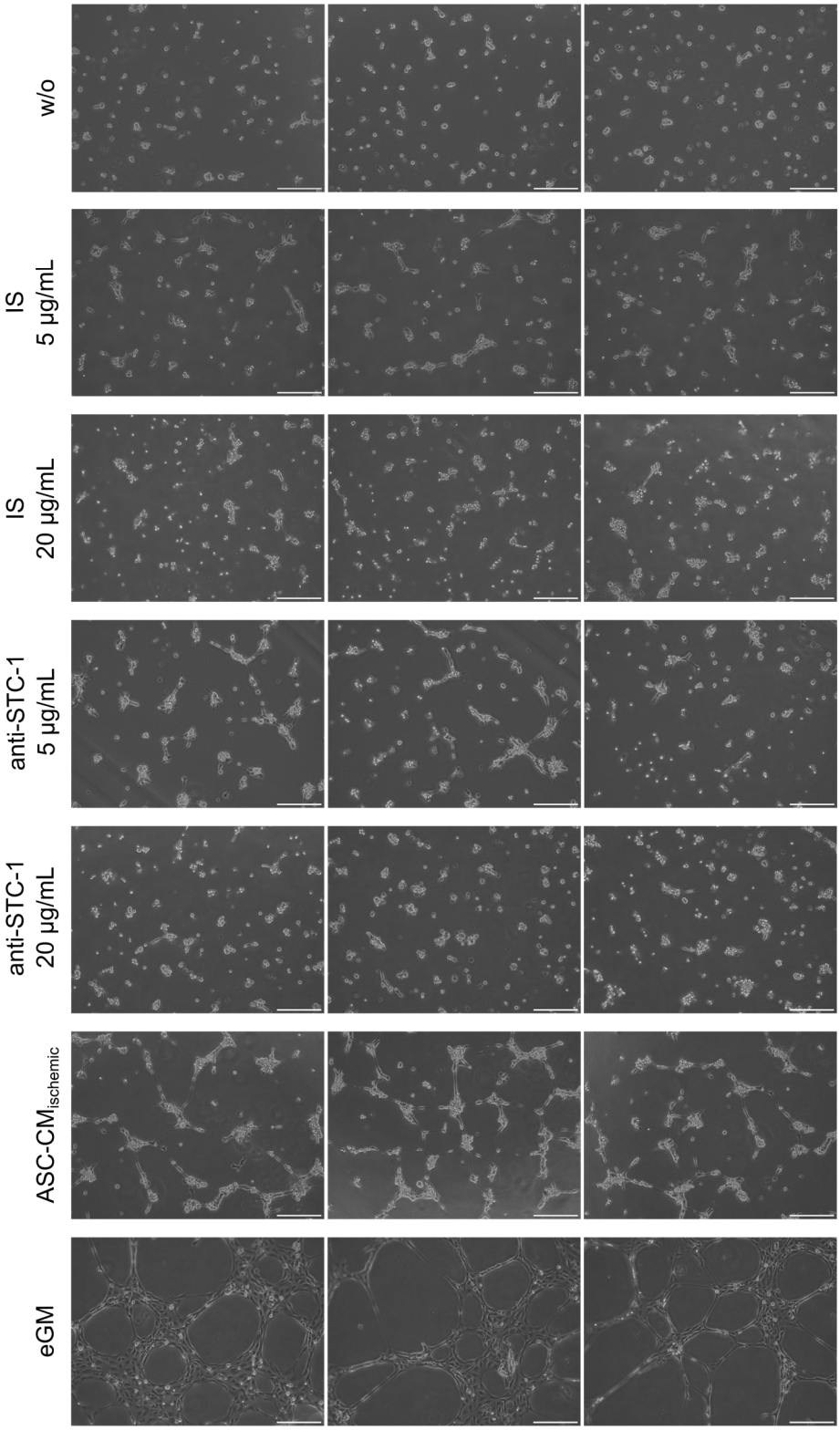


Figure 4.19: Effect of tube formation on HUVECs by specific inhibition of STC-1.

Tube formation by HUVECs in conditioned medium of ischemia-challenged ASCs supplemented with different concentrations of STC-1 antibody (5 and 20 µg/mL) after 6 hours on growth

factor-reduced Matrigel. Tube formation with the respective isotype IgG1 antibody added to the conditioned medium served as control. Representative micrographs were shown. Scale bar represents 200 µm. Abbreviations: w/o: basal medium without supplements; STC-1: Stanniocalcin-1; anti-STC-1: conditioned medium (CM) of ischemia-challenged ASCs supplemented with STC-1 antibody; IS: isotype; ASC-$CM_{ischemic}$: adipose-derived stem cell-conditioned medium of glucose/oxygen-deprived ASCs; eGM: endothelial growth medium.

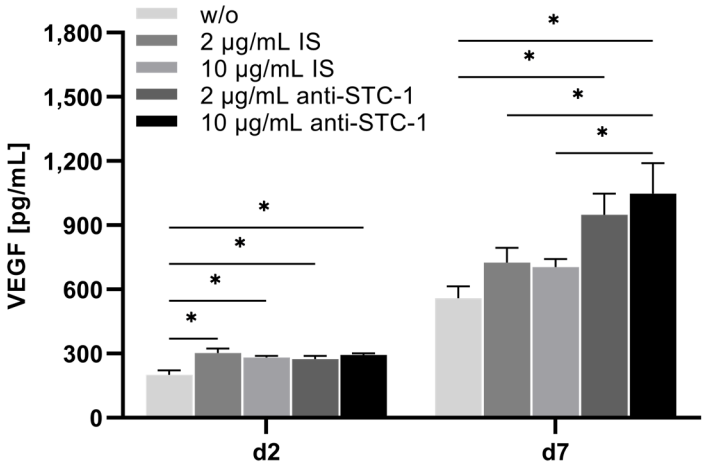


Figure 4.20: Effect of specific STC-1 inhibition on VEGF secretion in ASCs.
Measurement of the secreted amount of VEGF in the cell culture medium of ASCs treated with different concentrations of STC-1 neutralizing antibody and respective isotype controls. Isotype IgG1 antibody added to the basal medium served as control. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviations: VEGF: Vascular endothelial growth factor; STC-1: Stanniocalcin-1; anti-STC-1: basal medium supplemented with STC-1 antibody; IS: isotype; w/o: without.

4.3.3 Discussion

ASCs have gained popularity for tissue regenerative approaches as they potentially exert beneficial effects either through their capability to differentiate into cell types of the damaged tissue or through the secretion of trophic/immunomodulatory paracrine factors [108,127,178,336]. Some of these regenerative effects exerted by secreted factors were analyzed in the previous chapter 4.2, where conditioned medium of ischemia-challenged ASCs revealed angiogenic effects on endothelial cells. One factor that was significantly expressed on mRNA and protein level in ischemia-challenged ASCs was STC-1 (Figure 4.12 A and B). So far, STC-1 expression by ASCs has not been reported. STC-1 has however, previously been shown to influence cell survival, cellular metabolism and the secretion of pro-angiogenic factors of MSCs, breast cancer cells and HUVECs [349,350,388,443]. Furthermore, other studies described an up-regulation of VEGF by STC-1 expression [423,444]. Therefore, the focus of this chapter was to examine the effect of STC-1 on HUVEC tube-formation as a well-established *in vitro* assay system for angiogenesis and on the secretion of VEGF by ASCs.

First, the tube-formation and VEGF stimulation upon culture of HUVECs in rhSTC-1 supplemented medium was investigated. Increasing rhSTC-1 concentrations in the culture medium were tested. The lowest rhSTC-1 concentration (40 pg/mL) that was applied in this assay was comparable to the concentration of the previously measured endogenous STC-1 in ischemia-challenged-ASC-conditioned medium. It was found that exogenously added rhSTC-1 had no effect on tube formation, therefore STC-1 alone, probably does not trigger angiogenesis. However, culture of ASCs in rhSTC-1-supplemented medium resulted in increased VEGF secretion on day 2 of culture. These results indicate a possible effect on angiogenesis. As information on *in vivo* levels of circulating STC-1 in mammals are limited, it is not clear if the concentrations used in the assays presented in this thesis are physiologically relevant. Further, Thairi et al. [443] reported that cells may be responsive to endogenous STC-1 but may not necessarily be responsive to exogenously added STC-1, as endogenous rhSTC-1 may have an autocrine function, which cannot be mimicked by recombinant STC-1. Which may be one explanation why we could not demonstrate effects on tube formation of endothelial cells by rhSTC-1. However, a possible correlation between VEGF and STC-1 secretion in ASCs upon addition of rhSTC-1 could be revealed by ELISA.

To further address whether and, if yes, how STC-1 influences tube-formation of endothelial cells and VEGF secretion by ASCs, the inhibition of STC-1 by an antibody was attempted. The inhibition of STC-1 was assayed with 2 different anti-STC-1 antibody concentrations. Tube-like structures of endothelial cells could not be identified in the presence of STC-1 antibodies, but the same effect was also observed in the presence of the isotype control. These results indicated that the tube formation was inhibited by an unspecific effect in this experimental set-up. Moreover, VEGF concentrations were increased in both, the ASC culture media that were supplemented with the anti-STC-1 antibody and in the media that were supplemented with the isotype control antibody. This result did therefore not allow any conclusions about the function of STC-1 in this experimental set-up. By using a polyclonal antibody instead of an inhibitory antibody in these analyses, as none is commercially available, this antibody binds to several epitopes within the STC-1 protein and potentially exerts an inhibitory function. Unfortunately, due to the unspecific effects observed in these analyses it is not clear if this antibody indeed inhibits STC-1. Further experiments, such as the use of significantly lower antibody concentrations or the use of other readouts, are therefore needed in a further approach to identify an STC-1 inhibitory antibody.

Published literature shows, STC-1 induces and/or enhances the activity of other growth factors and cytokines contributing in this way to angiogenic effects [445–447]. Several studies reported high STC-1 expression levels in the tumor vasculature of breast adenocarcinomas and colon cancers, pointing to a potential role of STC-1 in tumor angiogenesis [448–450]. He et al. [444] demonstrated that STC-1 was not directly promoting angiogenesis, but rather

modulated the tissue microenvironment, which ultimately promoted sprouting of new blood vessels. Furthermore, these authors also showed that STC-1 regulates VEGF expression through PKCβII and ERK1/2 signalling pathways in gastric cancer cells.

In summary, a positive feedback-loop between STC-1 and VEGF stimulation in ASCs with significantly increased VEGF levels in the early culture phase could be demonstrated upon culture of cells in medium supplemented with rhSTC-1. The specific inhibition of STC-1 could not be analysed due to non-specific effects. More work is therefore required to determine the role of STC-1 and to investigate in more detail mechanisms and pathways by which STC-1 exerts its function. In this context, a siRNA-mediated knockdown of the STC-1 encoding gene could help to study the role of STC-1 in angiogenesis and in tissue regeneration.

4.4 Establishment of a 3D adipose tissue *in vitro* culture system to study ASC function in CAL

Autologous fat grafting, or lipotransfer, is one promising and commonly used treatment in aesthetic and reconstructive surgery due to the easy accessibility of fat and the prevention of complications by allergic or foreign body reactions [300,428]. In autologous fat grafting, adipose tissue obtained by clinical suction or excision is purified, and subsequently reimplanted into the same patient [354]. However, the main drawback of fat grafting is the unpredictable and rapid resorption of the injected material, resulting in a long-term volume loss of 25% to 80% that reduces the clinical efficacy of this technique [296,302,328,451,452]. To improve the outcome of autologous fat grafting a modified procedure, called cell-assisted lipotransfer (CAL), was established, e.g., in the field of cosmetic breast augmentation and reconstruction or in laryngoplasty for the functional reconstruction of the glottal gap after unilateral vocal fold paralysis [453,454]. CAL is a transplantation of fat grafts enriched with ASCs prior to transplantation and has been shown to promote a higher survival rate and persistency of the transplanted fat compared to adipose tissue alone [316,328,340]. For clinical applications, ASCs represent a promising tool for cell-based therapies due to their high abundance, easy harvesting and cultivation [106,352]. Moreover, they provide good regenerative properties, mainly through the secreted factors that promote, among others, vascularization, especially under ischemic conditions as they are prevalent in transplanted tissues [134,309,346,347,455], as shown in chapter 4.2

The cultivation of explanted human adipose tissue over extended time periods would represent a promising alternative to animal models to mimic functions and responses of cells and tissue in a more physiological manner for tissue regeneration applications and therefore also for CAL. Cell culture on 2D surfaces is not capable of mimicking the 3D *in vivo* adipose environment, while artificial 3D culture models are often composed of only one or two cell types and do not reflect the complexity of the native tissue. Therefore, the overall aim of the work presented in this chapter was first to develop a 3D, physiologically relevant, model of human adipose tissue *in vitro*. In this model, we assessed the viability and tissue structure under long-term conditions. For studying the function of ASCs in CAL, an adipose tissue-ASC co-culture model was further established to specifically investigate the secretory capacity of ASCs in this co-culture under ischemic conditions such as those occurring at the transplantation site. Thus, the fat tissue model developed in this work could contribute to a better understanding of the function of ASCs in CAL. In addition, the preparation method of adipose tissue for implantation in laryngoplasty for glottic insufficiency was optimised providing better viability and structure integrity of the implanted tissue.

4.4.1 Lipograft *in vitro* culture system - Experimental set-up

To better characterize ASC function in CAL, a human 3D adipose tissue *in vitro* model was first established. Adipose tissue was obtained from healthy female patients, following abdominoplasty that was conducted in the plastic surgery department, University Hospital of Wuerzburg. Adipose tissue samples were generated as 2x2 mm tissue fragments, called lipografts, and cultured with growth medium in agarose-coated plates to prevent the outgrowth and adherence of cells to the plastic surface of the culture plates. First, to determine the viability of the generated lipografts after cultivation for 14 days, a live/dead assay was performed and displayed vital adipose tissue (Figure 4.21 A). By means of a staining with a human-specific antibody against perilipin, a surface protein of lipid vacuoles [456], adipocyte integrity was demonstrated after the long-term culture period (Figure 4.21 B). To further assess the tissue architecture and composition of lipografts and to demonstrate the structure of adipocytes and blood vessels, the samples were analyzed by whole mount staining (WMS), as the 3D structure can hardly be represented by histological staining on sections. Concurrent CD31 and BODIPY staining of vascular structures and lipid droplets revealed the presence of capillaries running alongside adipocytes, representing vascularized tissue segments of the *in vitro* generated lipografts (Figure 4.21 C).

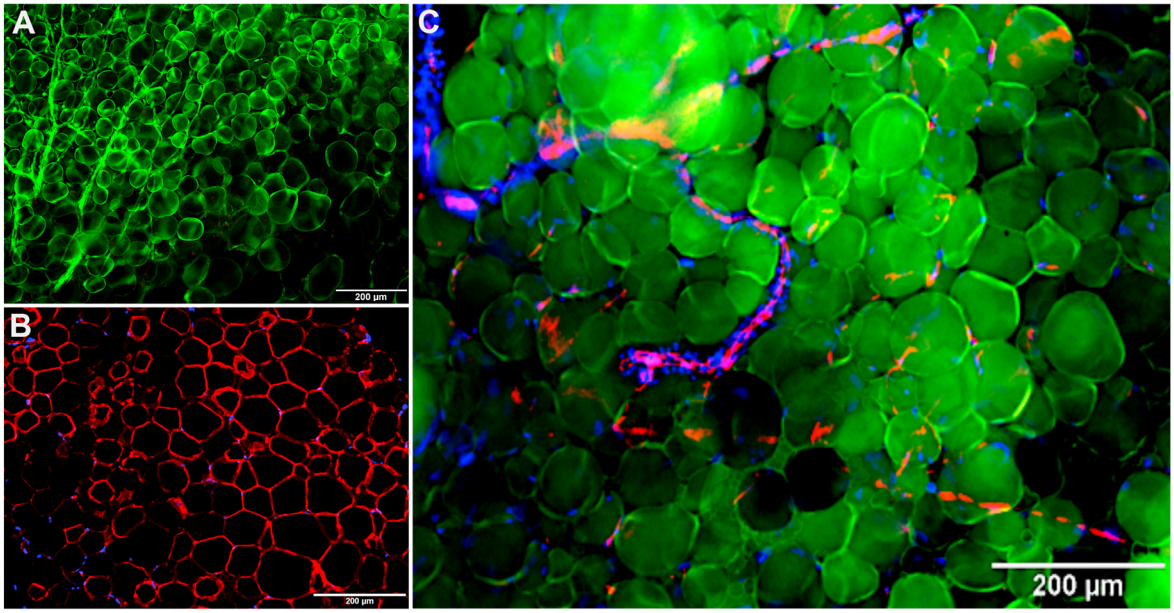


Figure 4.21: Characteristics of the lipografts, cultured *in vitro* under standard culture conditions for 14 days.
(**A**) Adipocytes and blood vessels stained with live/dead assay (green/red). (**B**) Staining against perilipin (red) was performed to highlight intact tissue structure. (**C**) Visualization of lipografts by whole mount staining. Interaction of vascular structures and adipocytes were demonstrated by CD31/BODIPY (red/green) staining. Nuclei were counterstained with DAPI (blue). Scale bar represents 200 µm.

4.4.2 Evaluation of the preparation method of adipose tissue for implantation in laryngoplasty- Pilot experiment

Using the successfully established lipograft *in vitro* culture system, the preparation method of adipose tissue for the use in laryngoplasty was evaluated in collaboration with the ENT clinic of Wuerzburg on the project “Cell-assisted lipotransfer for vocal fold augmentation”.

For the clinical application of adipose tissue for vocal fold augmentation, a strong mechanical processing and mincing of the adipose tissue material was routinely performed due to the injection of the tissue fragments through a cannula. However, the mechanical disintegration of the adipose tissue resulted in a strong decrease in viability as demonstrated by live/dead staining (Figure 4.22 A) over the cultivation period. This observation was in distinct contrast to the lipografts generated by controlled mincing of the tissue, which could be cultured for 14 days with good viability. Already directly after the preparation of the adipose tissue (d0), a large number of dead cells and damaged tissue (red) can be seen within the strongly processed injection material. These findings were corroborated by the assessment of the viability by a resazurin assay, which demonstrated significant differences at day 9 and day 14 of culture with a greatly reduced viability of the injection material at day 14 (Figure 4.22 B). Thus, the here established lipografts showed significantly enhanced viability rates compared to the current clinical preparation technique for the injection of adipose tissue in laryngoplasty.

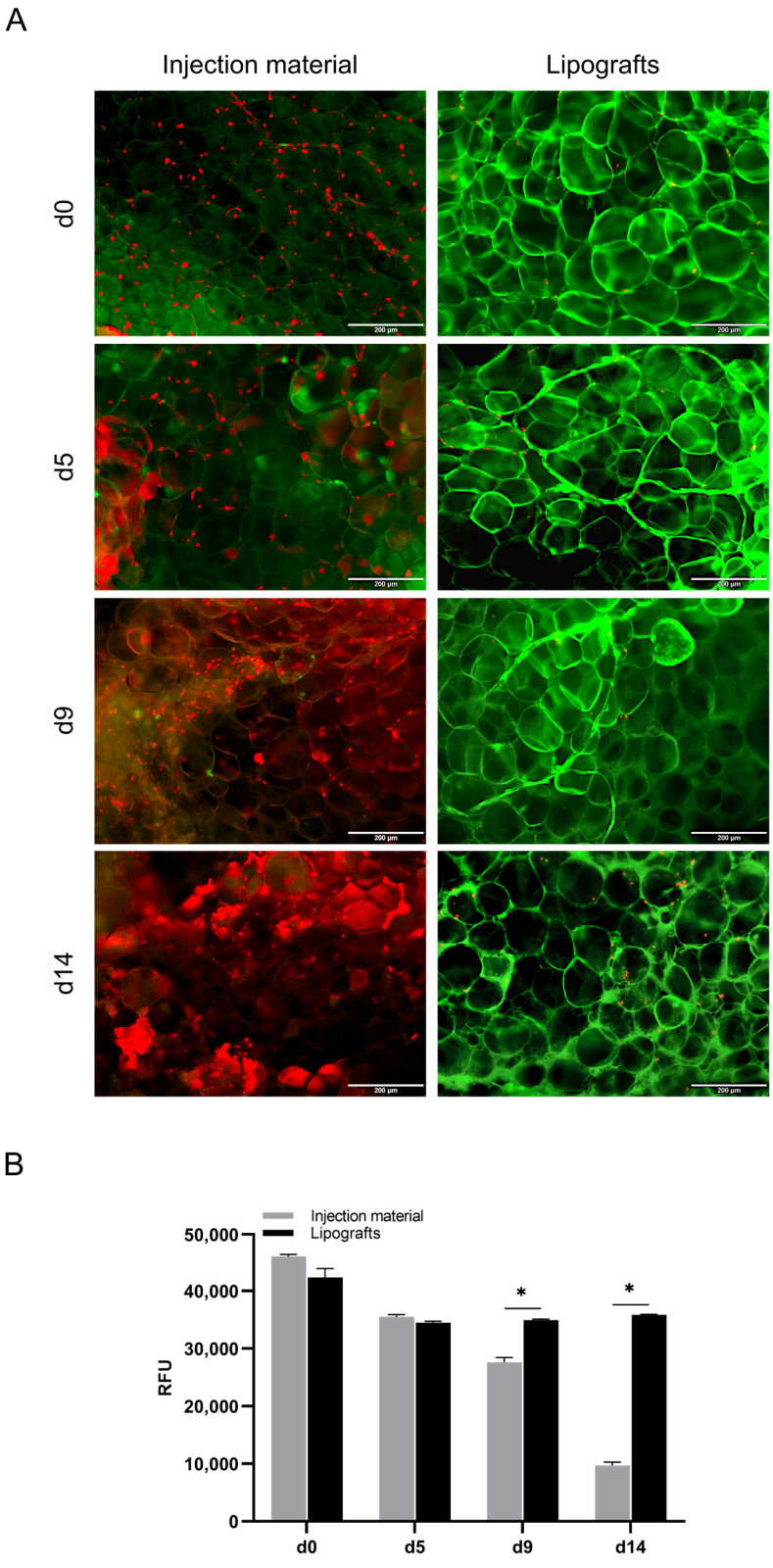


Figure 4.22: Viability of adipose tissue fragments resulting from 2 different preparation techniques.
(**A**) Microscopic images of live/dead staining (green/red). The respective prepared adipose tissue samples were cultured in agarose-coated well-plates under standard culture conditions and imaged at the indicated time points. Scale bar represents 200 µm. (**B**) Quantitative determination of viability by a resazurin assay. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviation: RFU: Relative fluorescence units.

We further examined the adipose tissue structure histologically by perilipin immunostaining (Figure 4.23). The results indicated that the strong mechanical treatment of the adipose tissue, as it is necessary for injection via cannula, also led to a strong damage of the tissue structure over time, characterized by disrupted adipocytes, already immediately after the preparation (d0) and further to necrotic areas with a complete degradation of the injection material within 14 days. In contrast, generated lipografts demonstrated perilipin positive, intact, and round adipocytes over a period of 14 days.

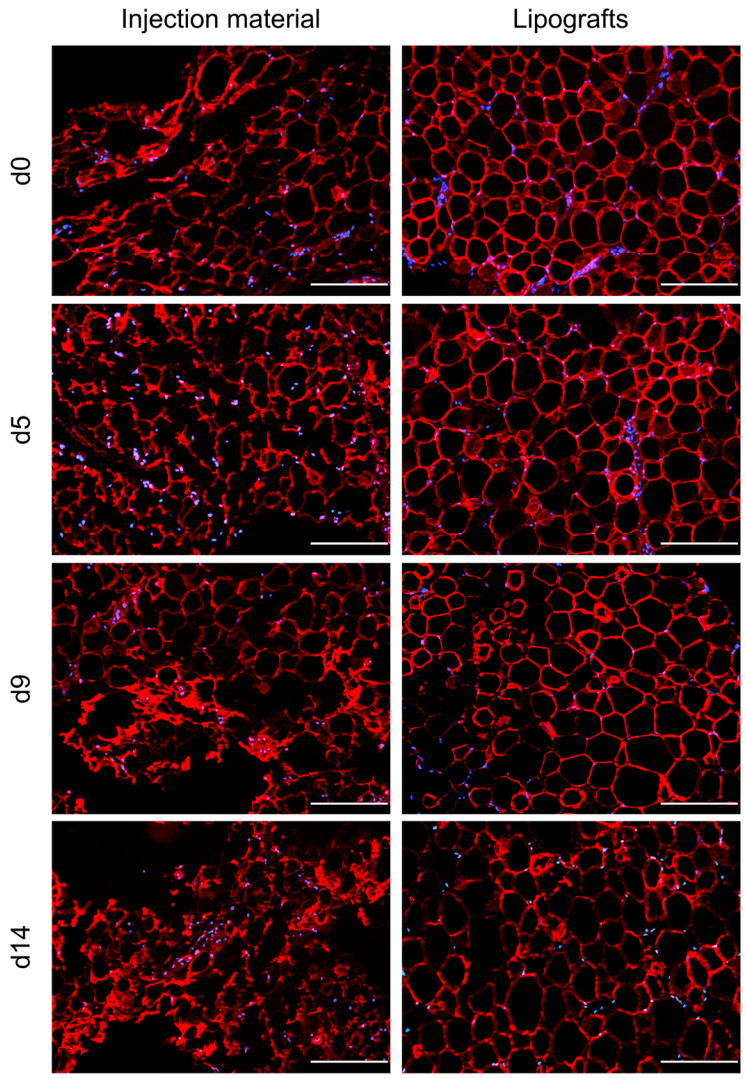


Figure 4.23: Immunohistological comparison between adipose tissue processed by the conventional clinical preparation technique of adipose tissue for injection via cannula and lipografts, generated as small adipose tissue fragments over a culture of 14 days.

The respective samples were fixed at each indicated time point and stained for perilipin (red) and counterstained with DAPI (blue). Representative micrographs at different time points were chosen for illustration. Scale bar represents 200 µm.

Taken together, we could demonstrate that the conventional, clinically applied preparation method of adipose tissue for injection via cannula leads to distinctly damaged and necrotic tissue. Therefore, lipografts due to their intact, vital, and well-vascularized tissue structure after preparation, could represent an alternative in laryngoplasty for the functional reconstruction of the glottal gap after unilateral vocal fold paralysis, if the implantation technique would be modified and adapted accordingly.

4.4.3 Viability of lipografts exposed to harsh ischemic conditions

To analyze the impact of harsh ischemic culture conditions, such as those occurring at the transplantation site, on lipografts viability, lipografts were cultured under the previously defined harsh ischemic condition with complete withdrawal of glucose and serum and an oxygen reduction to 0.2% over a period of 10 days. The live/dead assay demonstrated a strong decrease of the viability of lipografts under harsh ischemic conditions within 10 days, with decreasing numbers of vital adipocytes (green) and an increase in dead areas (red), likely due to the lack of glucose and serum in the culture medium (Figure 4.24. A). Moreover, adipocytes in lipografts under this harsh ischemic condition lost their typical round morphology from day 3 on, whereas lipografts showed vital and intact adipocytes for up to 10 days under standard culture conditions. The metabolic activity of the lipografts (determined using the resazurin assay) was also significantly impaired under harsh ischemic conditions with a reduction of approx. 62% at day 3 up to 84% after 10 days as compared to day 0 (Figure 4.24 B). The metabolic activity of the lipografts under control conditions was reduced by 40% compared to day 0 and subsequently nearly remained unaffected until the end of the culture.

Overall, these results confirmed that under harsh ischemic conditions the viability and metabolic activity of lipografts was strongly impaired probably due to the absence of glucose and serum in the culture medium and the associated lack of adequate nutrient supply.

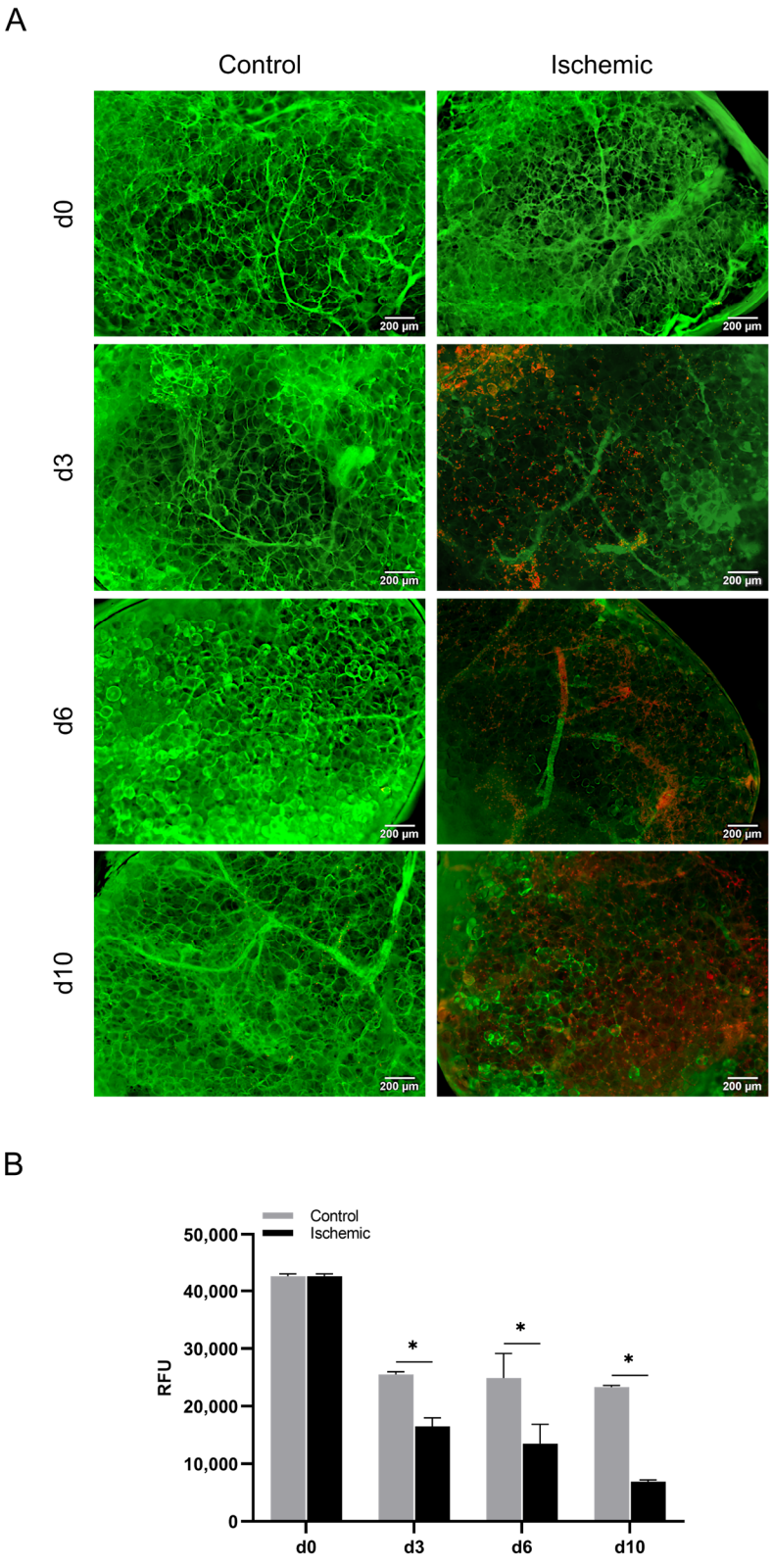


Figure 4.24: Viability and metabolic activity of lipografts cultured under harsh ischemic culture conditions.
(**A**) Live/dead staining of lipografts at different time points of the culture. (**B**) Metabolic activity as determined by resazurin assay. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviation: RFU: Relative fluorescence units.

4.4.4 Autologous lipograft-ASC co-culture system - Experimental set-up

In order to better characterize ASC function in CAL, an autologous transwell-based lipograft-ASC co-culture was established. For this purpose, lipografts were generated and ASCs were obtained from the same donor as described in Materials and Methods (see Chapter 3). Prior to assembling ASCs and lipografts, ASCs had to be propagated to sufficient cell numbers, which usually took 3-4 days. During this time, the lipografts were maintained with growth medium under standard culture conditions. Afterwards, ASCs were seeded in the lower 24-well chamber and co-cultured with 5 lipografts (approx. total volume of 50 µL) per insert to achieve a clinically relevant concentration of 10^6 ASCs/mL lipografts for CAL. This autologous co-culture system enables differential analysis of secreted cytokines and growth factors in an *in vitro* system mimicking the situation in CAL and can result in a better understanding of the effects of ASCs in this context.

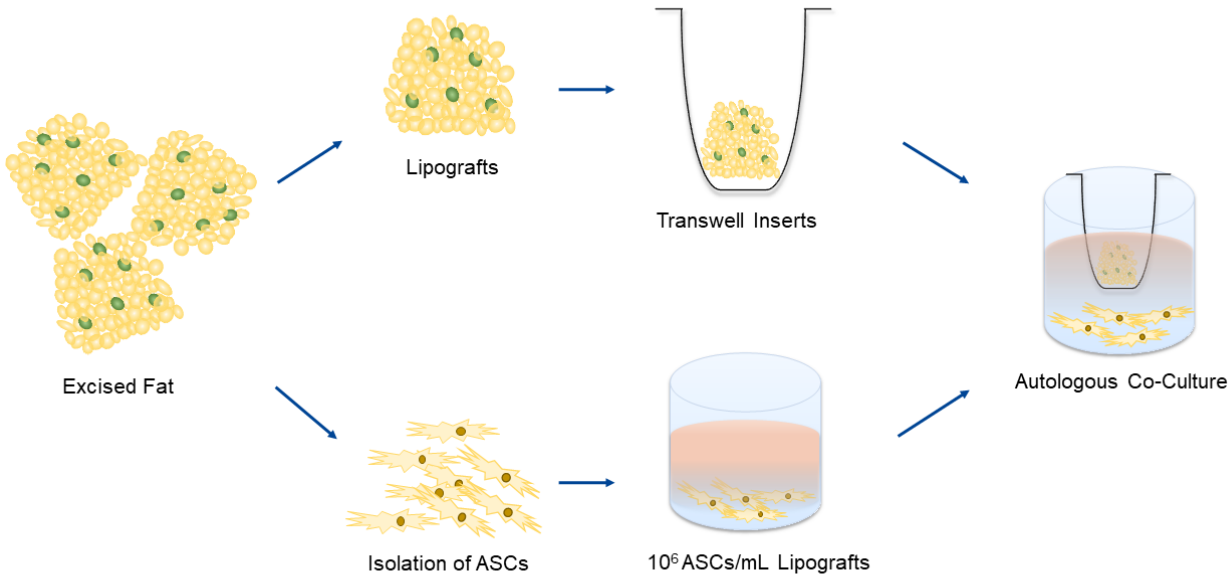


Figure 4.25: Experimental set-up of an autologous lipograft-ASC co-culture *in vitro*.
Establishment of the co-culture system with the simultaneous generation of lipografts and isolation of ASC followed by a transwell-based co-culture with ASCs in the lower chamber and lipografts in the transwell insert.

4.4.5 Secretion of growth factors and cytokines in the lipograft-ASC co-culture system

To further address the function of ASCs in CAL, we quantified the concentration of the selected factors IL-6, VEGF, and STC-1 in the culture supernatants of our established autologous co-culture system on day 4 of culture using ELISA. In order to further reveal the impact of ischemic stress conditions on their expression level, co-cultures were also subjected to the harsh deprivation condition (0 g/L glucose, 0% FBS, 0.2% O_2) and compared to the standard culture condition. For comparison, the secretion levels of lipograft and ASC monocultures were examined under the same conditions.

As shown in Figure 4.26 for the both donors studied, significantly increased levels of IL-6 were measured in the lipograft as well as in the co-culture compared to secreted amounts of ASCs alone under both standard and ischemic culture conditions. Thus, the secretion of IL-6 is mainly attributed to the lipografts themselves and indicates no (donor 1) or minor (donor 2) contribution of the ASCs. In clear contrast, the pro-angiogenic factor VEGF was significantly increased in lipografts added with ASCs. This effect was also observed under both standard and ischemic conditions, whereby the measured amounts in general were, as for IL-6, significantly increased under ischemic conditions for both donors. Finally, STC-1 secretion was not affected by the addition of ASCs under ischemic conditions but under control conditions, a significantly increased secreted amount of STC-1 could be observed for lipografts enriched with ASCs.

Taken together, lipografts from both donors studied secreted comparable amounts of the selected anti-apoptotic, angiogenic and immune-modulatory factors. The secretory response was in general significantly increased under harsh ischemic conditions compared to standard culture conditions. For IL-6 and STC-1, there was no evidence that the addition of ASCs to lipografts positively influences the secretion of these factors under ischemic conditions. In contrast, the secreted amounts of VEGF were shown to be significantly elevated in the lipograft-ASC co-culture compared to the lipograft monoculture, thus a contribution of the added ASCs to an angiogenic response in this system could be revealed.

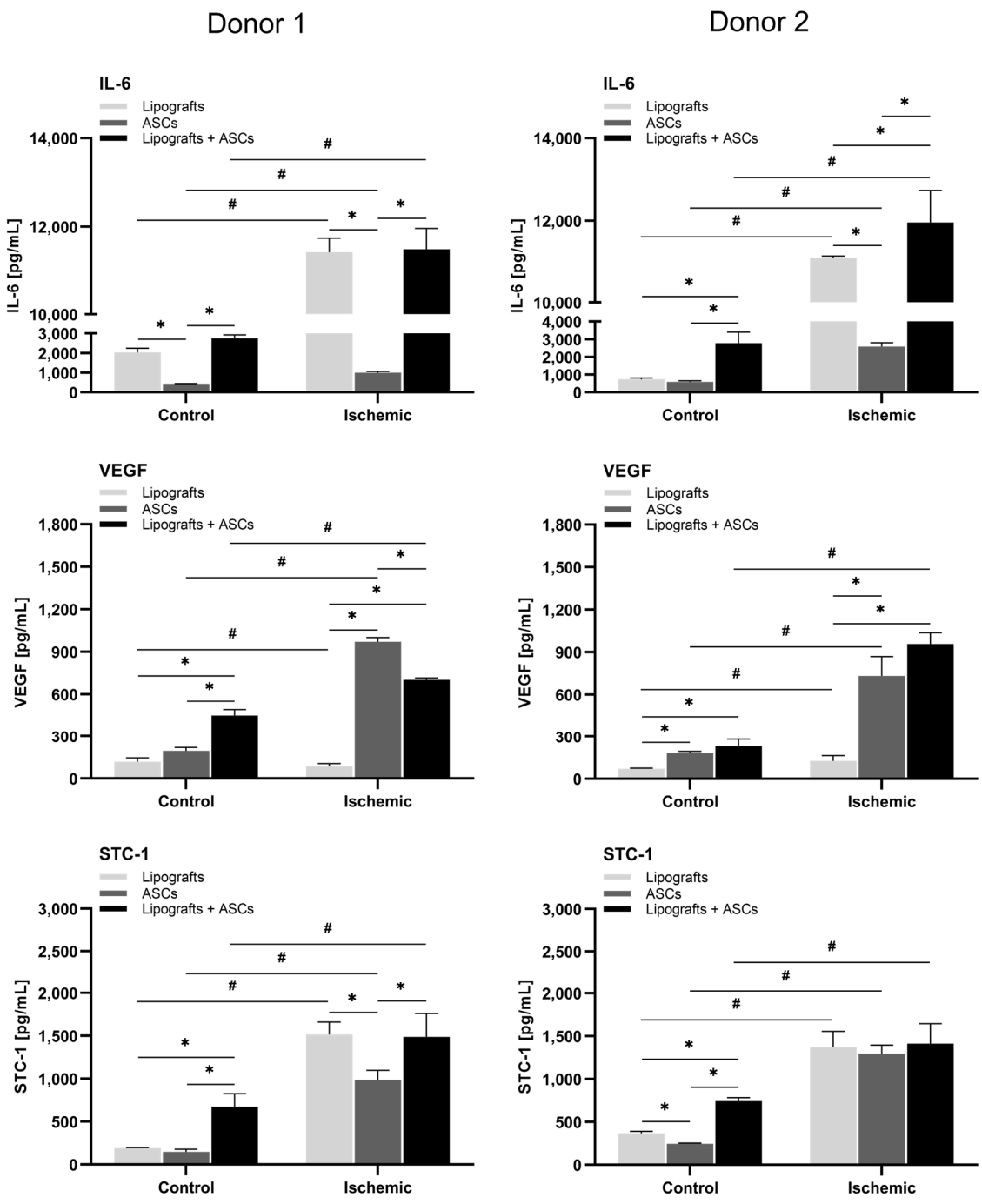


Figure 4.26: Secretion of IL-6, VEGF, and STC-1 in the established lipograft-ASC co-culture system compared to lipograft and ASC monoculture.

Secretion was assessed under standard and harsh ischemia-mimicking culture conditions after 4 days for 2 independent donors. Data are presented as means ± SD of n = 3; * $p < 0.05$. Abbreviation: IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; STC-1: Stanniocalcin-1.

4.4.6 Discussion

The positive effects of the enrichment of lipgrafts with ASC in CAL to improve long-term graft retention and lower post-operative complication were revealed in several studies [316,318,328,457]. However, substantial volume loss of the implanted cell-laden lipograft in the early phase after transplantation due to the adverse microenvironment to which implants are exposed has also been documented [197,198]. Therefore, it is of great importance to clarify the contribution of ASCs in this context and to which mechanisms the positive effects of the ASCs in CAL can be mainly attributed. How ASCs can exert beneficial effects in tissue regeneration was discussed in detail in the previous chapter. This includes especially the secretory activity through the secretion of angiogenic, anti-apoptotic, and immune-modulatory factors under ischemic conditions occurring at the transplantation site. In order to gain deeper insights into the functional roles and effects of added ASCs on lipografts, a novel human *in vitro* adipose tissue ASC co-culture model has been established.

The challenge in generating adipose tissue models *in vitro* is the mimicking of a 3D *in vivo* adipose environment and the reflection of the complexity of the native tissue [458,459]. Thus, first in this chapter, we specifically addressed the establishment of a long-term culture of intact adipose tissue as the basis for the co-culture with autologous ASCs. We were able to culture adipose tissue as small tissue fragments, called lipografts, in growth medium with very good viability over a period of 14 days, while preserving tissue structure. Whole mount staining demonstrated well-vascularized tissue with the presence of capillaries running alongside adipocytes. In the literature, there are also studies on the cultivation of adipose tissue *in vitro*, where the adipose tissue fragments are embedded into biocompatible gels, such as collagen type I [460–462], which represents another feasible approach for the preparation of 3D *in vitro* cultures. That vascularization and oxygenation of adipose tissue strongly impact clinical outcomes and that adipose tissue easily degenerates when exposed to ischemia is well reported in the literature [463–466]. Suga et al. [174] demonstrated in their work the effect of severe ischemia on adipose tissue. Using a mouse model of surgically induced ischemia, the authors observed a significantly reduced volume stability of the fat constructs under severe ischemic conditions over a period of 28 days. To mimic ischemic conditions, to which lipografts are exposed after implantation, lipografts were additionally cultured under severe oxygen and glucose deprivation. Thereby, it could be demonstrated a distinct reduction in viability within 10 days. This is well in accordance with the results by Suga et al. [174], as the strongly reduced viability of the tissue fragments, hence the degeneration of the tissue, that was demonstrated above *in vitro*, leads to necrosis *in vivo* and consequently to a significant volume loss. Thus, for the first time, the effect of ischemia on an adipose tissue model could be revealed *in vitro.*

Aiming to characterize ASC function in CAL, we started by investigating the secretion of the anti-apoptotic, immunomodulatory and angiogenic factors STC-1, IL-6, and VEGF in the co-culture compared to lipograft and ASC monoculture. To ensure relevant ASC concentrations in the co-culture, a cell concentration of 10^6 ASC in 1mL lipograft was applied, which is commonly used in the clinical set-up in CAL [428,467,468]. To be able to establish an autologous co-culture of ASCs and lipografts from the same donor, ASCs were isolated and propagated to sufficient cell numbers prior to the co-culture with the lipografts. The levels of IL-6 and STC-1 were markedly enhanced in the lipografts under ischemic conditions, while the enrichment with ASCs had no impact on their secretion. In contrast, significantly increased levels of the pro-angiogenic factor VEGF were shown in the lipograft-ASC co-culture compared to the lipograft monoculture. Based on the fact that VEGF secretion from lipografts themselves is very weak, these results suggest that added ASCs promote vascularization and thus may lead to improved lipograft maintenance that is crucial for the reported enhanced volume stability in CAL. In line with our findings, several studies reported promoted angiogenesis and enhanced long-term graft survival by using conditioned medium of VEGF-transfected ASCs or by applying VEGF-inducing preconditioning [469–471]. Therefore, the use of VEGF has great clinical promise and could be a therapeutic strategy in cell-based approaches. To better understand the beneficial effects of ASC in CAL, it would be highly relevant to identify other factors that are specifically secreted by ASCs in this co-culture system that may contribute to the positive effects in fat graft survival. For this purpose, a cytokine antibody array would also be here a good option to address a larger panel of cytokines. Furthermore, the impact of secreted factors of ASCs on parameter such as lipograft viability, integrity of adipocytes and blood vessels and maintenance of structure should be examined more specifically to get deeper insights into the beneficial role of ASCs in CAL.

In a pilot project, in cooperation with the ENT clinic on the project “Cell-assisted lipotransfer for vocal fold augmentation”, we additional evaluated the conventional preparation method of adipose tissue for lipoinjection therapy in the treatment of patients with glottic insufficiency and compared this clinical routine method with the lipografts we generated. The results showed a severe decrease in viability and tissue structure with the method commonly used in the clinic due to the need to prepare the tissue for injection via cannula. Accordingly, this form of preparation does not appear to be suitable for obtaining a viable injection material. These findings are in accordance with studies reporting different fat survival rates by different preparation methods based on the clinical settings [472]. Furthermore, after injection of the prepared tissue, the amount of fibrosis and the number of viable fat cells were reported to be one of the main factors, which determines the stability of the injected fat tissue [473,474]. Thus, based on these findings, a modification of the surgical procedure for vocal fold augmentation in terms of implantation of small fat pieces, comparable to the

lipografts described in this study, in a small subepithelial pocket in the vocal fold could contribute to a better and longer volume stability in the clinical application of CAL for vocal fold augmentation.

In summary, this chapter presented a 3D, physiologically relevant, long-term volume stable *in vitro* model of human adipose tissue. The establishment of such an *in vitro* model provides the basis for future studies on ASC function in CAL and tissue regeneration. First investigations in a transwell-based lipograft-ASC co-culture provided valuable insights into the secretory capacity of ASCs in the presence of lipografts. Further efforts are needed to identify more secreted factors that may be relevant for the beneficial effect of the ASCs in this system and to identify parameters that are able to predict the long-term stability of lipografts *in vivo.* Finally, based on the *in vitro* evaluation of the preparation method of adipose tissue for vocal fold augmentation in laryngoplasty, the modified preparation method of the adipose tissue by generating lipografts, could be a benefit for the treatment of patients with glottis insufficiency.

5 Conclusion and Outlook

The use of ASCs offers a potential tool for cell-based therapies due to their capacity to exert beneficial functions in tissue regeneration or in tissue repair. Apart from their ability to differentiate into mesenchymal lineages and to stimulate the recruitment of endogenous stem cells to the damaged area, ASCs also promote tissue regeneration by secreting a broad panel of angiogenic, anti-apoptotic and immune-modulatory factors. Nevertheless, there are several studies that have shown low survival of ASCs upon transplantation. One explanation for the massive cell death of transplanted cells is the ischemic environment to which the cells are exposed due to an initial lack of blood supply at the transplantation site. To date, studies on how ASCs respond to ischemic stress have been very rare. For a better understanding of the mechanisms underlying the beneficial effects of transplanted ASCs in regenerative approaches and to improve their therapeutic efficacy, a comprehensive characterization of ASCs under conditions occurring at the transplantation site is needed and represented the main task in this thesis.

To address this challenge, in a first approach, an *in vitro* model of ischemia that is characterized by glucose and oxygen deprivation was established. In this set-up, ASCs showed a drastically reduced viability in the absence of glucose with severe hypoxia. A further characterization of ASCs under ischemia-mimicking conditions also entailed the investigation on the secretory capacity. To investigate, whether ischemia-challenged ASCs were capable to maintain their secretory activity, gene expression analyses and secretion profiles of a set of factors with known angiogenic, anti-apoptotic and immune-modulatory properties were performed. All factors investigated were significantly elevated in the absence of glucose in combination with severe hypoxia compared to control conditions at early time points of culture. In this context it turned out that PSP, which is commonly used as a pH-indicator in cell culture media, markedly promoted the viability of ASCs under harsh ischemic conditions due to its estrogen activity. Additional investigations on the effect of E2 on ischemia-challenged ASCs revealed that PSP as well as E2 restored viability of ASCs under severe glucose/oxygen deprivation and indicated that estrogen-like compounds may modulate therapeutic effects of ASCs in tissue regeneration or tissue repair. In summary, it could be demonstrated that ASCs were able to maintain their secretory capacity in an *in vitro* model of ischemia with severe glucose and oxygen deprivation, at least at early time points.

While the modulation of the paracrine activity of ASCs by low oxygen concentrations is well documented, the response of ASCs to combined glucose and oxygen deprivation remains poorly understood. Therefore, the secretory response of ASCs under glucose starvation and hypoxia both separately and in combination to evaluate the influence of each parameter was further investigated. To study ASC secretion under glucose/oxygen deprivation more

comprehensively, an antibody array was conducted that revealed the secretion of a broad panel of angiogenic factors, growth factors, matrix-regulating proteins, chemokines, and others under glucose and oxygen deprivation. For selected factors of this panel a significantly elevated secretion was confirmed through measurements of the secreted protein amounts. Thus, it could be demonstrated that glucose deprivation in conjunction with hypoxia significantly increased the secretory activity of ASCs compared to the hypoxic condition alone. The angiogenic capacity of the secretome of ischemia-challenged ASCs was shown by enhanced viability and metabolic activity and increased tube formation of endothelial cells (HUVECs) exposed to conditioned medium of glucose/oxygen-deprived ASCs. Besides the angiogenic activity, stimulated proliferation, and migration of fibroblasts by the secreted factors of ischemia-challenged ASCs were further shown. In order to investigate anti-inflammatory effects of the secretome of ischemia-challenged ASC, future experiments may include the examination of conditioned medium from glucose/oxygen-deprived ASCs on proinflammatory M1 macrophages.

One of the highly expressed factors under ischemic conditions was STC-1, a peptide hormone which is involved in the reduction of apoptosis, enhanced resistance to hypoxia and metabolic stress, but a still unknown factor in ASCs. To assess a possible contribution of STC-1 to the angiogenic effect of glucose/oxygen-deprived ASCs, the influence of STC-1 on endothelial tube formation was investigated by the addition of recombinant human STC-1 (rhSTC-1) as well as by specific blocking of STC-1 in the conditioned medium of ASCs. In these experiments, a specific effect of STC-1 could not be observed. However, a positive correlation between VEGF and STC-1 secretion in ASCs upon addition of rhSTC-1 could be demonstrated by ELISA. Further investigations would be necessary to reveal mechanisms and pathways by which STC-1 exerts its function in this context. This may include a siRNA-mediated knockdown to assess the function of the newly detected STC-1 in ASCs regarding angiogenesis and tissue regeneration.

The secretory capacity of ASCs may also contribute to the beneficial effects of ASCs in CAL, where an enrichment of fat grafts with isolated ASCs is reported to enhance clinical efficacy through higher survival rates and persistence of the transplanted fat. A significant limitation for *in vitro* models of human adipose tissue is to mimic functions and responses in a more physiological manner. To address this challenge, a 3D, physiologically relevant, long-term volume stable *in vitro* culture system of adipose tissue fragments was established. Co-culture of these adipose tissue fragments with autologous ASCs showed generally higher secretory activity under ischemia-mimicking conditions and specifically higher VEGF concentrations due to the added ASCs. Future studies with the established *in vitro* model might include investigations on the contribution of the added ASCs to the stability and structure of

the fat tissue under ischemic conditions, for example preservation of blood vessels and viability and maintenance of adipocytes.

Preparation methods of adipose tissue for lipografting are based on clinical settings. The stability rate of the transplanted tissue depends on the fibrosis induced and on the number of viable fat cells after preparation. Thus, the clinically used preparation of adipose tissue for vocal fold augmentation in laryngoplasty was evaluated *in vitro*. While the commonly used preparation of the fat for injection by syringe led to a large number of dead cells and highly damaged tissue structure with necrotic areas, the preparation method of adipose tissue consisting of defined lipografts yielded an intact, vital, and well-vascularized tissue structure. This finding led to a direct modification of the preparation and implantation technique of lipografts in laryngoplasty in a conducted collaborative animal experiment on the function of added ASCs in CAL and represents a potential direct translation of the results of this work into clinical practice.

Taken together, the work of this thesis represents a comprehensive characterization of ASCs under ischemia-mimicking conditions. The findings regarding the remarkable secretory capacity under combined glucose and oxygen deprivation provide new insights into how ASCs may mediate regenerative effects in ischemic diseases or in tissue repair. First investigations in a newly established adipose tissue-ASC co-culture system revealed the secretory capacity in this system in a more physiological context and can form the basis for further research on ASC function in CAL.

References

1. Karastergiou, K.; Mohamed-Ali, V. The autocrine and paracrine roles of adipokines. *Mol. Cell. Endocrinol.* **2010**, *318*, 69–78, doi:10.1016/J.MCE.2009.11.011.

2. Trayhurn, P. Endocrine and signalling role of adipose tissue: New perspectives on fat. *Acta Physiol. Scand.* **2005**, *184*, 285–293, doi:10.1111/J.1365-201X.2005.01468.X.

3. Flynn, L.; Woodhouse, K.A. Adipose tissue engineering with cells in engineered matrices. *Organogenesis* **2008**, *4*, 228–235, doi:10.4161/ORG.4.4.7082.

4. Trayhurn, P. Adipocyte biology. *Obes. Rev.* **2007**, *8*, 41–44, doi:10.1111/J.1467-789X.2007.00316.X.

5. Frühbeck, G. Overview of adipose tissue and its role in obesity and metabolic disorders. *Methods Mol. Biol.* **2008**, *456*, 1–22, doi:10.1007/978-1-59745-245-8_1.

6. Shen, W.; Wang, Z.; Punyanita, M. Lei, J.; Sinav, A.; Kral, JG.; Imielinska,C.; Ross, R.; Heymsfield, SB. Adipose tissue quantification by imaging methods: a proposed classification. *Obes. Res.* **2003**, *11*, 5–16, doi:10.1038/OBY.2003.3.

7. Prunet-Marcassus, B.; Cousin, B.; Caton, D.; André, M.; Pénicaud, L.; Casteilla, L. From heterogeneity to plasticity in adipose tissues: Site-specific differences. *Exp. Cell Res.* **2006**, *312*, 727–736, doi:10.1016/J.YEXCR.2005.11.021.

8. Sun, K.; Kusminski, C.M.; Scherer, P.E. Adipose tissue remodeling and obesity. *J. Clin. Invest.* **2011**, *121*, 2094–2101, doi:10.1172/JCI45887.

9. Blüher, M. The distinction of metabolically “healthy” from “unhealthy” obese individuals. *Curr. Opin. Lipidol.* **2010**, *21*, 38–43, doi:10.1097/MOL.0B013E3283346CCC.

10. Maury, E.; Brichard, S.M. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol. Cell. Endocrinol.* **2010**, *314*, 1–16, doi:10.1016/J.MCE.2009.07.031.

11. Haki, W.; Tontonoz, P. Endocrine functions of adipose tissue. *Annu. Rev. Pathol.* **2007**, *2*, 31–56, doi:10.1146/ANNUREV.PATHOL.2.010506.091859.

12. Van Gaal, L.F.; Mertens, I.L.; De Block, C.E. Mechanisms linking obesity with cardiovascular disease. *Nat.* **2006**, *444*, 875–880, doi:10.1038/nature05487.

13. Burki, T. European Commission classifies obesity as a chronic disease. *Lancet Diabetes & Endocrinology*. **2021**, 9(7), 418, doi: 10.1016/S2213-8587(21)00145-5.

14. Bjorndal, B.; Burri, L.; Staalesen, V.; Skorve, J.; Berge, RK. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. *J. Obes.* **2011**, *2011*, doi:10.1155/2011/490650.

15. Kurth, B.M.; Rosario, A.S. Die Verbreitung von Übergewicht und Adipositas bei Kindern und Jugendlichen in Deutschland: Ergebnisse des bundesweiten Kinder- und Jugendgesundheitssurveys (KiGGS). *Bundesgesundheitsblatt - Gesundheitsforsch. - Gesundheitsschutz* **2007**, *50*, 736–743, doi:10.1007/S00103-007-0235-5.

16. Schwartz, M.W.; Seeley, R.J.; Zeltser, L.M.; Drewnowski, A.; Ravussin, E.; Redman, L.M.; Leibel, R.L. Obesity Pathogenesis: An Endocrine Society Scientific Statement. *Endocr. Rev.* **2017**, *38*, 267–296, doi:10.1210/ER.2017-00111.

17. Hopkins, M.; Blundell, J.E. Energy balance, body composition, sedentariness and appetite regulation: pathways to obesity. *Clin. Sci.* **2016**, *130*, 1615–1628, doi:10.1042/CS20160006.

18. Albuquerque, D.; Stice, E.; Rodriguez-Lopez,R.; Manco, L.; Nobrega, C. Current review of genetics of human obesity: from molecular mechanisms to an evolutionary perspective. *Mol. Genet. Genomics* **2015**, *290*, 1191–1221, doi:10.1007/S00438-015-1015-9.

19. Bucky, LP.; Percec, I. The science of autologous fat grafting: views on current and future approaches to neoadipogenesis. *Aesthetic Surg. J.* **2008**, *28*, 313–321, doi:10.1016/J.ASJ.2008.02.004.

20. Bucky, L.P.; Kanchwala, S.K. The role of autologous fat and alternative fillers in the aging face. *Plast. Reconstr. Surg.* **2007**, *120*, doi:10.1097/01.PRS.0000248866.57638.40.

21. Zielins, E.R.; Brett, E.A.; Longaker, M.T.; Wan, D.C. Autologous Fat Grafting: The Science Behind the Surgery. *Aesthetic Surg. J.* **2016**, *36*, 488–496, doi:10.1093/ASJ/SJW004.

22. Monfort, A.; Izeta, A.; Monfort, A.; Izeta, A. Strategies for Human Adipose Tissue Repair and Regeneration. *J. Cosmet. Dermatological Sci. Appl.* **2012**, *2*, 93–107, doi:10.4236/JCDSA.2012.222021.

23. Mahoney, C.M.; Imbarlina, C.; Yates, C.C.; Marra, K.G. Current Therapeutic Strategies for Adipose Tissue Defects/Repair Using Engineered Biomaterials and Biomolecule Formulations. *Front. Pharmacol.* **2018**, *0*, 507, doi:10.3389/FPHAR.2018.00507.

24. Ailhaud, G.; Grimaldi, P.; Négrel, R. Cellular and molecular aspects of adipose tissue development. *Annu. Rev. Nutr.* **1992**, *12*, 207–233, doi.org/10.1146/annurev.nu.12.070192.001231.

25. Niemelä, S.M.; Miettinen, S.; Konttinen, Y.; Waris, T.; Kellomäki, M.; Ashammakhi, N.A.; Ylikomi, T. Fat tissue: Views on reconstruction and exploitation. *J. Craniofac. Surg.* **2007**, *18*, 325–335, doi:10.1097/SCS.0B013E3180333B6A.

26. Frayn, K.N.; Karpe, F.; Fielding, B.A., Macdonald, A.; Coppack, SW. Integrative physiology of human adipose tissue. *Int. J. Obes. Relat. Metab. Disord.* **2003**, *27*, 875–888, doi:10.1038/SJ.IJO.0802326.

27. Wozniak, S.E.; Gee, L.L.; Wachtel, M.S.; Frezza, E.E. Adipose tissue: The new endocrine organ? a review article. *Dig. Dis. Sci.* **2009**, *54*, 1847–1856, doi:10.1007/S10620-008-0585-3.

28. Cannon, B.; Nedergaard, J. Brown adipose tissue: function and physiological significance. *Physiol. Rev.* **2004**, *84*, 277–359, doi:10.1152/PHYSREV.00015.2003.

29. Farmer, S.R. Molecular determinants of brown adipocyte formation and function. *Genes Dev.* **2008**, *22*, 1269–1275, doi:10.1101/GAD.1681308.

30. Nedergaard, J.; Bengtsson, T.; Cannon, B. Unexpected evidence for active brown adipose tissue in adult humans. *Am. J. Physiol. Endocrinol. Metab.* **2007**, *293*, doi:10.1152/AJPENDO.00691.2006.

31. Louis, F.; Matsusaki, M. Adipose tissue engineering. *Biomater. Organ Tissue Regen.* **2020**, 393–423, doi:10.1016/B978-0-08-102906-0.00008-8.

32. Jo, J.; Gavrilova, O.; Pack, S.; Jou, W.; Mullen, S.; Sumner, AE.; Cushman, SW.; Periwal, V. Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. *PLoS Comput. Biol.* **2009**, *5*, doi:10.1371/JOURNAL.PCBI.1000324.

33. Kubo, Y.; Kaidzu, S.; Nakajima, I.; Takenouchi, K., Nakamura, F. Organization of extracellular matrix components during differentiation of adipocytes in long-term culture. *In Vitro Cell Dev Biol Anim.* ***2000,*** 36(1):38-44, doi: 10.1290/1071-2690(2000)036<0038:OOEMCD>2.0.CO;2.

34. Nakajima, I.; Muroya, S.; Tanabe, R.I.; Chikuni, K. Extracellular matrix development during differentiation into adipocytes with a unique increase in type V and VI collagen. *Biol. Cell* **2002**, *94*, 197–203, doi:10.1016/S0248-4900(02)01189-9.

35. Halberg, N.; Wernstedt-Asterholm, I.; Scherer, P.E. The Adipocyte as an Endocrine Cell. *Endocrinol. Metab. Clin. North Am.* **2008**, *37*, 753–768, doi:10.1016/J.ECL.2008.07.002.

36. Mariam, E.C.; Wang, P. Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cell. Mol. Life Sci.* **2010**, *67*, 1277–1292, doi:10.1007/S00018-010-0263-4.

37. Avram, M.M.; Avram, A.S.; James, W.D. Subcutaneous fat in normal and diseased states. 3. Adipogenesis: From stem cell to fat cell. *J. Am. Acad. Dermatol.* **2007**, *56*, 472–492, doi:10.1016/J.JAAD.2006.06.022.

38. Rosen, E.D.; MacDougald, O.A. Adipocyte differentiation from the inside out. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 885–896, doi:10.1038/nrm2066.

39. Gregoire, F.M.; Smas, C.M.; Sul, HS. Understanding adipocyte differentiation. *Physiol. Rev.* **1998**, *78*, 783–809, doi:10.1152/PHYSREV.1998.78.3.783.

40. Diamond, F.B.; Eichler, D.C. Leptin and the adipocyte endocrine system. *Crit. Rev. Clin. Lab. Sci.* **2002**, *39*, 499–525, doi:10.1080/10408360290795565.

41. Zhang, Y.; Proenca, R.; Maffei, M.; Barone, M.; Leopold, L.; Friedman, J.M. Positional cloning of the mouse obese gene and its human homologue. *Nature* **1994**, *372*, 425–432, doi:10.1038/372425A0.

42. Fischer-Posovszky, P.; Wabitsch, M.; Hochberg, Z. Endocrinology of adipose tissue - an update. *Horm. Metab. Res.* **2007**, *39*, 314–321, doi:10.1055/S-2007-976539.

43. Lago, F.; Gómez, R.; Gómez-Reino, J.J.; Dieguez, C.; Gualillo, O. Adipokines as novel modulators of lipid metabolism. *Trends Biochem. Sci.* **2009**, *34*, 500–510, doi:10.1016/J.TIBS.2009.06.008.

44. Guerre-Millo, M. Adipose tissue hormones. *J. Endocrinol. Invest.* **2002**, *25*, 855–861, doi:10.1007/BF03344048.

45. Kadowaki, T.; Yamauchi, T. Adiponectin receptor signaling: a new layer to the current model. *Cell Metab.* **2011**, *13*, 123–124, doi:10.1016/J.CMET.2011.01.012.

46. Kershaw, E.E.; Flier, J.S. Adipose tissue as an endocrine organ. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 2548–2556, doi:10.1210/JC.2004-0395.

47. Friedman, J.M.; Halaas, J.L. Leptin and the regulation of body weight in mammals. *Nature* **1998**, *395*, 763–770, doi:10.1038/27376.

48. Friedman, J.M. Leptin and the regulation of body weigh. *Keio J. Med.* **2011**, *60*, 1–9, doi:10.2302/KJM.60.1.

49. Park, J.; Euhus, D.M.; Scherer, P.E. Paracrine and Endocrine Effects of Adipose Tissue on Cancer Development and Progression. *Endocr. Rev.* **2011**, *32*, 550, doi:10.1210/ER.2010-0030.

50. Kelesidis, T.; Kelesidis, I.; Chou, S.; Mantzoros, C.S. Narrative review: The role of leptin in human physiology: Emerging clinical applications. *Ann. Intern. Med.* **2010**, *152*, 93–100, doi:10.7326/0003-4819-152-2-201001190-00008.

51. Flier, J.S. The missing link with obesity? *Nature* **2001**, *409*, 292–293, doi:10.1038/35053251.

52. Zhou, B.; Lu, Y.; Hajifathalian, K.; Bentham, J.; Cesare, M. Di; Danaei, G.; Bixby, H.; Cowan, M.; Ali, M.; Taddei, C.; et al. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4·4 million participants. *Lancet* **2016**, *387*, 1513–1530, doi:10.1016/S0140-6736(16)00618-8.

53. Scherer, P.E.; Williams, S.; Fogliano, M.; Baldini, G.; Lodish, HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.* **1995**, *270*, 26746–26749, doi:10.1074/JBC.270.45.26746.

54. Hu, E.; Liang, P.; Spiegelman, BM.; AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J. Biol. Chem.* **1996**, *271*, 10697–10703, doi:10.1074/JBC.271.18.10697.

55. Berg, A.H.; Combs, T.P.; Du, X.; Brownlee, M.; Scherer, P.E. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat. Med. 2001 78* **2001**, *7*, 947–953, doi:10.1038/90992.

56. Maeda, N.; Shimomura, I.; Kishida, K.; Nishizawa, H.; Matsuda, M.; Nagaretani, H.; Furuyama, N.; Kondo, H.; Takahashi, M.; Arita, Y.; et al. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat. Med. 2002 87* **2002**, *8*, 731–737, doi:10.1038/nm724.

57. Yamauchi, T.; Kamon, J.; Minokoshi, Y.; Ito, Y.; Waki, H.; Uchida, S.; Yamashita, S.; Noda, M.; Kita, S.; Ueki, K.; et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med. 2002 811* **2002**, *8*, 1288–1295, doi:10.1038/nm788.

58. Yoon, M.J.; Lee, G.Y.; Chung, J.-J.; Ahn, Y.H.; Hong, S.H.; Kim, J.B. Adiponectin Increases Fatty Acid Oxidation in Skeletal Muscle Cells by Sequential Activation of AMP-Activated Protein Kinase, p38 Mitogen-Activated Protein Kinase, and Peroxisome Proliferator–Activated Receptor α. *Diabetes* **2006**, *55*, 2562–2570, doi:10.2337/DB05-1322.

59. Ahima, R.S.; Flier, J.S. Adipose tissue as an endocrine organ. *Trends Endocrinol. Metab.* **2000**, *11*, 327–332, doi:10.1016/S1043-2760(00)00301-5.

60. Cao, Y. Angiogenesis modulates adipogenesis and obesity. *J. Clin. Invest.* **2007**, *117*, 2362–2368, doi:10.1172/JCI32239.

61. Lijnen, H.R. Angiogenesis and obesity. *Cardiovasc. Res.* **2008**, *78*, 286–293, doi:10.1093/CVR/CVM007.

62. Lemoine, A.Y.; Ledoux, S.; Larger, E. Adipose tissue angiogenesis in obesity. *Thromb. Haemost.* **2013**, *110*, 661–669, doi:10.1160/TH13-01-0073.

63. Nishimura, S.; Manabe, I.; Nagasaki, M.; Hosoya, Y.; Yamashita, H.; Fujita, H.; Ohsugi, M.; Tobe, K.; Kadowaki, T.; Nagai, R.; Sugiura, S. Adipogenesis in obesity requires close interplay between differentiating adipocytes, stromal cells, and blood vessels. *Diabetes* **2007**, *56*, 1517–1526, doi:10.2337/DB06-1749.

64. Christiaens, V.; Lijnen, H.R. Angiogenesis and development of adipose tissue. *Mol. Cell. Endocrinol.* **2010**, *318*, 2–9, doi:10.1016/j.mce.2009.08.006.

65. Hausman, G.J.; Richardson, R.L. Adipose tissue angiogenesis. *J. Anim. Sci.* **2004**, *82*, 925–934, doi:10.2527/2004.823925X.

66. Stupack, D.G.; Cheresh, D.A. ECM remodeling regulates angiogenesis: endothelial integrins look for new ligands. *Sci. STKE* **2002**, *2002*, doi:10.1126/STKE.2002.119.PE7.

67. Carmeliet, P.; Jain, R.K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* **2011**, *473*, 298–307, doi:10.1038/NATURE10144.

68. Miranville, A.; Heeschen, C.; Sengenes, C.; Curat, CA.; Busse, R.; Bouloumie. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* **2004**, *110*, 349–355, doi:10.1161/01.CIR.0000135466.16823.D0.

69. Ribatti, D.; Conconi, MT.; Nussdorfer, GG. Nonclassic endogenous novel regulators of angiogenesis. *Pharmacol. Rev.* **2007**, *59*, 185–205, doi:10.1124/PR.59.2.3.

70. Cho, C.H.; Koh, Y.J.; Han, J.; Sung, H.K.; Lee, H.J.; Morisada, T.; Schwendener, R.A.; Brekken, R.A.; Kang, G.; Oike, Y.; et al. Angiogenic role of LYVE-1-positive macrophages in adipose tissue. *Circ. Res.* **2007**, *100*, doi:10.1161/01.RES.0000259564.92792.93.

71. Rehman, J.; Traktuev, D.; Li, J.; Merfeld-Clauss, S.; Temm-Grove, CJ.; Bovenkerk, JE.; Pell, CL.; Johnstone, BH.; Considine, RV.; March, KL. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* **2004**, *109*, 1292–1298, doi:10.1161/01.CIR.0000121425.42966.F1.

72. Saiki, A.; Watanabe, F.; Murano, T.; Miyashita, Y.; Shirai, K. Hepatocyte growth factor secreted by cultured adipocytes promotes tube formation of vascular endothelial cells in vitro. *Int. J. Obes. (Lond).* **2006**, *30*, 1676–1684, doi:10.1038/SJ.IJO.0803316.

73. Kilroy, G.E.; Foster, S.J.; Wu, X.; Ruiz, J.; Sherwood, S.; Heifetz, A.; Ludlow, J.W.; Stricker, D.M.; Potiny, S.; Green, P.; et al. Cytokine profile of human adipose-derived stem cells: Expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J. Cell. Physiol.* **2007**, *212*, 702–709, doi:10.1002/JCP.21068.

74. Grosfeld, A.; André, J.; Mouzon, S.H. De; Berra, E.; Pouysségur, J.; Guerre-Millo, M. Hypoxia-inducible factor 1 transactivates the human leptin gene promoter. *J. Biol. Chem.* **2002**, *277*, 42953–42957, doi:10.1074/JBC.M206775200.

75. Corvera, S.; Gealekman, O. Adipose tissue angiogenesis: impact on obesity and type-2 diabetes. *Biochim. Biophys. Acta* **2014**, *1842*, 463–472, doi:10.1016/J.BBADIS.2013.06.003.

76. Ribatti, D.; Crivellato, E. “Sprouting angiogenesis”, a reappraisal. *Dev. Biol.* **2012**, *372*, 157–165, doi:10.1016/J.YDBIO.2012.09.018.

77. Sierra-Honigman, M.R.; Nath, A.K.; Murakami, C.; Garcia-Cardena, G.; Papapetropoulos, A.; Sessa, WC.; Madge, LA.; Schechner, JS.; Schwabb, MB.; Polverini, PJ.; Flores-Riveros, JR. Biological action of leptin as an angiogenic factor. *Science* **1998**, *281*, 1683–1686, doi:10.1126/SCIENCE.281.5383.1683.

78. Cao, R.; Brakenhielm, E.; Wahlestedt, C.; Thyberg, J.; Cao, Y. Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 6390, doi:10.1073/PNAS.101564798.

79. Suganami, E.; Takagi, H.; Ohashi, H.; Suzuma, K.; Suzuma, I.; Oh, H.; Watanabe, D.; Ojima, T.; Suganami, T.; Fujio, Y.; et al. Leptin Stimulates Ischemia-Induced Retinal Neovascularization. *Diabetes* **2004**, *53*, 2443–2448, doi:10.2337/DIABETES.53.9.2443.

80. Fukumura, D.; Ushiyama, A.; Duda, GD.; Xu, L.; Tam, J.; Krishna, V.; Chatterjee, K.; Garkavtsev, I.; Jain, RK. Paracrine regulation of angiogenesis and adipocyte differentiation during in vivo adipogenesis. *Circ. Res.* **2003**, *93*, doi:10.1161/01.RES.0000099243.20096.FA.

81. Choi, J.H.; Bellas, E.; Gimble, J.M.; Vunjak-Novakovic, G.; Kaplan, D.L. Lipolytic function of adipocyte/endothelial cocultures. *Tissue Eng. - Part A* **2011**, *17*, 1437–1444, doi:10.1089/TEN.TEA.2010.0527.

82. Zuk, P.A.; Zhu, M.; Mizuno, H.; Huang, J.; William Futrell, J.; Katz, AJ.; Benhaim, P.; Lorenz, HP.; Hedrick, MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* **2001**, *7*, 211–228, doi:10.1089/107632701300062859.

83. Zuk, P.A.; Zhu, M.; Ashjian, P.; De Ugarte, D.A.; Huang, J.I.; Mizuno, H.; Alfonso, Z.C.; Fraser, J.K.; Benhaim, P.; Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **2002**, *13*, 4279–4295, doi:10.1091/MBC.E02-02-0105.

84. Gimble, J.M.; Guilak, F.; Bunnell, B.A. Clinical and preclinical translation of cell-based therapies using adipose tissue-derived cells. *Stem Cell Res. Ther.* **2010**, *1*, doi:10.1186/SCRT19.

85. Aust, L.; Devlin, B.; Foster, S.J.; Halvorsen, Y.D.C.; Hicok, K.; du Laney, T.; Sen, A.; Willingmyre, G.D.; Gimble, J.M. Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* **2004**, *6*, 7–14, doi:10.1080/14653240310004539.

86. Gimble, J.M.; Katz, A.J.; Bunnell, B.A. Adipose-derived stem cells for regenerative medicine. *Circ. Res.* **2007**, *100*, 1249–1260, doi:10.1161/01.RES.0000265074.83288.09.

87. Dubois, S.G.; Floyd, E.Z.; Zvonic, S.; Kilroy, G.; Wu, X.; Carling, S.; Di, Y.; Halvorsen, C.; Ravussin, E.; Gimble, J.M. Isolation of Human Adipose-derived Stem Cells from Biopsies and Liposuction Specimens. *Methods in molecular biology* **2008**, 449:69-79, doi:10.1007/978-1-60327-169-1_5.

88. Gruber, R.P.; Jazayeri, L. Discussion: Effects of adipose-derived stem cells on improving the viability of diced cartilage grafts. *Plast. Reconstr. Surg.* **2012**, *129*, 378–379, doi:10.1097/PRS.0B013E31822B61B8.

89. Fraser, J.K.; Wulur, I.; Alfonso, Z.; Hedrick, M.H. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol.* **2006**, *24*, 150–154, doi:10.1016/J.TIBTECH.2006.01.010.

90. Chu, D.-T.; Nguyen Thi Phuong, T.; Tien, N.L.B.; Tran, D.K.; Minh, L.B.; Thanh, V. Van; Gia Anh, P.; Pham, V.H.; Thi Nga, V. Adipose Tissue Stem Cells for Therapy: An Update on the Progress of Isolation, Culture, Storage, and Clinical Application. *J. Clin. Med.* **2019**, *8*, 917, doi:10.3390/jcm8070917.

91. Bourin, P.; Bunell, B.A.; Casteilla, L.; Dominici, M.; Katz, A.J.; March, K.L.; Redl, H.; Rubin, J.P.; Yoshimura, K.; Gimble, J.M. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* **2013**, *15*, 641–648, doi:10.1016/J.JCYT.2013.02.006.

92. Rainey Daher, S.; Johnstone, B.H.; Phinney, D.G., March, K.L. Adipose stromal/stem cells: basic and translational advances: the IFATS collection. *Stem Cells* **2008**, *26*, 2664–2665, doi:10.1634/STEMCELLS.2008-0927.

93. Fraser, J.K.; Wulur, I.; Alfonso, Z.; Hedrick, M.H. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol.* **2006**, *24*, 150–154, doi:10.1016/j.tibtech.2006.01.010.

94. Mitchell, J.B.; McIntosh, K.; Zvonic, S.; Garret, S.; Floyd, Z.E.; Kloster, A.; Halvorsen, Y.D.; Storms, R.W.; Goh, B.; Kilroy, G.; Wu, X.; Gimble, J.M. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* **2006**, *24*, 376–385, doi:10.1634/STEMCELLS.2005-0234.

95. Guilak, F.; Lott, K.E.; Awad, H.A.; Cao, Q.; Hicok, K.C.; Fermor, B.; Gimble, J.M. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J. Cell. Physiol.* **2006**, *206*, 229–237, doi:10.1002/JCP.20463.

96. Tsuji, W. Adipose-derived stem cells: Implications in tissue regeneration. *World J. Stem Cells* **2014**, *6*, 312, doi:10.4252/WJSC.V6.I3.312.

97. Gonda, K.; Shigeura, T.; Sato, T.; Matsumoto, D.; Suga, H.; Inoue, K.; Aoi, N.; Kato, H.; Sato, K.; Murase, S.; et al. Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation. *Plast. Reconstr. Surg.* **2008**, *121*, 401–410, doi:10.1097/01.PRS.0000298322.70032.BC.

98. Raposio, E.; Simonacci, F.; Perrotta, R.E. Adipose-derived stem cells: Comparison between two methods of isolation for clinical applications. *Ann. Med. Surg.* **2017**, *20*, 87, doi:10.1016/J.AMSU.2017.07.018.

99. Bellei, B.; Migliano, E.; Tedesco, M.; Caputo, S.; Picardo, M. Maximizing non-enzymatic methods for harvesting adipose-derived stem from lipoaspirate: technical considerations and clinical implications for regenerative surgery. *Sci. Rep.* **2017**, *7*, doi:10.1038/S41598-017-10710-6.

100. Katz, A.J.; Tholpady, A.; Tholpady, S.S.; Shang, H.; Ogle, R.C. Cell Surface and Transcriptional Characterization of Human Adipose-Derived Adherent Stromal (hADAS) Cells. *Stem Cells* **2005**, *23*, 412–423, doi:10.1634/STEMCELLS.2004-0021.

101. Yoshimura, K.; Shigeura, T.; Matsumoto, D.; Sato, T.; Takaki, Y.; Aiba-Kojima, E.; Sato, K.; Inoue, K.; Nagase, T.; Koshima, I.; et al. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J. Cell. Physiol.* **2006**, *208*, 64–76, doi:10.1002/JCP.20636.

102. Zimmerlin, L.; Donnenberg, V.S.; Pfeifer, M.E.; Meyer, E.M.; Peault, B.; Rubin, J.P. Stromal vascular progenitors in adult human adipose tissue. *Cytometry. A* **2010**, *77*, 22–30, doi:10.1002/CYTO.A.20813.

103. Zannettino, A.C.W.; Paton, S.; Arthur, A.; Khor, F.; Itescu, S.; Gimble, J.M.; Gronthos, S. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J. Cell. Physiol.* **2008**, *214*, 413–421, doi:10.1002/JCP.21210.

104. Traktuev, D.O.; Merfeld-Clauss, S.; Li, J.; Kolonin, M.; Arap, W.; Pasqualini, R.; Johnstone, B.H.; March, K.L. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. *Circ. Res.* **2008**, *102*, 77–85, doi:10.1161/CIRCRESAHA.107.159475.

105. Crandall, D.L.; Hausman, G.J.; Kral, J.G. A review of the microcirculation of adipose tissue: Anatomic, metabolic, and angiogenic perspectives. *Microcirculation* **1997**, *4*, 211–232, doi:10.3109/10739689709146786.

106. Bunnell, B.A.; Flaat, M.; Gagliardi, C.; Patel, B.; Ripoll, C. Adipose-derived stem cells: Isolation, expansion and differentiation. *Methods* **2008**, *45*, 115–120, doi:10.1016/j.ymeth.2008.03.006.

107. Radtke, C.; Schmitz, B.; Spies, M.; Kocsis, J.D.; Vogt, P.M. Peripheral glial cell differentiation from neurospheres derived from adipose mesenchymal stem cells. *Int. J. Dev. Neurosci.* **2009**, *27*, 817–823, doi:10.1016/J.IJDEVNEU.2009.08.006.

108. Kapur, S.K.; Katz, A.J. Review of the adipose derived stem cell secretome. *Biochimie* **2013**, *95*, 2222–2228, doi:10.1016/j.biochi.2013.06.001.

109. Mao, A.S.; Mooney, D.J. Regenerative medicine: Current therapies and future directions. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 14452, doi:10.1073/PNAS.1508520112.

110. Mason, C.; Dunnill P. A brief definition of regenerative medicine. *Regen. Med.* **2008**, *3*, 1–5, doi:10.2217/17460751.3.1.1.

111. Guilak, F. Functional tissue engineering: The role of biomechanics in reparative medicine. *Ann. N. Y. Acad. Sci.* **2002**, *961*, 193–195, doi:10.1111/J.1749-6632.2002.TB03080.X.

112. Sundelacruz, S.; Kaplan, D.L. Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine. *Semin. Cell Dev. Biol.* **2009**, *20*, 646–655, doi:10.1016/J.SEMCDB.2009.03.017.

113. Horwitz, E.M.; Le Blanc, K.; Dominici, M.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.C.; Deans, R.J.; Krause, D.S.; Keating, A. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* **2005**, *7*, 393–395, doi:10.1080/14653240500319234.

114. Blanpain, C.; Lowry, W.E.; Geoghegan, A.; Polak, L.; Fuchs, E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* **2004**, *118*, 635–648, doi:10.1016/J.CELL.2004.08.012.

115. Si, Z.; Wang, X.; Sun, C.; Kang, Y.; Xu, J.; Wang, X.; Hui, Y. Adipose-derived stem cells: Sources, potency, and implications for regenerative therapies. *Biomed. Pharmacother.* **2019**, *114*, 108765.

116. Casadei, A.; Epis, R.; Ferroni, L.; Tocco, I.; Gardin, C.; Bressan, E.; Sivolella, S.; Vindigni, V.; Pinton, P.; Mucci, G.; et al. Adipose tissue regeneration: A state of the art. *J. Biomed. Biotechnol.* **2012**, *2012*, doi:10.1155/2012/462543.

117. Mizuno, H. Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review. *J. Nippon Med. Sch.* **2009**, *76*, 56–66, doi:10.1272/JNMS.76.56.

118. Zuk, P. Adipose-Derived Stem Cells in Tissue Regeneration: A Review. *ISRN Stem Cells* **2013**, *2013*, 35, doi:10.1155/2013/713959.

119. Lu, F.; Mizuno, H.; A Uysal, C.; Cai, X.; Ogawa, R.; Hyakusoku, H. Improved viability of random pattern skin flaps through the use of adipose-derived stem cells. *Plast. Reconstr. Surg.* **2008**, *121*, 50–58, doi:10.1097/01.PRS.0000293876.10700.B8.

120. Karathanasis, V.; Petrakis, S.; Topouridou, K.; Koliakou, E.; Koliakos, G.; Demiri, E. Intradermal injection of GFP-producing adipose stromal cells promotes survival of random-pattern skin flaps in rats. *Eur. J. Plast. Surg. 2013 365* **2013**, *36*, 281–288, doi:10.1007/S00238-013-0810-Y.

121. Kim, W.S.; Park, B.S.; Kim, H.K.; Park, J.S.; Kim, K.J.; Choi, J.S.; Chung, S.J.; Kim, D.D.; Sung, J.H. Evidence supporting antioxidant action of adipose-derived stem cells: protection of human dermal fibroblasts from oxidative stress. *J. Dermatol. Sci.* **2008**, *49*, 133–142, doi:10.1016/J.JDERMSCI.2007.08.004.

122. Ohnishi, S.; Sumiyoshi, H.; Kitamura, S.; Nagaya, N. Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. *FEBS Lett.* **2007**, *581*, 3961–3966, doi:10.1016/J.FEBSLET.2007.07.028.

123. Vriend, L. The Therapeutic Use Of Adipose Tissue-Derived Stromal Cells (ASCs) In Scar Tissue And Its Potential In Scar Remodeling. *J. Stem Cells Res. Dev. Ther.* **2020**, *6*, 1–7, doi:10.24966/SRDT-2060/100036.

124. Richardson, S.M.; Kalamegam, G.; Pushparaj, P.N.; Matta, C.; Memic, A.; Khademhosseini, A.; Mobasheri, R.; Poletti, F.L.; Hoyland, J.A.; Mobasheri, A. Mesenchymal stem cells in regenerative medicine: Focus on articular cartilage and intervertebral disc regeneration. *Methods* **2016**, *99*, 69–80, doi:10.1016/J.YMETH.2015.09.015.

125. Marigo, I.; Dazzi, F. The immunomodulatory properties of mesenchymal stem cells. *Semin. Immunopathol.* **2011**, *33*, 593–602, doi:10.1007/S00281-011-0267-7.

126. Benvenuto, F.; Ferrari, S.; Gerdoni, E.; Gualandi, F.; Frassoni, F.; Pistoia, V.; Mancardi, G.; Uccelli, A. Human mesenchymal stem cells promote survival of T cells in a quiescent state. *Stem Cells* **2007**, *25*, 1753–1760, doi:10.1634/STEMCELLS.2007-0068.

127. Chen, L.; Tredget, E.E.; Wu, P.Y.G.; Wu, Y.Y.G. Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing. *PLoS One* **2008**, *3*, 1886, doi:10.1371/journal.pone.0001886.

128. Timper, K.; Seboek, D.; Eberhardt, M.; Linscheid, P.; Christ-Crain, M.; Keller, U.; Müller, B.; Zulewski, H. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochem. Biophys. Res. Commun.* **2006**, *341*, 1135–1140, doi:10.1016/J.BBRC.2006.01.072.

129. Almalki, S.G.; Agrawal, D.K. Key transcription factors in the differentiation of mesenchymal stem cells. *Differentiation* **2016**, *92*, 41–51, doi:10.1016/J.DIFF.2016.02.005.

130. Rigotti, G.; Marchi, A.; Galie, M.; Baroni, G.; Benati, D.; Krampera, M.; Pasini, A.; Sbarbati, A. Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. *Plast. Reconstr. Surg.* **2007**, *119*, 1409–1422, doi:10.1097/01.PRS.0000256047.47909.71.

131. Mazolla, R.F.; Cantarella, G.; Torretta, S.; Sbarbati, A.; Lazzari, L.; Pignataro, L. Autologous fat injection to face and neck: from softtissue augmentation to regenerative medicine. *Acta Otorhinolaryngol. Ital.* **2011**, *31*, 59.

132. Dealy, E.; Garson, S.; Tousson, G.; Sinna, R.; Fat injection to the breast: technique, results, and indications based on 880 procedures over 10 years. *Aesthetic Surg. J.* **2009**, *29*, 360–376, doi:10.1016/J.ASJ.2009.08.010.

133. Rosen, E.D.; Spiegelman, B.M. Molecular regulation of adipogenesis. *Annu. Rev. Cell Dev. Biol.* **2000**, *16*, 145–171, doi:10.1146/ANNUREV.CELLBIO.16.1.145.

134. Bauer-Kreisel, P.; Goepferich, A.; Blunk, T. Cell-delivery therapeutics for adipose tissue regeneration. *Adv. Drug Deliv. Rev.* **2010**, *62*, 798–813, doi:10.1016/j.addr.2010.04.003.

135. Hwang, C.S.; Loftus, T.M.; Mandrup, S.; Lane, M.D. Adipocyte differentiation and leptin expression. *Annu. Rev. Cell Dev. Biol.* **1997**, *13*, 231–259, doi:10.1146/ANNUREV.CELLBIO.13.1.231.

136. Flynn, L.; Woodhouse, K.A. Adipose tissue engineering with cells in engineered matrices. *Organogenesis* **2008**, *4*, 228–235, doi:10.4161/org.4.4.7082.

137. Ali, A.T.; Hochfeld, W.E.; Myburgh, R.; Pepper, M.S. Adipocyte and adipogenesis. *Eur. J. Cell Biol.* **2013**, *92*, 229–236, doi:10.1016/J.EJCB.2013.06.001.

138. Fève, B. Adipogenesis: Cellular and molecular aspects. *Best Pract. Res. Clin. Endocrinol. Metab.* **2005**, *19*, 483–499, doi:10.1016/J.BEEM.2005.07.007.

139. Otto, T.C.; Lane, M.D. Adipose development: From stem cell to adipocyte. *Crit. Rev. Biochem. Mol. Biol.* **2005**, *40*, 229–242, doi:10.1080/10409230591008189.

140. Cawthorn, W.P.; Scheller, E.L.; MacDougald, O.A. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. *J. Lipid Res.* **2012**, *53*, 227–246, doi:10.1194/JLR.R021089.

141. Tang, Q.Q.; Lane, M.D. Adipogenesis: From stem cell to adipocyte. *Annu. Rev. Biochem.* **2012**, *81*, 715–736, doi:10.1146/ANNUREV-BIOCHEM-052110-115718.

142. Lowe, C.E.; O'Rahilly, S.; Rochford, J.J. Adipogenesis at a glance. *J. Cell Sci.* **2011**, *124*, 2681–2686, doi:10.1242/jcs.079699.

143. Bourgeois, C.; Gorwood, J.; Barrail-Tran, A.; Lagathu, C.; Capeau, J.; Desjardins, D.; Grand, R. Le; Damouche, A.; Béréziat, V.; Lambotte, O. Specific Biological Features of Adipose Tissue, and Their Impact on HIV Persistence. *Front. Microbiol.* **2019**, *10*, doi:10.3389/FMICB.2019.02837.

144. de Villiers, D.; Potgieter, M.; Ambele, M.A.; Adam, L.; Durandt, C.; Pepper, M.S. The role of reactive oxygen species in adipogenic differentiation. *Adv. Exp. Med. Biol.* **2018**, *1083*, 125–144, doi:10.1007/5584_2017_119.

145. Christodoulides, C.; Lagathu, C.; Sethi, J.K.; Vidal-Puig, A. Adipogenesis and WNT signalling. *Trends Endocrinol. Metab.* **2009**, *20*, 16–24, doi:10.1016/J.TEM.2008.09.002.

146. Farmer, S.R. Transcriptional control of adipocyte formation. *Cell Metab.* **2006**, *4*, 263–273, doi:10.1016/J.CMET.2006.07.001.

147. Rosen, E.D.; Hsu, C.-H.; Wang, X.; Sakai, S.; Freeman, M.W.; Gonzalez, F.J.; Spiegelman, B.M. C/EBPα induces adipogenesis through PPARγ: a unified pathway. *Genes Dev.* **2002**, *16*, 22, doi:10.1101/GAD.948702.

148. Won Park, K.; Halperin, D.S.; Tontonoz, P. Before They Were Fat: Adipocyte Progenitors. *Cell Metab.* **2008**, *8*, 454–457, doi:10.1016/J.CMET.2008.11.001.

149. Gentile, P.; Orlandi, A.; Giovanna Scioli, M.; Di Pasquali, C.; Bocchini, I. Concise review: adipose-derived stromal vascular fraction cells and platelet-rich plasma: basic and clinical implications for tissue engineering therapies in regenerative surgery. *Stem Cells Transl. Med.* **2012**, *1*, 230–236, doi:10.5966/SCTM.2011-0054.

150. Gesta, S.; Tseng, Y.H.; Kahn, C.R. Developmental origin of fat: tracking obesity to its source. *Cell* **2007**, *131*, 242–256, doi:10.1016/J.CELL.2007.10.004.

151. Tontonoz, P.; Spiegelman, B.M. Fat and beyond: The diverse biology of PPARγ. *Annu. Rev. Biochem.* **2008**, *77*, 289–312, doi:10.1146/ANNUREV.BIOCHEM.77.061307.091829.

152. Wu, Z.; Wang, S. Role of kruppel-like transcription factors in adipogenesis. *Dev. Biol.* **2013**, *373*, 235–243, doi:10.1016/J.YDBIO.2012.10.031.

153. Oishi, Y.; Manabe, I.; Tobe, K.; Tsushima, K.; Shindo, T.; Fujiu, K.; Nishimura, G.; Maemura, K.; Yamauchi, T.; Kubota, N.; Suzuki, R.; Kitamura, T.; Akira, S.; Kadowaki, T.; Nagai, R. Krüppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. *Cell Metab.* **2005**, *1*, 27–39, doi:10.1016/J.CMET.2004.11.005.

154. Zhang, Y.; Khan, D.; Delling, J.; Tobiasch, E. Mechanisms Underlying the Osteo- and Adipo-Differentiation of Human Mesenchymal Stem Cells. *Sci. World J.* **2012**, *2012*, 14, doi:10.1100/2012/793823.

155. Bacakova, L.; Zarubova, J.; Travnickova, M.; Musilkova, J.; Pajorova, J.; Slepicka, P.; Kasalkova, N.S.; Svorcik, V.; Kolska, Z.; Motarjemi, H.; et al. Stem cells: their source, potency and use in regenerative therapies with focus on adipose-derived stem cells – a review. *Biotechnol. Adv.* **2018**, *36*, 1111–1126, doi:10.1016/J.BIOTECHADV.2018.03.011.

156. Naderi, N.; Wilde, C.; Haque, T.; Francis, W.; Seifalian, A.M.; Thornton, C.A.; Xia, Z.; Whitaker, I.S. Adipogenic differentiation of adipose-derived stem cells in 3-dimensional spheroid cultures (microtissue): Implications for the reconstructive surgeon. *J. Plast. Reconstr. Aesthetic Surg.* **2014**, *67*, 1726–1734, doi:10.1016/J.BJPS.2014.08.013.

157. Wei, Y.; Sun, X.; Wang, W.; Hu, Y. Adipose-derived stem cells and chondrogenesis. *Cytotherapy* **2007**, *9*, 712–716, doi:10.1080/14653240701620596.

158. Dubey, N.; Mishra, V.; Dubey, R.; Deng, Y.-H.; Tsai, F.-C.; Deng, W.-P. Revisiting the Advances in Isolation, Characterization and Secretome of Adipose-Derived Stromal/Stem Cells. *Int. J. Mol. Sci.* **2018**, *19*, 2200, doi:10.3390/ijms19082200.

159. Khan, W.S.; Tew, S.R.; Adesida, A.B.; Hardingham, T.E. Human infrapatellar fat pad-derived stem cells express the pericyte marker 3G5 and show enhanced chondrogenesis after expansion in fibroblast growth factor-2. *Arthritis Res. Ther.* **2008**, *10*, R74, doi:10.1186/AR2448.

160. Hildner, F.; Albrecht, C.; Gabriel, C.; Redl, H.; van Griensven, M. State of the art and future perspectives of articular cartilage regeneration: a focus on adipose-derived stem cells and platelet-derived products. *J. Tissue Eng. Regen. Med.* **2011**, *5*, doi:10.1002/TERM.386.

161. Tapp, H.; Hanley, E.N.; Patt, J.C.; Gruber, H.E. Adipose-Derived Stem Cells: Characterization and Current Application in Orthopaedic Tissue Repair. *Exp. Biol. Med.* **2008**, *234*, 1–9, doi:10.3181/0805/MR-170.

162. Maioli, M.; Basoli, V.; Santaniello, S.; Cruciani, S.; Palmerio Delitala, A.; Pinna, R.; Milia, E.; Grillari-Voglauer, R.; Fontani, V.; Rinaldi, S.; Muggironi, R.; Pigliaru, G. et al. Osteogenesis from Dental Pulp Derived Stem Cells: A Novel Conditioned Medium Including Melatonin within a Mixture of Hyaluronic, Butyric, and Retinoic Acids. *Stem Cells Int.* **2016**, *2016*, 2056416–2056416, doi:10.1155/2016/2056416.

163. Halvorsen, Y.-D.C.; Franklin, D.; Bond, A.L.; Hitt, D.C.; Auchter, C.; Boskey, A.L.; Paschalis, E.P.; Wilkison, W.O.; Gimble, J.M. Extracellular Matrix Mineralization and Osteoblast Gene Expression by Human Adipose Tissue–Derived Stromal Cells. *Tissue Eng*. **2004**, *7*, 729–741, doi:10.1089/107632701753337681.

164. Grottkau, B.E.; Lin, Y. Osteogenesis of Adipose-Derived Stem Cells. *Bone Res.* **2013**, *1*, 133–145, doi:10.4248/BR201302003.

165. Friedenstein, A.J.; Chailakhyan, R.K.; Gerasimov, U. V. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Prolif.* **1987**, *20*, 263–272, doi:10.1111/J.1365-2184.1987.TB01309.X.

166. Elabd, C.; Chiellini, C.; Massoudi, A.; Cochet, O.; Zaragosi, L.E.; Trojana, C.; Michiels, J.F.; Weiss, P. Carle, G et al. Human adipose tissue-derived multipotent stem cells differentiate in vitro and in vivo into osteocyte-like cells. *Biochem. Biophys. Res. Commun.* **2007**, *361*, 342–348, doi:10.1016/J.BBRC.2007.06.180.

167. Halvorsen, Y.D.C.; Franklin, D.; Bond, A.L.; Hitt, D.C.; Auchter, C.; Boskey, A.L.; Paschalis, E.P.; Wilkison, W.O.; Gimble, J.M. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. *Tissue Eng.* **2001**, *7*, 729–741, doi:10.1089/107632701753337681.

168. Radtke, C.; Schmitz, B.; Spies, M.; Kocsis, J.D.; Vogt, P.M. Peripheral glial cell differentiation from neurospheres derived from adipose mesenchymal stem cells. *Int. J. Dev. Neurosci.* **2009**, *27*, 817–823, doi:10.1016/J.IJDEVNEU.2009.08.006.

169. Lindroos, B.; Suuronen, R.; Miettinen, S. The Potential of Adipose Stem Cells in Regenerative Medicine. *Stem Cell Rev. Reports* **2011**, *7*, 269–291, doi:10.1007/S12015-010-9193-7.

170. Ren, L.; Chen, L.; Qi, H.; Li, F.; Gong, F. In vitro Differentiation of Human Adipose Tissue-Derived Stem Cells into Islet-Like Clusters Promoted by Islet Neogenesis-Associated Protein Pentadecapeptide. *Cells Tissues Organs* **2014**, *199*, 329–341, doi:10.1159/000362500.

171. Seo, M.J.; Suh, S.Y.; Bae, Y.C.; Jung, J.S. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochem. Biophys. Res. Commun.* **2005**, *328*, 258–264, doi:10.1016/J.BBRC.2004.12.158.

172. Bonora-Centelles, A.; Jover, R.; Mirabet, V.; Lahoz, A.; Carbonell, F.; Castell, J.V.; Gomez-Lechon, M.J. Sequential hepatogenic transdifferentiation of adipose tissue-derived stem cells: relevance of different extracellular signaling molecules, transcription factors involved, and expression of new key marker genes. *Cell Transplant.* **2009**, *18*, 1319–1340, doi:10.3727/096368909X12483162197321.

173. Timper, K.; Seboek, D.; Eberhardt, M.; Linscheid, P.; Christ-Crain, M.; Keller, U.; Müller, B.; Zulewski, H. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochem. Biophys. Res. Commun.* **2006**, *341*, 1135–1140, doi:10.1016/J.BBRC.2006.01.072.

174. Suga, H.; Eto, H.; Aoi, N.; Kato, H.; Araki, J.; Doi, K.; Higashino, T.; Yoshimura, K. Adipose tissue remodeling under ischemia: Death of adipocytes and activation of stem/ progenitor cells. *Plast. Reconstr. Surg.* **2010**, *126*, 1911–1923, doi:10.1097/PRS.0b013e3181f4468b.

175. Dai, R.; Wang, Z.; Samanipour, R.; Koo, K.I.; Kim, K. Adipose-Derived Stem Cells for Tissue Engineering and Regenerative Medicine Applications. *Stem Cells Int.* **2016**, *2016*, doi:10.1155/2016/6737345.

176. Tabata, Y. Biomaterial technology for tissue engineering applications. *J. R. Soc. Interface* **2009**, *6 Suppl 3*, doi:10.1098/RSIF.2008.0448.FOCUS.

177. O'Brien, F.J. Biomaterials & scaffolds for tissue engineering. *Mater. Today* **2011**, *14*, 88–95, doi:10.1016/S1369-7021(11)70058-X.

178. Salgado, A.J.; Reis, R.L.; Sousa, N.; Gimble, J.M. Adipose Tissue Derived Stem Cells Secretome: Soluble Factors and Their Roles in Regenerative Medicine. *Curr Stem Cell Res. Ther.* ***2010****, 5(2):103-10,* doi: 10.2174/157488810791268564.

179. Li, X.; Ma, T.; Sun, J.; Shen, M.; Xue, X.; Chen, Y.; Zhang, Z. Harnessing the secretome of adipose-derived stem cells in the treatment of ischemic heart diseases. *Stem Cell Res. Ther.* **2019**, *10*, 1–13, doi:10.1186/s13287-019-1289-7.

180. Banas, A.; Teratani, T.; Yamamoto, Y.; Tokuhara, M.; Takeshita, F.; Osaki, M.; Kawamata, M.; Kato, T.; Okochi, H.; Ochiya, T. IFATS Collection: In Vivo Therapeutic Potential of Human Adipose Tissue Mesenchymal Stem Cells After Transplantation into Mice with Liver Injury. *Stem Cells* **2008**, *26*, 2705–2712, doi:10.1634/STEMCELLS.2008-0034.

181. Trzyna, A.; Pikuła, B.; Ludwin, A.; Kocan, B.; Banaś-Ząbczyk, A. The influence of an electromagnetic field on adipose-derived stem/stromal cells' growth factor secretion: modulation of FGF-2 production by in vitro exposure. *Arch. Biol. Sci.* **2020**, *72*, 339–347, doi:10.2298/ABS200321028T.

182. An, Y.; Zhao, J.; Nie, F.; Qin, Z.; Xue, H.; Wang, G.; Li, D. Exosomes from Adipose-Derived Stem Cells (ADSCs) Overexpressing miR-21 Promote Vascularization of Endothelial Cells. *Sci. Reports 2019 91* **2019**, *9*, 1–10, doi:10.1038/s41598-019-49339-y.

183. Chang, C.-L.; Chen, H.-H.; Chen, K.-H.; Chiang, J.Y.; Li, Y.-C.; Lin, H.-S.; Sung, P.-H.; Yip, H.-K. Adipose-derived mesenchymal stem cell-derived exosomes markedly protected the brain against sepsis syndrome induced injury in rat. *Am. J. Transl. Res.* **2019**, *11*, 3955.

184. Chen, Z.; Xue, S.; Zhang, S.; Cheng, K.; Ye, Q. Exosomes from donor-derived adipose mesenchymal stem cells prolong the survival of vascularized composite allografts. *J. Cell. Physiol.* **2021**, *236*, 5895–5905, doi:10.1002/JCP.30274.

185. Yu, H.; Lu, K.; Zhu, J.; Wang, J. Stem cell therapy for ischemic heart diseases. *Br. Med. Bull.* **2017**, *121*, 135–154, doi:10.1093/BMB/LDW059.

186. Cesselli, D.; Beltrami, A.P.; Rigo, S.; Bergamin, N.; D'Aurizio, F.; Verardo, R.; Piazza, S.; Klaric, E.; Fanin, R.; Toffoletto, B.; et al. Multipotent progenitor cells are present in human peripheral blood. *Circ. Res.* **2009**, *104*, 1225–1234, doi:10.1161/CIRCRESAHA.109.195859.

187. Ding, D.-C.; Chou, H.-L.; Hung, W.-T.; Liu, H.-W.; Chu, T.-Y. Human adipose-derived stem cells cultured in keratinocyte serum free medium: Donor's age does not affect the proliferation and differentiation capacities. *J. Biomed. Sci. 2013 201* **2013**, *20*, 1–11, doi:10.1186/1423-0127-20-59.

188. Wu, H.; Li, J.Z.; Xie, B.D.; Tian, H.; Fang, S.H.; Jiang, S.L.; Kang, K. Lower senescence of adipose-derived stem cells than donor-matched bone marrow stem cells for surgical ventricular restoration. *Stem Cells Dev.* **2018**, *27*, 612–623, doi:10.1089/SCD.2017.0271.

189. Samsonraj, R.M.; Raghunath, M.; Nurcombe, V.; Hui, J.H.; Wijnen, A.J. van; Cool, S.M. Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine. *Stem Cells Transl. Med.* **2017**, *6*, 2173–2185, doi:10.1002/SCTM.17-0129.

190. Ruetze, M.; Richter, W. Adipose-derived stromal cells for osteoarticular repair: trophic function versus stem cell activity. *Expert Rev. Mol. Med.* **2014**, *16*, doi:10.1017/ERM.2014.9.

191. Rehman, J.; Traktuev, D.; Li, J.; Merfeld-Clauss, S.; Temm-Grove, C.J.; Bovenkerk, J.E.; Pell, C.L.; Johnstone, B.H.; Considine, R. V.; March, K.L. Secretion of Angiogenic and Antiapoptotic Factors by Human Adipose Stromal Cells. *Circulation* **2004**, *109*, 1292–1298, doi:10.1161/01.CIR.0000121425.42966.F1.

192. Gir, P.; Oni, G.; Brown, S.A.; Mojallal, A.; Rohrich, R.J. Human adipose stem cells: Current clinical applications. *Plast. Reconstr. Surg.* **2012**, *129*, 1277–1290, doi:10.1097/PRS.0B013E31824ECAE6.

193. Sterodimas, A.; De Faria, J.; Nicaretta, B.; Pitanguy, I. Tissue engineering with adipose-derived stem cells (ADSCs): Current and future applications. *J. Plast. Reconstr. Aesthetic Surg.* **2010**, *63*, 1886–1892, doi:10.1016/J.BJPS.2009.10.028.

194. Moriyama, H.; Moriyama, M.; Ozawa, T.; Tsuruta, D.; Iguchi, T.; Tamada, S.; Nakatani, T.; Nakagawa, K.; Hayakawa, T. Notch signaling enhances stemness by regulating metabolic pathways through modifying p53, nf-κb, and hif-1α. *Stem Cells Dev.* **2018**, *27*, 935–947, doi:10.1089/scd.2017.0260.

195. Nakagami, H.; Maeda, K.; Morishita, R.; Iguchi, S.; Nishikawa, T.; Takami, Y.; Kikuchi, Y.; Saito, Y.; Tamai, K.; Ogihara, T.; et al. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 2542–2547, doi:10.1161/01.ATV.0000190701.92007.6d.

196. Phino, A.G.; Cibrao, J.R.; Silva, N.A.; Monteira, S.; Salgado, A.J. Cell Secretome: Basic Insights and Therapeutic Opportunities for CNS Disorders. *Pharmaceuticals (Basel).* **2020**, *13*, doi:10.3390/PH13020031.

197. Mooney, D.J.; Vandenburgh, H. Cell Delivery Mechanisms for Tissue Repair. *Cell Stem Cell* **2008**, *2*, 205–213, doi:10.1016/j.stem.2008.02.005.

198. Copland, I.B.; Lord-Dufour, S.; Cuerquis, J.; Coutu, D.L.; Annabi, B.; Wang, E.; Galipeau, J. Improved Autograft Survival of Mesenchymal Stromal Cells by Plasminogen Activator Inhibitor 1 Inhibition. *Stem Cells* **2009**, *27*, 467–477, doi:10.1634/stemcells.2008-0520.

199. Han, M.K.; Kim, M.; Bae, S.Y.; Kang, L.; Han, S.Y.; Lee, Y.S.; Rha, J.H.; Kim, S.U.; Roh, J.K. VEGF protects human cerebral hybrid neurons from in vitro ischemia. *Neuroreport* **2004**, *15*, 847–850, doi:10.1097/00001756-200404090-00022.

200. Stein, I.; Neeman, M.; Shweiki, D.; Itin, A.; Keshet, E. Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. *Mol. Cell. Biol.* **1995**, *15*, 5363–5368, doi:10.1128/mcb.15.10.5363.

201. Shweiki, D.; Neeman, M.; Itin, A.; Keshet, E. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: Implications for tumor angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 768–772, doi:10.1073/pnas.92.3.768.

202. Cai, L.; Johnstone, B.H.; Cook, T.G.; Liang, Z.; Traktuev, D.; Cornetta, K.; Ingram, D.A.; Rosen, E.D.; March, K.L. Suppression of hepatocyte growth factor production impairs the ability of adipose-derived stem cells to promote ischemic tissue revascularization. *Stem Cells* **2007**, *25*, 3234–3243, doi:10.1634/STEMCELLS.2007-0388.

203. Ieda, Y.; Fujita, J.; Ieda, M.; Yagi, T.; Kawada, H.; Ando, K.; Fukuda, K. G-CSF and HGF: Combination of vasculogenesis and angiogenesis synergistically improves recovery in murine hind limb ischemia. *J. Mol. Cell. Cardiol.* **2007**, *42*, 540–548, doi:10.1016/J.YJMCC.2006.11.015.

204. Cooke, J.P. Inflammation and Its Role in Regeneration and Repair. *Circ. Res.* **2019**, *124*, 1166–1168, doi:10.1161/CIRCRESAHA.118.314669.

205. Ivanova-Todorova, E.; Bochev, I.; Mourdjeva, M.; Dimitrov, R.; Bukarev, D.; Kyurkchiev, S.; Tivchev, P.; Altunkova, I.; Kyurkchiev, D.S. Adipose tissue-derived mesenchymal stem cells are more potent suppressors of dendritic cells differentiation compared to bone marrow-derived mesenchymal stem cells. *Immunol. Lett.* **2009**, *126*, 37–42, doi:10.1016/J.IMLET.2009.07.010.

206. Ceccarelli, S.; Pontecorvi, P.; Anastasiadou, E.; Napoli, C.; Marchese, C. Immunomodulatory Effect of Adipose-Derived Stem Cells: The Cutting Edge of Clinical Application. *Front. Cell Dev. Biol.* **2020**, *8*, 236, doi:10.3389/fcell.2020.00236.

207. Kocan, B.; Maziarz, A.; Tabarkiewicz, J.; Ochiya, T.; Banaś-Ząbczyk, A. Trophic Activity and Phenotype of Adipose Tissue-Derived Mesenchymal Stem Cells as a Background of Their Regenerative Potential. *Stem Cells Int.* **2017**, *2017*, doi:10.1155/2017/1653254.

208. Delarosa, O.; Lombardo, E.; Beraza, A.; Mancheño-Corvo, P.; Ramirez, C.; Menta, R.; Rico, L.; Camarillo, E.; García, L.; Abad, J.L.; et al. Requirement of IFN-γ-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. *Tissue Eng. - Part A* **2009**, *15*, 2795–2806, doi:10.1089/TEN.TEA.2008.0630.

209. Forcheron, F.; Agay, D.; Scherthan, H.; Riccobono, D.; Herodin, F.; Meineke, V.; Drouet, M. Autologous Adipocyte Derived Stem Cells Favour Healing in a Minipig Model of Cutaneous Radiation Syndrome. *PLoS One* **2012**, *7*, 31694, doi:10.1371/JOURNAL.PONE.0031694.

210. Mi Jeong, L.; Jaeyoon, K.; Min Young, K.; Yoe-Sik, B.; Sung Ho, R.; Taehoon, G.L.; Jae Ho, K. Proteomic analysis of tumor necrosis factor-alpha-induced secretome of human adipose tissue-derived mesenchymal stem cells. *J. Proteome Res.* **2010**, *9*, 1754–1762, doi:10.1021/PR900898N.

211. Jiang, D.; Qi, Y.; Walker, N.G.; Sindrilaru, A.; Hainzl, A.; Wlaschek, M.; MacNeil, Scharffetter-Kochanek, K. The effect of adipose tissue derived MSCs delivered by a chemically defined carrier on full-thickness cutaneous wound healing. *Biomaterials* **2013**, *34*, 2501–2515, doi:10.1016/J.BIOMATERIALS.2012.12.014.

212. Blaber, S.P.; Webster, R.A.; Hill, C.J.; Breen, E.J.; Kuah, D.; Vesey, G.; Herbert, B.R. Analysis of in vitro secretion profiles from adipose-derived cell populations. *J. Transl. Med.* **2012**, *10*, 1–16, doi:10.1186/1479-5876-10-172.

213. Ma, H.; Kuen Lam, P.; Sum Siu, W.; See Wai Tong, C.; Ki Yan Lo, K.; Man Koon, C.; Xiao Wu, X.; Li, X.; Cheng, W.; Ting Shum, W.; et al. Adipose Tissue-Derived Mesenchymal Stem Cells (ADMSCs) and ADMSC-Derived Secretome Expedited Wound Healing in a Rodent Model-A Preliminary Study. *Clinical, Cosmetic and Investigational Dermatology* **2021**, doi:10.2147/CCID.S298105.

214. Saparov, A.; Ogay, V.; Nurgozhin, T.; Jumabay, M.; Chen, W.C.W. Preconditioning of human mesenchymal stem cells to enhance their regulation of the immune response. *Stem Cells Int.* **2016**, *2016*, doi:10.1155/2016/3924858.

215. Wang, Y.; Chen, X.; Cao, W.; Shi, Y. Plasticity of mesenchymal stem cells in immunomodulation: Pathological and therapeutic implications. *Nat. Immunol.* **2014**, *15*, 1009–1016, doi:10.1038/NI.3002.

216. Hassanshahi, A.; Hassanshahi, M.; Khabbazi, S.; Hosseini-Khah, Z.; Peymanfar, Y.; Ghalamkari, S.; Su, Y.; Xian, C.J. Adipose-derived stem cells for wound healing. *J. Cell. Physiol.* **2019**, *234*, 7903–7914, doi:10.1002/jcp.27922.

217. Zhao, J.; Hu, L.; Liu, J.; Gong, N.; Chen, L. The effects of cytokines in adipose stem cell-conditioned medium on the migration and proliferation of skin fibroblasts in vitro. *Biomed Res. Int.* **2013**, *2013*, doi:10.1155/2013/578479.

218. Ebrahimian, T.G.; Pouzoulet, F.; Squiban, C.; Buard, V.; André, M.; Cousin, B.; Gourmelon, P.; Benderitter, M.; Casteilla, L.; Tamarat, R. Cell therapy based on adipose tissue-derived stromal cells promotes physiological and pathological wound healing. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 503–510, doi:10.1161/ATVBAHA.108.178962.

219. Luckett, L.R.; Gallucci, R.M. Interleukin-6 (IL-6) modulates migration and matrix metalloproteinase function in dermal fibroblasts from IL-6KO mice. *Br. J. Dermatol.* **2007**, *156*, 1163–1171, doi:10.1111/j.1365-2133.2007.07867.x.

220. Lin, Z.-Q.; Kondo, T.; Ishida, Y.; Takayasu, T.; Mukaida, N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J. Leukoc. Biol.* **2003**, *73*, 713–721, doi:10.1189/jlb.0802397.

221. Michel, G.; Kemény, L.; Peter, R.U.; Beetz, A.; Ried, C.; Arenberger, P.; Ruzicka, T. Interleukin-8 receptor-mediated chemotaxis of normal human epidermal cells. *FEBS Lett.* **1992**, *305*, 241–243, doi:10.1016/0014-5793(92)80677-9.

222. Chicharro, D.; Carillo, J.M.; Rubio, M.; Cugat, R.; Cuervo, B.; Guil, S.; et al. Combined plasma rich in growth factors and adipose-derived mesenchymal stem cells promotes the cutaneous wound healing in rabbits. *BMC Vet. Res.* **2018**, *14*, doi:10.1186/S12917-018-1577-Y.

223. Egashira, Y.; Sugitani, S.; Suzuki, Y.; Mishiro, K.; Tsumura, K.; Shimazawa, M.; et al. The conditioned medium of murine and human adipose-derived stem cells exerts neuroprotective effects against experimental stroke model. *Brain Res.* **2012**, *1461*, 87–95, doi:10.1016/J.BRAINRES.2012.04.033.

224. O'Halloran, N.; Courtney, D.; Kerin, M.J.; Lowery, A.J. Adipose-Derived Stem Cells in Novel Approaches to Breast Reconstruction: Their Suitability for Tissue Engineering and Oncological Safety. *Breast Cancer (Auckl).* **2017**, *11*, doi:10.1177/1178223417726777.

225. de Miranda, M.C.; Ferreira, A. da F.; de Melo, M.I.A.; Kunrath-Lima, M.; Goes, A.M. de; Rodrigues, M.A.; Gomes, D.A.; Faria, J.A.Q.A. Adipose-derived stem/stromal cell secretome modulates breast cancer cell proliferation and differentiation state towards aggressiveness. *Biochimie* **2021**, *191*, 69–77, doi:10.1016/j.biochi.2021.08.010.

226. Ryu, H.; Oh, J.E.; Rhee, K.J.; Baik, S.K.; Kim, J.; Kang, S.J.; Sohn, J.H.; Choi, E.; Shin, H.C.; Kim, Y.M.; et al. Adipose tissue-derived mesenchymal stem cells cultured at high density express IFN-β and suppress the growth of MCF-7 human breast cancer cells. *Cancer Lett.* **2014**, *352*, 220–227, doi:10.1016/J.CANLET.2014.06.018.

227. Zimmerlin, L.; Donnenberg, A.D.; Rubin, J.P.; Basse, P.; Landreneau,R.J.; Donnenberg, V.S. Regenerative therapy and cancer: in vitro and in vivo studies of the interaction between adipose-derived stem cells and breast cancer cells from clinical isolates. *Tissue Eng. Part A* **2011**, *17*, 93–106, doi:10.1089/TEN.TEA.2010.0248.

228. Zaborowski, M.P.; Balaj, L.; Breakefield, X.O.; Lai, C.P. Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Bioscience* **2015**, *65*, 783–797, doi:10.1093/BIOSCI/BIV084.

229. Yanez-Mo, M.; Siljander, P.R-M.; Andreu, Z.; Bedina Zavec, A.; Borras, F.E.; Buzas, E.I.; Buzas, K.; et al. Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. vesicles* **2015**, *4*, 1–60, doi:10.3402/JEV.V4.27066.

230. Greening, D.W.; Simpson, R.J. Understanding extracellular vesicle diversity–current status. *Expert Rev. Proteomics* **2018**, *15*, 887–910, doi:10.1080/14789450.2018.1537788.

231. Vlassov, A. V.; Magdaleno, S.; Setterquist, R.; Conrad, R. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim. Biophys. Acta - Gen. Subj.* **2012**, *1820*, 940–948, doi:10.1016/J.BBAGEN.2012.03.017.

232. Bebelman, M.P.; Smit, M.J.; Pegtel, D.M.; Baglio, S.R. Biogenesis and function of extracellular vesicles in cancer. *Pharmacol. Ther.* **2018**, *188*, 1–11, doi:10.1016/J.PHARMTHERA.2018.02.013.

233. Raposo, G.; Stoorvogel, W. Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* **2013**, *200*, 373–383, doi:10.1083/JCB.201211138.

234. Borges, F.T.; Reis, L.A.; Schor, N. Extracellular vesicles: structure, function, and potential clinical uses in renal diseases. *Brazilian J. Med. Biol. Res.* **2013**, *46*, 824–830, doi:10.1590/1414-431X20132964.

235. Xing, X.; Han, S.; Cheng, G.; Ni, Y.; Li, Z.; Li, Z. Proteomic Analysis of Exosomes from Adipose-Derived Mesenchymal Stem Cells: A Novel Therapeutic Strategy for Tissue Injury. *Biomed Res. Int.* **2020**, *2020*, doi:10.1155/2020/6094562.

236. Ni, J.; Li, H.; Zhou, Y.; Gu, B.; Xu, Y.; Fu, Q.; Peng, X.; et al. Therapeutic Potential of Human Adipose-Derived Stem Cell Exosomes in Stress Urinary Incontinence - An in Vitro and in Vivo Study. *Cell. Physiol. Biochem.* **2018**, *48*, 1710–1722, doi:10.1159/000492298.

237. Li, X.; Xie, X.; Lian, W.; Shi, R.; Han, S.; Zhang, H.; Lu,L.; Li, M. Exosomes from adipose-derived stem cells overexpressing Nrf2 accelerate cutaneous wound healing by promoting vascularization in a diabetic foot ulcer rat model. *Exp. Mol. Med.* **2018**, *50*, doi:10.1038/S12276-018-0058-5.

238. Kang, T.; Jones, T.M.; Naddell, C.; Bacanamwo, M.; Calvert, J.W.; Thompson, W.E.; Bond, V.C.; Chen, Y.E.; Liu, D. Adipose-Derived Stem Cells Induce Angiogenesis via Microvesicle Transport of miRNA-31. *Stem Cells Transl. Med.* **2016**, *5*, 440–450, doi:10.5966/SCTM.2015-0177.

239. Togliatto, G.; Dentelli, P.; Gili, M.; Gallo, S.; Deregibus, C.; et al. Obesity reduces the pro-angiogenic potential of adipose tissue stem cell-derived extracellular vesicles (EVs) by impairing miR-126 content: impact on clinical applications. *Int. J. Obes. (Lond).* **2016**, *40*, 102–111, doi:10.1038/IJO.2015.123.

240. Baraniak, P.R.; McDevitt, T.C. Stem cell paracrine actions and tissue regeneration. *Regen. Med.* **2010**, *5*, 121–143, doi:10.2217/RME.09.74.

241. Paquet, J.; Deschepper, M.; Moya, A.; Logeart-Avramoglou, D.; Boisson-Vidal, C.; Petite, H. Oxygen Tension Regulates Human Mesenchymal Stem Cell Paracrine Functions. *Stem Cells Transl. Med.* **2015**, *4*, 809–821, doi:10.5966/sctm.2014-0180.

242. Chang, C.-P.; Chio, C.-C.; Cheong, C.-U.; Chao, C.-M.; Cheng, B.-C.; Lin, M.-T. Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury. *Clin. Sci.* **2013**, *124*, 165–176, doi:10.1042/CS20120226.

243. Antebi, B.; Rodriguez, L.A.; Walker, K.P.; Asher, A.M.; Kamucheka, R.M.; Alvarado, L.; Mohammadipoor, A.; Cancio, L.C. Short-term physiological hypoxia potentiates the therapeutic function of mesenchymal stem cells. *Stem Cell Res. Ther. 2018 91* **2018**, *9*, 1–15, doi:10.1186/S13287-018-1007-X.

244. Weiser, B.; Prantl, L.; Schubert, T.E.O.; Zellner, J.; Fischbach-Teschl, C.; Spruss, T.; Seitz, A.K.; Tessmar, J.; Goepferich, A.; Blunk, T. *In Vivo* Development and Long-Term Survival of Engineered Adipose Tissue Depend on *In Vitro* Precultivation Strategy. *Tissue Eng. Part A* **2008**, *14*, 275–284, doi:10.1089/tea.2007.0130.

245. Lu, F.; Li, J.; Gao, J.; Ogawa, R.; Ou, C.; Yang, B.; Fu, B. Improvement of the survival of human autologous fat transplantation by using VEGF-transfected adipose-derived stem cells. *Plast. Reconstr. Surg.* **2009**, *124*, 1437–1446, doi:10.1097/PRS.0b013e3181babbb6.

246. Riis, S.; Stensballe, A.; Emmersen, J.; Pennisi, C.P.; Birkelund, S.; Zachar, V.; Fink, T. Mass spectrometry analysis of adipose-derived stem cells reveals a significant effect of hypoxia on pathways regulating extracellular matrix. *Stem Cell Res. Ther.* **2016**, *7*, doi:10.1186/S13287-016-0310-7.

247. Armstrong, J.P.K.; Holme, M.N.; Stevens, M.M. Re-Engineering Extracellular Vesicles as Smart Nanoscale Therapeutics. *ACS Nano* **2017**, *11*, 69–83, doi:10.1021/ACSNANO.6B07607.

248. Chu, D.T.; Phuong, T.N.T.; Le Bao Tien, N.; Tran, D.K.; Minh, L.B.; Van Thanh, V.; et al. Adipose Tissue Stem Cells for Therapy: An Update on the Progress of Isolation, Culture, Storage, and Clinical Application. *J. Clin. Med.* **2019**, *8*, doi:10.3390/JCM8070917.

249. Lalu, M.M.; Mcintyre, L.; Pugliese, C.; Fergusson, D.; Winston, B.W.; Marshall, J.C.; Granton, J.; Stewart, D.J. Safety of Cell Therapy with Mesenchymal Stromal Cells (SafeCell): A Systematic Review and Meta-Analysis of Clinical Trials. *PLoS One* **2012,** *7*(10):e47559, doi:10.1371/journal.pone.0047559.

250. Li, P.; Guo, X. A review: therapeutic potential of adipose-derived stem cells in cutaneous wound healing and regeneration. *Stem Cell Res. Ther.* **2018**, *9*, 1–7, doi:10.1186/s13287-018-1044-5.

251. Han, G.; Ceilley, R. Chronic Wound Healing: A Review of Current Management and Treatments. *Adv. Ther.* **2017**, *34*, 599–610, doi:10.1007/S12325-017-0478-Y.

252. Kato, T.; Khanh, V.C.; Sato, K.; Takeuchi, K.; Carolina, E.; Yamashita, T.; Sugaya, H.; Yoshioka, T.; Mishima, H.; Ohneda, O. SDF-1 improves wound healing ability of glucocorticoid-treated adipose tissue-derived mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* **2017**, *493*, 1010–1017, doi:10.1016/J.BBRC.2017.09.100.

253. Wang, M.; Crisostomo, P.R.; Herring, C.; Meldrum, K.K.; Meldrum, D.R. Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2006**, *291*, doi:10.1152/AJPREGU.00280.2006.

254. Rigotti, G.; Marchi, A.; Galiè, M.; Baroni, G.; Benati, D.; Krampera, M.; Pasini, A.; Sbarbati, A. Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: A healing process mediated by adipose-derived adult stem cells. *Plast. Reconstr. Surg.* **2007**, *119*, 1409–1422, doi:10.1097/01.PRS.0000256047.47909.71.

255. da Pinheiro, C.H.J.; de Queiroz, J.C.F.; Guimarães-Ferreira, L.; Vitzel, K.F.; Nachbar, R.T.; de Sousa, L.G.O.; de Souza-Jr, A.L.; Nunes, M.T.; Curi, R. Local Injections of Adipose-Derived Mesenchymal Stem Cells Modulate Inflammation and Increase Angiogenesis Ameliorating the Dystrophic Phenotype in Dystrophin-Deficient Skeletal Muscle. *Stem Cell Rev. Reports* **2012**, *8*, 363–374, doi:10.1007/S12015-011-9304-0.

256. Hong, S.J.; Jia, S.X.; Xie, P.; Xu, W.; Leung, K.P.; Mustoe, T.A.; Galiano, R.D. Topically delivered adipose derived stem cells show an activated-fibroblast phenotype and enhance granulation tissue formation in skin wounds. *PLoS One* **2013**, *8*, doi:10.1371/JOURNAL.PONE.0055640.

257. González, M.A.; Gonzalez-Rey, E.; Rico, L.; Büscher, D.; Delgado, M. Adipose-Derived Mesenchymal Stem Cells Alleviate Experimental Colitis by Inhibiting Inflammatory and Autoimmune Responses. *Gastroenterology* **2009**, *136*, 978–989, doi:10.1053/J.GASTRO.2008.11.041.

258. Yun, I.S.; Jeon, Y.R.; Lee, W.J.; Lee, J.W.; Rah, D.K.; Tark, K.C.; Lew, D.H. Effect of human adipose derived stem cells on scar formation and remodeling in a pig model: A pilot study. *Dermatologic Surg.* **2012**, *38*, 1678–1688, doi:10.1111/J.1524-4725.2012.02495.X.

259. da Silva, R.A.; de Camargo Andrade, A.F.; da Silva Feltran, G.; Fernandes, C.J. da C.; de Assis, R.I.F.; Ferreira, M.R.; Andia, D.C.; Zambuzzi, W.F. The role of triiodothyronine hormone and mechanically-stressed endothelial cell paracrine signalling synergism in gene reprogramming during hBMSC-stimulated osteogenic phenotype in vitro. *Mol. Cell. Endocrinol.* **2018**, *478*, 151–167, doi:10.1016/J.MCE.2018.08.008.

260. Bertolini, F.; Lohsiriwat, V.; Petit, J.Y.; Kolonin, M.G. Adipose tissue cells, lipotransfer and cancer: A challenge for scientists, oncologists and surgeons. *Biochim. Biophys. Acta - Rev. Cancer* **2012**, *1826*, 209–214, doi:10.1016/J.BBCAN.2012.04.004.

261. Fang, B.; Song, Y.; Lin, Q.; Zhang, Y.; Cao, Y.; Zhao, R.C.; Ma, Y. Human adipose tissue-derived mesenchymal stromal cells as salvage therapy for treatment of severe refractory acute graft-vs.-host disease in two children. *Pediatr. Transplant.* **2007**, *11*, 814–817, doi:10.1111/J.1399-3046.2007.00780.X.

262. Puissant, B.; Barreau, C.; Bourin, P.; Clavel, C.; Corre, J.; Bousquet, C.; Taureau, C.; Cousin, B.; Abbal, M.; Laharrague, P.; et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. *Br. J. Haematol.* **2005**, *129*, 118–129, doi:10.1111/J.1365-2141.2005.05409.X.

263. Gonzalez-Rey, E.; Gonzalez, M.A.; Varela, N.; O'Valle, F.; Hernandez-Cortes, P.; Rico, L.; Büscher, D.; Delgado, M. Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis. *Ann. Rheum. Dis.* **2010**, *69*, 241–248, doi:10.1136/ARD.2008.101881.

264. Gonzalez-Rey, E.; Anderson, P.; González, M.A.; Rico, L.; Büscher, D.; Delgado, M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* **2009**, *58*, 929–939, doi:10.1136/GUT.2008.168534.

265. Garcia-Olmo, D.; Garcia-Arranz, M.; Herreros, D. Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. *Expert Opin. Biol. Ther.* **2008**, *8*, 1417–1423, doi:10.1517/14712598.8.9.1417.

266. García-Olmo, D.; García-Arranz, M.; Herreros, D.; Pascual, I.; Peiro, C.; Rodríguez-Montes, J.A. A phase I clinical trial of the treatment of crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis. Colon Rectum* **2005**, *48*, 1416–1423, doi:10.1007/S10350-005-0052-6.

267. Mesimäki, K.; Lindroos, B.; Törnwall, J.; Mauno, J.; Lindqvist, C.; Kontio, R.; Miettinen, S.; Suuronen, R. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. *Int. J. Oral Maxillofac. Surg.* **2009**, *38*, 201–209, doi:10.1016/J.IJOM.2009.01.001.

268. Sándor, G.K.; Tuovinen, V.J.; Wolff, J.; Patrikoski, M.; Jokinen, J.; Nieminen, E.; Mannerström, B.; Lappalainen, O.P.; Seppänen, R.; Miettinen, S. Adipose stem cell tissue-engineered construct used to treat large anterior mandibular defect: A case report and review of the clinical application of good manufacturing practice-level adipose stem cells for bone regeneration. *J. Oral Maxillofac. Surg.* **2013**, *71*, 938–950, doi:10.1016/J.JOMS.2012.11.014.

269. Lendeckel, S.; Jödicke, A.; Christophis, P.; Heidinger, K.; Wolff, J.; Fraser, J.K.; Hedrick, M.H.; Berthold, L.; Howaldt, H.P. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: Case report. *J. Cranio-Maxillofacial Surg.* **2004**, *32*, 370–373, doi:10.1016/J.JCMS.2004.06.002.

270. Sàndor, G.K.B.; Nish, I.A.; Carmichael, R.P. Comparison of conventional surgery with motorized trephine in bone harvest from the anterior iliac crest. *Oral Surgery, Oral Med. Oral Pathol. Oral Radiol. Endodontology* **2003**, *95*, 150–155, doi:10.1067/MOE.2003.42.

271. Zhang, H.N.; Li, L.; Leng, P.; Wang, Y.Z.; Lü, C.Y. Uninduced adipose-derived stem cells repair the defect of full-thickness hyaline cartilage. *Chinese J. Traumatol. (English Ed.)* **2009**, *12*, 92–97, doi:10.3760/CMA.J.ISSN.1008-1275.2009.02.006.

272. Bahrani, H.; Razmkhah, M.; Ashraf, M.J.; Tanideh, N.; Chenari, N.; Khademi, B.; Ghaderi, A. Differentiation of adipose-derived stem cells into ear auricle cartilage in rabbits. *J. Laryngol. Otol.* **2012**, *126*, 770–774, doi:10.1017/S0022215112001065.

273. Glass,G.E.; Ferretti, P. Adipose-Derived Stem Cells in Aesthetic Surgery. *Aesthetic Surg. J.* **2019**, *39*, 423–438, doi:10.1093/ASJ/SJY160.

274. Pak, J. Regeneration of human bones in hip osteonecrosis and human cartilage in knee osteoarthritis with autologous adipose-tissue-derived stem cells: a case series. *J. Med. Case Rep.* **2011**, *5*, doi:10.1186/1752-1947-5-296.

275. Lee, S.C.; Kim, J.O.; Kim, S.-J. Secretome from human adipose-derived stem cells protects mouse liver from hepatic ischemia-reperfusion injury. *Surgery* **2015**, 157(5):934-43, doi:10.1016/j.surg.2014.12.016.

276. Griffith, L.G. Emerging design principles in biomaterials and scaffolds for tissue engineering. *Ann. N. Y. Acad. Sci.* **2002**, *961*, 83–95, doi:10.1111/J.1749-6632.2002.TB03056.X.

277. Fuchs, J.R.; Nasseri, B.A.; Vacanti, J.P. Tissue engineering: A 21st century solution to surgical reconstruction. *Ann. Thorac. Surg.* **2001**, *72*, 577–591, doi:10.1016/S0003-4975(01)02820-X.

278. Miller, A.M.; Steiner, C.A.; Barrett, M.L.; Fingar, K.R.; Elixhauser, A. Breast Reconstruction Surgery for Mastectomy in Hospital Inpatient and Ambulatory Settings, 2009-2014. *Healthcare Cost and Utilization Project (HCUP) Statistical Briefs* [Internet]. **2009**.

279. Kappy, N.S.; Chang, S.; Harris, W.M.; Plastini, M.; Ortiz, T.; Zhang, P.; Hazelton, J.P.; Carpenter, J.P.; Brown, S.A. Human adipose-derived stem cell treatment modulates cellular protection in both in vitro and in vivo traumatic brain injury models. *J. Trauma Acute Care Surg.* **2018**, *84*, 745–751, doi:10.1097/TA.0000000000001770.

280. Tajiri, N.; Acosta, S.A.; Shahaduzzaman, M.; Ishikawa, H.; Shinozuka, K.; Pabon, M.; Hernandez-Ontiveros, D.; Kim, D.W.; Metcalf, C.; Staples, M.; et al. Intravenous Transplants of Human Adipose-Derived Stem Cell Protect the Brain from Traumatic Brain Injury-Induced Neurodegeneration and Motor and Cognitive Impairments: Cell Graft Biodistribution and Soluble Factors in Young and Aged Rats. *J. Neurosci.* **2014**, *34*, 313, doi:10.1523/JNEUROSCI.2425-13.2014.

281. Gugliandolo, A.; Bramanti, P.; Mazzon, E. Mesenchymal Stem Cells: A Potential Therapeutic Approach for Amyotrophic Lateral Sclerosis? *Stem Cells Int.* **2019**, *2019*, doi:10.1155/2019/3675627.

282. Choi, H.S.; Kim, H.J.; Oh, J.-H.; Park, H-G.; Chan Ra, J.; Chang, K-A. Therapeutic potentials of human adipose-derived stem cells on the mouse model of Parkinson's disease. *Neurobiol. Aging* **2015**, *36*, 2885–2892, doi:10.1016/J.NEUROBIOLAGING.2015.06.022.

283. Ohta, Y.; Hamaguchi, A.; Ootaki, M.; Watanabe, M.; Takeba, Y.; Iiri, T.; Matsumoto, N.; Takenaga. Intravenous infusion of adipose-derived stem/stromal cells improves functional recovery of rats with spinal cord injury. *Cytotherapy* **2017**, *19*, 839–848, doi:10.1016/J.JCYT.2017.04.002.

284. Peroglio, M.; Douma, L.S.; Caprez, T.S.; Janki, M.; Benneker, L.M.; Alini, M.; Grad, S. Intervertebral disc response to stem cell treatment is conditioned by disc state and cell carrier: An ex vivo study. *J. Orthop. Transl.* **2017**, *9*, 43, doi:10.1016/J.JOT.2017.03.003.

285. Song, K.; Gu, T.; Shuang, F.; Tang, J.; Ren, D.; Qin, J. Adipose-derived stem cells improve the viability of nucleus pulposus cells in degenerated intervertebral discs. *Mol. Med. Rep.* **2015**, *12*, 4664–4668, doi:10.3892/MMR.2015.3895.

286. Chu, D.-T.; Nguyen Thi Phuong, T.; Tien, N.L.B.; Tran, D.K.; Minh, L.B.; Thanh, V. Van; Gia Anh, P.; Pham, V.H.; Thi Nga, V. Adipose Tissue Stem Cells for Therapy: An Update on the Progress of Isolation, Culture, Storage, and Clinical Application. *J. Clin. Med.* **2019**, *8*, 917, doi:10.3390/jcm8070917.

287. Liu, G.-Y.; Liu, J.; Wang, Y.-L.; Liu, Y.; Shao, Y.; Han, Y.; Qin, Y.-R.; Xiao, F.-J.; Li, P.-F.; Zhao, L.-J.; et al. Adipose-Derived Mesenchymal Stem Cells Ameliorate Lipid Metabolic Disturbance in Mice. *Stem Cells Transl. Med.* **2016**, *5*, 1162–1170, doi:10.5966/SCTM.2015-0239.

288. Perin, E.C.; Sanz-Ruiz, R.; Sánchez, P.L.; Lasso, J.; Pérez-Cano, R.; Alonso-Farto, J.C.; Pérez-David, E.; Fernández-Santos, M.E.; Serruys, P.W.; Duckers, H.J.; et al. Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial. *Am. Heart J.* **2014**, *168*, 88-95.e2, doi:10.1016/J.AHJ.2014.03.022.

289. Bajek, A.; Gurtowska, N.; Olkowska, J.; Kazmierski, • Lukasz; Maj, M.; Drewa, • Tomasz Adipose-Derived Stem Cells as a Tool in Cell-Based Therapies. *Arch. Immunol. Ther. Exp.* **2016,** 64, 443–454, doi: 10.1007/s00005-016-0394-x.

290. Laloze, J.; Varin, A.; Gilhodes, J.; Bertheuil, N.; Grolleau, J.L.; Brie, J.; Usseglio, J.; Sensebe, L.; Filleron, T.; Chaput, B. Cell-assisted lipotransfer: Friend or foe in fat grafting? Systematic review and meta-analysis. *J Tissue Eng Regen Med* **2018**, 12(2):e1237-e1250, doi: 10.1002/term.2524.

291. Kakagia, D.; Pallua, N. Autologous Fat Grafting: In Search of the Optimal Technique. *Surg Innov* **2014**, 21(3):327-36, doi: 10.1177/1553350613518846.

292. Abu-Ghname, A.; Perdanasari, A.T.; Reece, E.M. Principles and Applications of Fat Grafting in Plastic Surgery. *Semin Plast Surg.* **2019**, *33*, 147–154, doi:10.1055/s-0039-1693438.

293. Pu, L.L.Q.; Yoshimura, K.; Coleman, S.R. Fat Grafting: Current Concept, Clinical Application, and Regenerative Potential, Part 1. *Clin. Plast. Surg.* **2015**, 42, ix-x, doi: 10.1016/j.cps.2015.02.001.

294. Coleman, S.R. Structural fat grafts: The ideal filler? *Clin. Plast. Surg.* **2001**, *28*, 111–119, doi:10.1016/S0094-1298(20)32343-9.

295. Hong, S.J.; Lee, J.H.; Hong, S.M.; Park, C.H. Enhancing the viability of fat grafts using new transfer medium containing insulin and β-fibroblast growth factor in autologous fat transplantation. *J. Plast. Reconstr. Aesthetic Surg.* **2010**, *63*, 1202–1208, doi:10.1016/J.BJPS.2009.05.040.

296. Gir, P.; Brown, S.A.; Oni, G.; Kashefi, N.; Mojallal, A.; Rohrich, R.J. Fat grafting: Evidence-based review on autologous fat harvesting, processing, reinjection, and storage. *Plast. Reconstr. Surg.* **2012**, *130*, 249–258.

297. Bucky, L.P.; Percec, I. The Science of Autologous Fat Grafting: Views on Current and Future Approaches to Neoadipogenesis. *Aesthetic Surg. J.* **2008**, *28*, 313–321, doi: 10.1016/j.asj.2008.02.004.

298. Pu, L.L.Q.; Yoshimura, K.; Coleman, S.R. Fat Grafting: Current Concept, Clinical Application, and Regenerative Potential, Part 2. Preface. *Clin. Plast. Surg.* **2015**, *42*, xiii–xiv, doi:10.1016/J.CPS.2015.05.001.

299. Klinger, M.; Caviggioli, F.; Klinger, F.M.; Giannasi, S.; Bandi, V.; Banzatti, B.; Forcellini, D.; Maione, L.; Catania, B.; Vinci, V. Autologous fat graft in scar treatment. *J. Craniofac. Surg.* **2013**, *24*, 1610–1615, doi:10.1097/SCS.0B013E3182A24548.

300. Simonacci, F.; Bertozzi, N.; Grieco, M.P.; Grignaffini, E.; Raposio, E. Procedure, applications, and outcomes of autologous fat grafting. *Ann. Med. Surg.* **2017**, *20*, 49–60, doi: 10.1016/j.amsu.2017.06.059.

301. Chan, C.W.; McCulley, S.J.; Macmillan, R.D. Autologous fat transfer – a review of the literature with a focus on breast cancer surgery. *J. Plast. Reconstr. Aesthetic Surg.* **2008**, *61*, 1438–1448, doi:10.1016/J.BJPS.2008.08.006.

302. Ersek, R.A.; Chang, P.; Salisbury, M.A. Lipo layering of autologous fat: An improved technique with promising results. *Plast. Reconstr. Surg.* **1998**, *101*, 820–826, doi:10.1097/00006534-199803000-00038.

303. Shiffman, M.A.; Mirrafati, S. Fat transfer techniques: The effect of harvest and transfer methods on adipocyte viability and review of the literature. *Dermatologic Surg.* **2001**, *27*, 819–826, doi:10.1046/J.1524-4725.2001.01062.X.

304. Tiryaki, T.; Findikli, N.; Tiryaki, D. Staged stem cell-enriched tissue (SET) injections for soft tissue augmentation in hostile recipient areas: A preliminary report. *Aesthetic Plast. Surg.* **2011**, *35*, 965–971, doi:10.1007/S00266-011-9716-X.

305. Gutowski, K. Current applications and safety of autologous fat grafts: a report of the ASPS fat graft task force. *Plast. Reconstr. Surg.* **2009**, *124*, 272–280, doi:10.1097/PRS.0B013E3181A09506.

306. Tabit, C.J.; Slack, G.C.; Fan, K.; Wan, D.C.; Bradley, J.P. Fat grafting versus adipose-derived stem cell therapy: Distinguishing indications, techniques, and outcomes. *Aesthetic Plast. Surg.* **2012**, *36*, 704–713, doi:10.1007/S00266-011-9835-4.

307. Coleman, S.R. Long-Term survival of fat transplants: Controlled demonstrations. *Aesthetic Plast. Surg.* **1995**, *19*, 421–425, doi:10.1007/BF00453875.

308. Parrish, J.N.; Metzinger, S.E. Autogenous fat grafting and breast augmentation: a review of the literature. *Aesthetic Surg. J.* **2010**, *30*, 549–556, doi:10.1177/1090820X10380859.

309. Matsumoto, D.; Sato, K.; Gonda, K.; Takaki, Y.; Shigeura, T.; Sato, T.; Aiba-Kojima, E.; Iizuka, F.; Inoue, K.; Suga, H.; et al. Cell-assisted lipotransfer: Supportive use of human adipose-derived cells for soft tissue augmentation with lipoinjection. *Tissue Eng.* **2006**, *12*, 3375–3382, doi:10.1089/ten.2006.12.3375.

310. Considine, R. V.; Nyce, M.R.; Morales, L.M.; Magosin, S.A.; Sinha, M.K.; Bauer, T.L.; Rosato, E.L.; Colberg, J.; Caro, J.F. Paracrine stimulation of preadipocyte-enriched cell cultures by mature adipocytes. *Am. J. Physiol. - Endocrinol. Metab.* **1996**, *270*, doi:10.1152/AJPENDO.1996.270.5.E895.

311. Planat-Benard, V.; Silvestre, J-S.; Cousin, B.; André, M.; Nibbelink, M.; et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation* **2004**, *109*, 656–663, doi:10.1161/01.CIR.0000114522.38265.61.

312. Moseley, T.A.; Zhu, M.; Hedrick, M.H. Adipose-derived stem and progenitor cells as fillers in plastic and reconstructive surgery. *Plast. Reconstr. Surg.* **2006**, *118*, doi:10.1097/01.PRS.0000234609.74811.2E.

313. Yoshimura, K.; Suga, H.; Eto, H. Adipose-derived stem/progenitor cells: Roles in adipose tissue remodeling and potential use for soft tissue augmentation. *Regen. Med.* **2009**, *4*, 265–273, doi: 10.2217/17460751.4.2.265.

314. Kondo, K.; Shintani, S.; Shibata, R.; Murakami, H.; Murakami, R.; Imaizumi, M.; Kitagawa, Y.; Murohara, T. Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 61–66, doi:10.1161/ATVBAHA.108.166496.

315. Kim, W.S.; Park, B.S.; Sung, J.H. The wound-healing and antioxidant effects of adipose-derived stem cells. *Expert Opin. Biol. Ther.* **2009**, *9*, 879–887, doi:10.1517/14712590903039684.

316. Yoshimura, K.; Sato, K.; Aoi, N.; Kurita, M.; Hirohi, T.; Harii, K. Cell-assisted lipotransfer for cosmetic breast augmentation: Supportive use of adipose-derived stem/stromal cells. *Aesthetic Plast. Surg.* **2008**, *32*, 48–55, doi:10.1007/s00266-007-9019-4.

317. Huang, S.; Zhao, W.; Wang, Z.; Tao, K.; Liu, X.; Chang, P. Potential drawbacks in cell-assisted lipotransfer: A systematic review of existing reports (Review). *Mol. Med. Rep.* **2016**, *13*, 1063, doi:10.3892/MMR.2015.4682.

318. Toyserkani, N.M.; Quaade, M.L.; Sørensen, J.A. Cell-Assisted Lipotransfer: A Systematic Review of Its Efficacy. *Aesthetic Plast. Surg.* **2016**, *40*, 309–318, doi: 10.1007/s00266-016-0613-1

319. Zhou, Y.; Wang, J.; Li, H.; Liang, X.; Bae, J.; Huang, X.; Li, Q. Efficacy and safety of cell-assisted lipotransfer: A systematic review and meta-analysis. *Plast. Reconstr. Surg.* **2016**, *137*, 44e-57e, doi:10.1097/PRS.0000000000001981.

320. Tissiani, L.A.L.; Alonso, N. A prospective and controlled clinical trial on stromal vascular fraction enriched fat grafts in secondary breast reconstruction. *Stem Cells Int.* **2016**, *2016*, doi:10.1155/2016/2636454.

321. Calabrese, C.; Orzalesi, L.; Casella, D.; Cataliotti, L. Breast reconstruction after nipple/areola-sparing mastectomy using cell-enhanced fat grafting. *Ecancermedicalscience* **2009**, *3*, doi:10.3332/ECANCER.2009.116.

322. Domenis, R.; Lazzaro, L.; Calabrese, S.; Mangoni, D.; Gallelli, A.; Bourkoula, E.; Manini, I.; Bergamin, N.; Toffoletto, B.; Beltrami, C.A.; et al. Adipose tissue derived stem cells: in vitro and in vivo analysis of a standard and three commercially available cell-assisted lipotransfer techniques. *Stem Cell Res. Ther.* **2015**, *6*, doi:10.1186/SCRT536.

323. Yoshimura, K.; Sato, K.; Aoi, N.; Kurita, M.; Hirohi, T.; Harii, K. Cell-assisted lipotransfer for cosmetic breast augmentation: supportive use of adipose-derived stem/stromal cells. *Aesthetic Plast. Surg.* **2008**, *32*, 48–55, doi:10.1007/S00266-007-9019-4.

324. Grabin, S.; Antes, G.; Stark, G.B.; Motschall, E.; Buroh, S.; Lampert, F.M. Cell-assisted lipotransfer. *Dtsch. Arztebl. Int.* **2015**, *112*, 255–261, doi:10.3238/arztebl.2015.0255.

325. Daniels, E. Cytori Therapeutics, Inc. *Regen. Med.* **2007**, *2*, 317–320, doi:10.2217/17460751.2.3.317.

326. Gentile, P.; Scioli, M.G.; Orlandi, A.; Cervelli, V. Breast Reconstruction with Enhanced Stromal Vascular Fraction Fat Grafting: What Is the Best Method? *Plast. Reconstr. Surg. Glob. Open* **2015**, *3*, doi:10.1097/GOX.0000000000000285.

327. Doi, K.; Tanaka, S.; Iida, H.; Eto, H.; Kato, H.; Aoi, N.; Kuno, S.; Hirohi, T.; Yoshimura, K. Stromal vascular fraction isolated from lipo-aspirates using an automated processing system: Bench and bed analysis. *J. Tissue Eng. Regen. Med.* **2013**, *7*, 864–870, doi:10.1002/TERM.1478.

328. Kølle, S.F.T.; Fischer-Nielsen, A.; Mathiasen, A.B.; Elberg, J.J.; Oliveri, R.S.; Glovinski, P. V.; Kastrup, J.; Kirchhoff, M.; Rasmussen, B.S.; Talman, M.L.M.; et al. Enrichment of autologous fat grafts with ex-vivo expanded adipose tissue-derived stem cells for graft survival: A randomised placebo-controlled trial. *Lancet* **2013**, *382*, 1113–1120, doi:10.1016/S0140-6736(13)61410-5.

329. Koh, K.S.; Oh, T.S.; Kim, H.; Chung, I.W.; Lee, K.W.; Lee, H.B.; Park, E.J.; Jung, J.S.; Shin, I.S.; Ra, J.C.; et al. Clinical application of human adipose tissue-derived mesenchymal stem cells in progressive hemifacial atrophy (Parry-Romberg disease) with microfat grafting techniques using 3-dimensional computed tomography and 3-dimensional camera. *Ann. Plast. Surg.* **2012**, *69*, 331–337, doi:10.1097/SAP.0B013E31826239F0.

330. Claro, F.; Figueiredo, J.C.A.; Zampar, A.G.; Pinto-Neto, A.M. Applicability and safety of autologous fat for reconstruction of the breast. *Br. J. Surg.* **2012**, *99*, 768–780, doi:10.1002/BJS.8722.

331. Largo, R.D.; Tchang, L.A.H.; Mele, V.; Scherberich, A.; Harder, Y.; Wettstein, R.; Schaefer, D.J. Efficacy, safety and complications of autologous fat grafting to healthy breast tissue: A systematic review. *J. Plast. Reconstr. Aesthetic Surg.* **2014**, *67*, 437–448, doi:10.1016/J.BJPS.2013.11.011.

332. Donnenberg, V.S.; Zimmerlin, L.; Rubin, J.P.; Donnenberg, A.D. Regenerative Therapy After Cancer: What Are the Risks? *Tissue Eng. Part B. Rev.* **2010**, *16*, 567, doi:10.1089/TEN.TEB.2010.0352.

333. Eterno, V.; Zambelli, A.; Pavesi, L.; Villani, L.; Zanini, V.; Petrolo, G.; Manera, S.; Tuscano, A.; Amato, A.; Eterno, V.; et al. Adipose-derived mesenchymal stem cells (ASCs) may favour breast cancer recurrence via HGF/c-Met signaling. *Oncotarget* **2013**, *5*, 613–633, doi:10.18632/ONCOTARGET.1359.

334. Yu, J.M.; Jun, E.S.; Bae, Y.C.; Jung, J.S. Mesenchymal stem cells derived from human adipose tissues favor tumor cell growth in vivo. *Stem Cells Dev.* **2008**, *17*, 463–473, doi:10.1089/SCD.2007.0181.

335. van Poll, D.; Parekkadan, B.; Borel Rinkes, I.H.M.; Tilles, A.W.; Yarmush, M.L. Mesenchymal Stem Cell Therapy for Protection and Repair of Injured Vital Organs. *Cell. Mol. Bioeng.* **2008**, *1*, 42–50, doi:10.1007/s12195-008-0001-2.

336. L., P.K.; Kandoi, S.; Misra, R.; S., V.; K., R.; Verma, R.S. The mesenchymal stem cell secretome: A new paradigm towards cell-free therapeutic mode in regenerative medicine. *Cytokine Growth Factor Rev.* **2019**, *46*, 1–9, doi:10.1016/j.cytogfr.2019.04.002.

337. Orabi, H.; Goulet, C.R.; Fradette, J.; Bolduc, S. Adipose-Derived Stem Cells — Are They the Optimal Cell Source for Urinary Tract Regeneration? *Cells Biomater. Regen. Med.* **2014**, 3–36, doi:10.5772/59223.

338. Sabol, R.A.; Bowles, A.C.; Côté, A.; Wise, R.; Pashos, N.; Bunnell, B.A. Therapeutic Potential of Adipose Stem Cells. *Adv Exp Med Biol* **2021***,* 1341:15-25, doi: 10.1007/5584_2018_248.

339. Bateman, M.E.; Strong, A.L.; Gimble, J.M.; Bunnell, B.A. Concise Review: Using Fat to Fight Disease: A Systematic Review of Nonhomologous Adipose-Derived Stromal/Stem Cell Therapies. *Stem Cells* **2018**, *36*, 1311–1328, doi:10.1002/stem.2847.

340. Vyas, K.S.; Vasconez, H.C.; Morrison, S.; Mogni, B.; Linton, S.; Hockensmith, L.; Kabir, T.; Zielins, E.; Najor, A.; Bakri, K.; et al. Fat Graft Enrichment Strategies: A Systematic Review. *Plast. Reconstr. Surg.* **2020**, *145*, 827–841, doi:10.1097/PRS.0000000000006557.

341. Deschepper, M.; Oudina, K.; David, B.; Myrtil, V.; Collet, C.; Bensidhoum, M.; Logeart-Avramoglou, D.; Petite, H. Survival and function of mesenchymal stem cells (MSCs) depend on glucose to overcome exposure to long-term, severe and continuous hypoxia. *J. Cell. Mol. Med.* **2011**, *15*, 1505–1514, doi:10.1111/j.1582-4934.2010.01138.x.

342. Zhao, L.; Johnson, T.; Liu, D. Therapeutic angiogenesis of adipose-derived stem cells for ischemic diseases. *Stem Cell Research & Therapy* **2017**, 125, doi:10.1186/s13287-017-0578-2.

343. Suga, H.; Glotzbach, J.P.; Sorkin, M.; Longaker, M.T.; Gurtner, G.C. Paracrine mechanism of angiogenesis in adipose-derived stem cell transplantation. *Ann. Plast. Surg.* **2014**, *72*, 234–241, doi:10.1097/SAP.0b013e318264fd6a.

344. Becquart, P.; Cambon-Binder, A.; Monfoulet, L.E.; Bourguignon, M.; Vandamme, K.; Bensidhoum, M.; Petite, H.; Logeart-Avramoglou, D. Ischemia is the prime but not the only cause of human multipotent stromal cell death in tissue-engineered constructs in vivo. *Tissue Eng. - Part A* **2012**, *18*, 2084–2094, doi:10.1089/ten.tea.2011.0690.

345. Potier, E.; Ferreira, E.; Meunier, A.; Sedel, L.; Logeart-Avramoglou, D.; Petite, H. Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue Eng.* **2007**, *13*, 1325–1331, doi:10.1089/ten.2006.0325.

346. Schäffler, A.; Büchler, C. Concise Review: Adipose Tissue-Derived Stromal Cells-Basic and Clinical Implications for Novel Cell-Based Therapies. *Stem Cells* **2007**, *25*, 818–827, doi:10.1634/stemcells.2006-0589.

347. Matsuda, K.; Falkenberg, K.J.; Woods, A.A.; Choi, Y.S.; Morrison, W.A.; Dilley, R.J. Adipose-Derived Stem Cells Promote Angiogenesis and Tissue Formation for In Vivo Tissue Engineering. *Tissue Eng Part A* **2013**, 19 (11-12):1327-35, doi:10.1089/ten.tea.2012.0391.

348. Kim, S.J.; Ko, J.H.; Yun, J.H.; Kim, J.A.; Kim, T.E.; Lee, H.J.; Kim, S.H.; Park, K.H.; Oh, J.Y. Stanniocalcin-1 Protects Retinal Ganglion Cells by Inhibiting Apoptosis and Oxidative Damage. *PLoS One* **2013**, *8*, e63749, doi:10.1371/journal.pone.0063749.

349. Block, G.J.; Ohkouchi, S.; Fung, F.; Frenkel, J.; Gregory, C.; Pochampally, R.; Dimattia, G.; Sullivan, D.E.; Prockop, D.J. Multipotent Stromal Cells (MSCs) are Activated to Reduce Apoptosis in Part by Upregulation and Secretion of Stanniocalcin-1. *Stem Cells* **2009**, *27*, 670–681, doi:10.1002/stem.20080742.

350. Chen, F.; Zhang, Z.; Pu, F. Role of stanniocalcin-1 in breast cancer (Review). *Oncol. Lett.* **2019**, *18*, 3946–3953, doi:10.3892/ol.2019.10777.

351. McCudden, C.R.; James, K.A.; Hasilo, C.; Wagner, G.F. Characterization of mammalian stanniocalcin receptors: Mitochondrial targeting of ligand and receptor for regulation of cellular metabolism. *J. Biol. Chem.* **2002**, *277*, 45249–45258, doi:10.1074/jbc.M205954200.

352. Philips, B.J.; Marra, K.G.; Rubin, J.P. Healing of grafted adipose tissue: current clinical applications of adipose-derived stem cells for breast and face reconstruction. *Wound Repair Regen.* **2014**, *22*, 11–13, doi: 10.1111/wrr.12164.

353. 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. *Tissue Eng. - Part A* **2015**, *21*, 1343–1353, doi:10.1089/ten.tea.2014.0299.

354. Choi, J.H.; Gimble, J.M.; Lee, K.; Marra, K.G.; Rubin, J.P.; Yoo, J.J.; Vunjak-Novakovic, G.; Kaplan, D.L. Adipose tissue engineering for soft tissue regeneration. *Tissue Eng. - Part B Rev.* **2010**, *16*, 413–426, doi:10.1089/ten.teb.2009.0544.

355. Mazini, L.; Rochette, L.; Amine, M.; Malka, G. Regenerative capacity of adipose derived stem cells (ADSCs), comparison with mesenchymal stem cells (MSCs). *Int. J. Mol. Sci.* **2019**, *20*, 1–30, doi:10.3390/ijms20102523.

356. Bajek, A.; Gurtowska, N.; Olkowska, J.; Kazmierski, L.; Maj, M.; Drewa, T. Adipose-Derived Stem Cells as a Tool in Cell-Based Therapies. *Arch. Immunol. Ther. Exp. (Warsz).* **2016**, *64*, 443–454, doi: 10.1007/s00005-016-0394-x.

357. Lindroos, B.; Suuronen, R.; Miettinen, S. The Potential of Adipose Stem Cells in Regenerative Medicine. *Stem Cell Rev. Rep.* **2011**, *7*, 269–291, doi: 10.1007/s12015-010-9193-7.

358. English, K. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol. Cell Biol.* **2013**, 91, 19–26, doi: 10.1038/icb.2012.56.

359. Klinker, M.W. Mesenchymal stem cells in the treatment of inflammatory and autoimmune diseases in experimental animal models. *World J. Stem Cells* **2015**, *7*, 556, doi:10.4252/wjsc.v7.i3.556.

360. Fu, Y.; Karbaat, L.; Wu, L.; Leijten, J.; Both, S.K.; Karperien, M. Trophic Effects of Mesenchymal Stem Cells in Tissue Regeneration. *Tissue Eng. - Part B Rev.* **2017**, *23*, 515–528, doi:10.1089/ten.teb.2016.0365.

361. Wakabayashi, K.; Nagai, A.; Sheikh, A.M.; Shiota, Y.; Narantuya, D.; Watanabe, T.; Masuda, J.; Kobayashi, S.; Kim, S.U.; Yamaguchi, S. Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. *J. Neurosci. Res.* **2010**, *88*, 1017–1025, doi:10.1002/jnr.22279.

362. Caplan, A.I.; Dennis, J.E. Mesenchymal stem cells as trophic mediators. *J. Cell. Biochem.* **2006**, *98*, 1076–1084, doi: 10.1002/jcb.20886.

363. Cai, L.; Johnstone, B.H.; Cook, T.G.; Tan, J.; Fishbein, M.C.; Chen, P.-S.; March, K.L. IFATS Collection: Human Adipose Tissue-Derived Stem Cells Induce Angiogenesis and Nerve Sprouting Following Myocardial Infarction, in Conjunction with Potent Preservation of Cardiac Function. *Stem Cells* **2009**, *27*, 230–237, doi:10.1634/stemcells.2008-0273.

364. Gnecchi, M.; Zhang, Z.; Ni, A.; Dzau, V.J. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res.* **2008**, *103* (11):1204-19, doi:10.1161/CIRCRESAHA.108.176826.

365. Daley, G.Q.; Scadden, D.T. Prospects for Stem Cell-Based Therapy. *Cell* **2008**, *132*, 544–548, doi:10.1016/j.cell.2008.02.009.

366. Giannoni, P.; Scaglione, S.; Daga, A.; Ilengo, C.; Cilli, M.; Quarto, R. Short-time survival and engraftment of bone marrow stromal cells in an ectopic model of bone regeneration. *Tissue Eng. - Part A* **2010**, *16* (2), 489-499, doi: 10.1089=ten.tea.2009.0041.

367. Mylotte, L.A.; Duffy, A.M.; Murphy, M.; O'Brien, T.; Samali, A.; Barry, F.; Szegezdi, E. Metabolic Flexibility Permits Mesenchymal Stem Cell Survival in an Ischemic Environment. *Stem Cells* **2008**, *26*, 1325–1336, doi:10.1634/stemcells.2007-1072.

368. Mischen, B.T.; Follmar, K.E.; Moyer, K.E.; Buehrer, B.; Olbrich, K.C.; Levin, L.S.; Klitzman, B.; Erdmann, D. Metabolic and functional characterization of human adipose-derived stem cells in tissue engineering? *Plast. Reconstr. Surg.* **2008**, *122*, 725–738, doi:10.1097/PRS.0b013e318180ec9f.

369. Faghih, H.; Javeri, A.; Taha, M.F. Impact of early subcultures on stemness, migration and angiogenic potential of adipose tissue-derived stem cells and their resistance to in vitro ischemic condition. *Cytotechnology* **2017**, *69*, 885–900, doi:10.1007/s10616-017-0104-5.

370. Moya, A.; Paquet, J.; Deschepper, M.; Larochette, N.; Oudina, K.; Denoeud, C.; Bensidhoum, M.; Logeart-Avramoglou, D.; Petite, H. Human Mesenchymal Stem Cell Failure to Adapt to Glucose Shortage and Rapidly Use Intracellular Energy Reserves Through Glycolysis Explains Poor Cell Survival After Implantation. *Stem Cells* **2018**, *36*, 363–376, doi:10.1002/stem.2763.

371. Deschepper, M.; Manassero, M.; Oudina, K.; Paquet, J.; Monfoulet, L.E.; Bensidhoum, M.; Logeart-Avramoglou, D.; Petite, H. Proangiogenic and prosurvival functions of glucose in human mesenchymal stem cells upon transplantation. *Stem Cells* **2013**, *31*, 526–535, doi:10.1002/stem.1299.

372. Brahimi-Horn, M.C.; Pouysségur, J. Oxygen, a source of life and stress. *FEBS Lett.* **2007**, *581*, 3582–3591, doi: 10.1016/j.febslet.2007.06.018.

373. Ferrara, N.; Kerbel, R.S. Angiogenesis as a therapeutic target. *Nature* **2005**, *438*, 967–974, doi: 10.1038/nature04483.

374. Chen, S.; Sang, N. Hypoxia-Inducible Factor-1: A Critical Player in the Survival Strategy of Stressed Cells. *J. Cell. Biochem.* **2016**, *117*, 267–278, doi:10.1002/jcb.25283.

375. Berthois, Y.; Katzenellenbogen, J.A.; Katzenellenbogen, B.S. Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **1986**, *83*, 2496–2500, doi:10.1073/pnas.83.8.2496.

376. Hofland, L.J.; van Koetsveld, P.; Koper, J.W.; den Holder, A.; Lamberts, S.W.J. Weak estrogenic activity of phenol red in the culture medium: its role in the study of the regulation of prolactin release in vitro. *Mol. Cell. Endocrinol.* **1987**, *54*, 43–50, doi:10.1016/0303-7207(87)90138-9.

377. Si, Z.; Wang, X.; Sun, C.; Kang, Y.; Xu, J.; Wang, X.; Hui, Y. Adipose-derived stem cells: Sources, potency, and implications for regenerative therapies. *Biomed. Pharmacother.* **2019**, *114*, doi:10.1016/j.biopha.2019.108765.

378. Izadpanah, R.; Trygg, C.; Patel, B.; Kriedt, C.; Dufour, J.; Gimble, J.M.; Bunnell, B.A. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J. Cell. Biochem.* **2006**, *99*, 1285–1297, doi:10.1002/jcb.20904.

379. Minguell, J.J.; Erices, A.; Conget, P. Mesenchymal stem cells. *Exp. Biol. Med.* **2001**, *226*, 507–520, doi: 10.1177/153537020122600603.

380. Russ, A.L.; Haberstroh, K.M.; Rundell, A.E. Experimental strategies to improve in vitro models of renal ischemia. *Exp. Mol. Pathol.* **2007**, *83*, 143–159, doi:10.1016/j.yexmp.2007.03.002.

381. Zhu, W.; Chen, J.; Cong, X.; Hu, S.; Chen, X. Hypoxia and Serum Deprivation-Induced Apoptosis in Mesenchymal Stem Cells. *Stem Cells* **2006**, *24*, 416–425, doi:10.1634/stemcells.2005-0121.

382. Choi, J.R.; Yong, K.W.; Wan Safwani, W.K.Z. Effect of hypoxia on human adipose-derived mesenchymal stem cells and its potential clinical applications. *Cell. Mol. Life Sci.* **2017**, *74*, 2587–2600, doi:10.1007/s00018-017-2484-2.

383. Wang, B.; Stuart Wood, I.; Trayhurn, P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflugers Arch* **2007,** *455* (3):479-92*,* doi:10.1007/s00424-007-0301-8.

384. Ferrara, N. Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int.* **1999**, *56*, 794–814, doi:10.1046/j.1523-1755.1999.00610.x.

385. Karaman, S.; Leppänen, V.M.; Alitalo, K. Vascular endothelial growth factor signaling in development and disease. *Dev.* **2018**, *145*, doi:10.1242/dev.151019.

386. Pu, C.M.; Liu, C.W.; Liang, C.J.; Yen, Y.H.; Chen, S.H.; Jiang-Shieh, Y.F.; Chien, C.L.; Chen, Y.C.; Chen, Y.L. Adipose-Derived Stem Cells Protect Skin Flaps against Ischemia/Reperfusion Injury via IL-6 Expression. *J. Invest. Dermatol.* **2017**, *137*, 1353–1362, doi:10.1016/j.jid.2016.12.030.

387. Fan, Y.; Ye, J.; Shen, F.; Zhu, Y.; Yeghiazarians, Y.; Zhu, W.; Chen, Y.; Lawton, M.T.; Young, W.L.; Yang, G.Y. Interleukin-6 stimulates circulating blood-derived endothelial progenitor cell angiogenesis in vitro. *J. Cereb. Blood Flow Metab.* **2008**, *28*, 90–98, doi:10.1038/sj.jcbfm.9600509.

388. Yeung, B.H.Y.; Law, A.Y.S.; Wong, C.K.C. Evolution and roles of stanniocalcin. *Mol. Cell. Endocrinol.* **2012**, *349*, 272–280, doi:10.1016/j.mce.2011.11.007.

389. Westberg, J.A.; Serlachius, M.; Lankila, P.; Penkowa, M.; Hidalgo, J.; Andersson, L.C. Hypoxic preconditioning induces neuroprotective stanniocalcin-1 in brain via IL-6 signaling. *Stroke* **2007**, *38*, 1025–1030, doi:10.1161/01.STR.0000258113.67252.fa.

390. Kassab, C.; Kerrigan, B.P.; Caruso, H.; Al Enazy, S.; Heimberger, A.B. Immunomodulatory Methods. *Nervous System Drug Delivery* **2019**, 297-334, doi:10.1016/B978-0-12-813997-4.00015-3.

391. Sharma, H.S.; Das, D.K. Role of cytokines in myocardial ischemia and reperfusion. *Mediators Inflamm.* **1997**, *6*, 175–183, doi: 10.1080/09629359791668.

392. Frangogiannis, N.G.; Youker, K.A.; Rossen, R.D.; Gwechenberger, M.; Lindsey, M.H.; Mendoza, L.H.; Michael, L.H.; Ballantyne, C.M.; Smith, C.W.; Entman, M.L. Cytokines and the microcirculation in ischemia and reperfusion. *J Mol Cell Cardiol.* **1998**, *30* (12):2567-76, doi: 10.1006/jmcc.1998.0829.

393. Nilupul Perera, M.; Ma, H.K.; Arakawa, S.; Howells, D.W.; Markus, R.; Rowe, C.C.; Donnan, G.A. Inflammation following stroke. *J. Clin. Neurosci.* **2006**, *13*, 1–8, doi: 10.1016/j.jocn.2005.07.005.

394. Rothwell, N.J.; Relton, J.K. Involvement of cytokines in acute neurodegeneration in the CNS. *Neurosci. Biobehav. Rev.* **1993**, *17*, 217–227, doi:10.1016/S0149-7634(05)80152-6.

395. Eltzschig, H.K.; Eckle, T. Ischemia and reperfusion-from mechanism to translation. *Nat. Med.* **2011**, *17*, 1391–1401, doi: 10.1038/nm.2507.

396. Strey, C.W.; Markiewski, M.; Mastellos, D.; Tudoran, R.; Spruce, L.A.; Greenbaum, L.E.; Lambris, J.D. The proinflammatory mediators C3a and C5a are essential for liver regeneration. *J. Exp. Med.* **2003**, *198*, 913–923, doi:10.1084/jem.20030374.

397. Danobeitia, J.S.; Djamali, A.; Fernandez, L.A. The role of complement in the pathogenesis of renal ischemia-reperfusion injury and fibrosis. *Fibrogenes. Tissue Repair* **2014**, *7*, 16, doi: 10.1186/1755-1536-7-16.

398. Ricardo, S.D.; Van Goor, H.; Eddy, A.A. Macrophage diversity in renal injury and repair. *J. Clin. Invest.* **2008**, *118*, 3522–3530, doi: 10.1172/JCI36150.

399. Feng, C.; Hu, J.; Liu, C.; Liu, S.; Liao, G.; Song, L.; Zeng, X. Association of 17-β estradiol with adipose-derived stem cells: New strategy to produce functional myogenic differentiated cells with a nano-scaffold for tissue engineering. *PLoS One* **2016**, *11*, doi:10.1371/journal.pone.0164918.

400. Mayes, J.S.; Watson, G.H. Direct effects of sex steroid hormones on adipose tissues and obesity. *Obes. Rev.* **2004**, *5*, 197–216, doi: 10.1111/j.1467-789X.2004.00152.x.

401. Zhang, W.; Schmull, S.; Du, M.; Liu, J.; Lu, Z.; Zhu, H.; Xue, S.; Lian, F. Estrogen Receptor α and β in Mouse: Adipose-Derived Stem Cell Proliferation, Migration, and Brown Adipogenesis In Vitro. *Cell. Physiol. Biochem.* **2016**, *38*, 2285–2299, doi:10.1159/000445583.

402. Celojevic, D.; Petersen, A.; Karlsson, J.-O.; Behndig, A.; Zetterberg, M. Effects of 17β-estradiol on proliferation, cell viability and intracellular redox status in native human lens epithelial cells. *Mol Vis.* **2011,** 17:1987-96.

403. Boutilier, R.G. Mechanisms of cell survival in hypoxia and hypothermia. *J Exp Biol.* **2001***, 204*(Pt 18):3171-81.

404. Nuschke, A.; Rodrigues, M.; Wells, A.W.; Sylakowski, K.; Wells, A. Mesenchymal stem cells/multipotent stromal cells (MSCs) are glycolytic and thus glucose is a limiting factor of in vitro models of MSC starvation. *Stem Cell Res. Ther.* **2016**, *7*, 1–9, doi:10.1186/s13287-016-0436-7.

405. Brighton, C.T.; Krebs, A.G. Oxygen tension of healing fractures in the rabbit. *J. Bone Joint Surg. Am.* **1972**, *54*, 323–332, doi:10.2106/00004623-197254020-00010.

406. Kim, W.S.; Han, J.; Hwang, S.J.; Sung, J.H. An update on niche composition, signaling and functional regulation of the adipose-derived stem cells. *Expert Opin. Biol. Ther.* **2014**, *14*, 1091–1102, doi:10.1517/14712598.2014.907785.

407. Li, X.; Ma, T.; Sun, J.; Shen, M.; Xue, X.; Chen, Y.; Zhang, Z. Harnessing the secretome of adipose-derived stem cells in the treatment of ischemic heart diseases. *Stem Cell Res. Ther.* **2019**, *10*, 196, doi: 10.1186/s13287-019-1289-7.

408. Ferreira, J.R.; Teixeira, G.Q.; Santos, S.G.; Barbosa, M.A.; Almeida-Porada, G.; Gonçalves, R.M. Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-conditioning. *Front. Immunol.* **2018**, *9*, 2837, doi:10.3389/fimmu.2018.02837.

409. Meiliana, A.; Dewi, N.M.; Wijaya, A. Mesenchymal stem cell secretome: Cell-free therapeutic strategy in regenerative medicine. *Indones. Biomed. J.* **2019**, *11*, 113–124, doi:10.18585/inabj.v11i2.839.

410. He, X.; Zhong, X.; Ni, Y.; Liu, M.; Liu, S.; Lan, X. Effect of ASCs on the graft survival rates of fat particles in rabbits. *J. Plast. Surg. Hand Surg.* **2013**, *47*, 3–7, doi:10.3109/2000656X.2012.730488.

411. Frazier, T.P.; Gimble, J.M.; Kheterpal, I.; Rowan, B.G. Impact of low oxygen on the secretome of human adipose-derived stromal/stem cell primary cultures. *Biochimie* **2013**, *95*, 2286–2296, doi:10.1016/j.biochi.2013.07.011.

412. Madrigal, M.; Rao, K.S.; Riordan, N.H. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J. Transl. Med.* **2014**, *12:260,* doi: 10.1186/s12967-014-0260-8.

413. Pirkmajer, S.; Chibalin, A. V. Serum starvation: Caveat emptor. *Am. J. Physiol. - Cell Physiol.* **2011**, *301*, 272–279, doi:10.1152/ajpcell.00091.2011.

414. Moya, A.; Larochette, N.; Paquet, J.; Deschepper, M.; Bensidhoum, M.; Izzo, V.; Kroemer, G.; Petite, H.; Logeart-Avramoglou, D. Quiescence Preconditioned Human Multipotent Stromal Cells Adopt a Metabolic Profile Favorable for Enhanced Survival under Ischemia. *Stem Cells* **2017**, *35*, 181–196, doi:10.1002/stem.2493.

415. Li, C.; Ye, L.; Yang, L.; Yu, X.; He, Y.; Chen, Z.; Li, L.; Zhang, D. Rapamycin Promotes the Survival and Adipogenesis of Ischemia-Challenged Adipose Derived Stem Cells by Improving Autophagy. *Cell. Physiol. Biochem.* **2018**, *44*, 1762–1774, doi:10.1159/000485783.

416. Pan, J.S.C.; Huang, L.; Belousova, T.; Lu, L.; Yang, Y.; Reddel, R.; Chang, A.; Ju, H.; DiMattia, G.; Tong, Q.; et al. Stanniocalcin-1 inhibits renal ischemia/reperfusion injury via an AMP-activated protein kinase-dependent pathway. *J. Am. Soc. Nephrol.* **2015**, *26*, 364–378, doi:10.1681/ASN.2013070703.

417. Higuera, G.A.; Fernandes, H.; Spitters, T.W.G.M.; van de Peppel, J.; Aufferman, N.; Truckenmueller, R.; Escalante, M.; Stoop, R.; van Leeuwen, J.P.; de Boer, J.; et al. Spatiotemporal proliferation of human stromal cells adjusts to nutrient availability and leads to stanniocalcin-1 expression in vitro and in vivo. *Biomaterials* **2015**, *61*, 190–202, doi:10.1016/j.biomaterials.2015.05.017.

418. Bironaite, D.; Westberg, J.A.; Andersson, L.C.; Venalis, A. A variety of mild stresses upregulate stanniocalcin-1 (STC-1) and induce mitohormesis in neural crest-derived cells. *J. Neurol. Sci.* **2013**, *329*, 38–44, doi:10.1016/j.jns.2013.03.011.

419. Bakopoulou, A.; Kritis, A.; Andreadis, D.; Papachristou, E.; Leyhausen, G.; Koidis, P.; Geurtsen, W.; Tsiftsoglou, A. Angiogenic Potential and Secretome of Human Apical Papilla Mesenchymal Stem Cells in Various Stress Microenvironments. *Stem Cells Dev.* **2015**, *24*, 2496–2512, doi:10.1089/scd.2015.0197.

420. Choi, S.J.; Shin, I.J.; Je, K.H.; Min, E.K.; Kim, E.J.; Kim, H.S.; Choe, S.; Kim, D.E.; Lee, D.K. Hypoxia Antagonizes Glucose Deprivation on Interleukin 6 Expression in an Akt Dependent, but HIF-1/2α Independent Manner. *PLoS One* **2013**, *8*, 1–10, doi:10.1371/journal.pone.0058662.

421. Fougeray, S.; Bouvier, N.; Beaune, P.; Legendre, C.; Anglicheau, D.; Thervet, E.; Pallet, N. Metabolic stress promotes renal tubular inflammation by triggering the unfolded protein response. *Cell Death Dis.* **2011**, *2*, e143, doi:10.1038/cddis.2011.26.

422. He, L.F.; Wang, T.T.; Gao, Q.Y.; Zhao, G.F.; Huang, Y.H.; Yu, L.K.; Hou, Y.Y. Stanniocalcin-1 promotes tumor angiogenesis through up-regulation of VEGF in gastric cancer cells. *J. Biomed. Sci.* **2011**, *18*, 39, doi:10.1186/1423-0127-18-39.

423. Law, A.Y.S.; Wong, C.K.C. Stanniocalcin-1 and -2 promote angiogenic sprouting in HUVECs via VEGF/VEGFR2 and angiopoietin signaling pathways. *Mol. Cell. Endocrinol.* **2013**, *374*, 73–81, doi:10.1016/j.mce.2013.04.024.

424. Lee, S.H.; Jin, S.Y.; Song, J.S.; Seo, K.K.; Cho, K.H. Paracrine effects of adipose-derived stem cells on keratinocytes and dermal fibroblasts. *Ann. Dermatol.* **2012**, *24*, 136–143, doi:10.5021/ad.2012.24.2.136.

425. DiPietro, L.A.; Reintjes, M.G.; Low, Q.E.H.; Levi, B.; Gamelli, R.L. Modulation of macrophage recruitment into wounds by monocyte chemoattractant protein-1. *Wound Repair Regen.* **2001**, *9*, 28–33, doi:10.1046/j.1524-475X.2001.00028.x.

426. Mazur, S.; Zołocińska, A.; Siennicka, K.; Janik-Kosacka, K.; Chrapusta, A.; Pojda, Z. Safety of adipose-derived cell (stromal vascular fraction – SVF) augmentation for surgical breast reconstruction in cancer patients. *Adv. Clin. Exp. Med.* **2018**, *27*, 1085–1090, doi:10.17219/acem/70798.

427. Waked, K.; Colle, J.; Doornaert, M.; Cocquyt, V.; Blondeel, P. Systematic review: The oncological safety of adipose fat transfer after breast cancer surgery. *Breast* **2017**, *31*, 128–136, doi: 10.1016/j.breast.2016.11.001.

428. Matsumoto, D.; Sato, K.; Gonda, K.; Takaki, Y.; Shigeura, T.; Sato, T.; Aiba-Kojima, E.; Iizuka, F.; Inoue, K.; Suga, H.; et al. Cell-Assisted Lipotransfer: Supportive Use of Human Adipose-Derived Cells for Soft Tissue Augmentation with Lipoinjection. *Tissue Eng.* **2006,** 12, 3375-82, doi: 10.1089/ten.2006.12.3375.

429. Lendeckel, S.; Jödicke, A.; Christophis, P.; Heidinger, K.; Wolff, J.; Fraser, J.K.; Hedrick, M.H.; Berthold, L.; Howaldt, H.-P. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. *J. Cranio-Maxillofacial Surg.* **2004**, *32*, 370–373, doi:10.1016/j.jcms.2004.06.002.

430. Qayyum, A.A.; Mathiasen, A.B.; Mygind, N.D.; Kühl, J.T.; Jørgensen, E.; Helqvist, S.; Elberg, J.J.; Kofoed, K.F.; Vejlstrup, N.G.; Fischer-Nielsen, A.; et al. Adipose-Derived Stromal Cells for Treatment of Patients with Chronic Ischemic Heart Disease (MyStromalCell Trial): A Randomized Placebo-Controlled Study. *Stem Cells Int.* **2017**, *2017*, doi:10.1155/2017/5237063.

431. Kocan, B.; Maziarz, A.; Tabarkiewicz, J.; Ochiya, T.; Banaś-Ząbczyk, A. Trophic Activity and Phenotype of Adipose Tissue-Derived Mesenchymal Stem Cells as a Background of Their Regenerative Potential. *Stem Cells Int.* **2017**, 2017:1653254, doi:10.1155/2017/1653254.

432. Wu, Y.; Hoogduijn, M.J.; Baan, C.C.; Korevaar, S.S.; Kuiper, R. De; Yan, L.; Wang, L.; Besouw, N.M.V. Adipose Tissue-Derived Mesenchymal Stem Cells Have a Heterogenic Cytokine Secretion Profile. *Stem Cells Int.* **2017**, *2017*, doi:10.1155/2017/4960831.

433. Bussolino, F.; Mantovani, A.; Persico, G. Molecular mechanisms of blood vessel formation. *Trends Biochem. Sci.* **1997**, *22*, 251–254, doi: 10.1016/s0968-0004(97)01074-8.

434. Naito, H.; Iba, T.; Takakura, N. Mechanisms of new blood-vessel formation and proliferative heterogeneity of endothelial cells. *Int. Immunol.* **2021**, *32*, 295–305, doi:10.1093/intimm/dxaa008.

435. Chang, A.C.M.; Janosi, J.; Hulsbeek, M.; de Jong, D.; Jeffrey, K.J.; Noble, J.R.; Reddel, R.R. A novel human cDNA highly homologous to the fish hormone stanniocalcin. *Mol. Cell. Endocrinol.* **1995**, *112*, 241–247, doi:10.1016/0303-7207(95)03601-3.

436. Hanssen, R.G.J.M.; Aarden, E.M.; van der Venne, W.P.H.G.; Pang, P.K.T.; Wendelaar Bonga, S.E. Regulation of secretion of the teleost fish hormone stanniocalcin: Effects of extracellular calcium. *Gen. Comp. Endocrinol.* **1991**, *84*, 155–163, doi:10.1016/0016-6480(91)90075-H.

437. Westberg, J.A.; Serlachius, M.; Lankila, P.; Andersson, L.C. Hypoxic preconditioning induces elevated expression of stanniocalcin-1 in the heart. *Am. J. Physiol. - Hear. Circ. Physiol.* **2007**, *293*, doi:10.1152/ajpheart.00017.2007.

438. Block, G.J.; Ohkouchi, S.; Fung, F.; Frenkel, J.; Gregory, C.; Pochampally, R.; DiMattia, G.; Sullivan, D.E.; Prockop, D.J. Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. *Stem Cells* **2009**, *27*, 670–681, doi:10.1002/stem.20080742.

439. Kahn, J.; Mehraban, F.; Ingle, G.; Xin, X.; Bryant, J.E.; Vehar, G.; Schoenfeld, J.; Grimaldi, C.J.; Peale, F.; Draksharapu, A.; et al. Gene expression profiling in an in vitro model of angiogenesis. *Am. J. Pathol.* **2000**, *156*, 1887–1900, doi:10.1016/S0002-9440(10)65062-6.

440. Liu, G.; Yang, G.; Chang, B.; Mercado-Uribe, I.; Huang, M.; Zheng, J.; Bast, R.C.; Lin, S.H.; Liu, J. Stanniocalcin 1 and ovarian tumorigenesis. *J. Natl. Cancer Inst.* **2010**, *102*, 812–827, doi:10.1093/jnci/djq127.

441. Holmes, D.I.R.; Zachary, I.C. Vascular endothelial growth factor regulates Stanniocalcin-1 expression via Neuropilin-1-dependent regulation of KDR and synergism with fibroblast growth Factor-2. *Cell. Signal.* **2008**, *20*, 569–579, doi:10.1016/j.cellsig.2007.11.009.

442. Liu, D.; Jia, H.; Holmes, D.I.R.; Stannard, A.; Zachary, I. Vascular Endothelial Growth Factor-Regulated Gene Expression in Endothelial Cells: KDR-Mediated Induction of Egr3 and the Related Nuclear Receptors Nur77, Nurr1, and Nor1. *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 2002–2007, doi:10.1161/01.ATV.0000098644.03153.6F.

443. Tahiri, R. The role of Stanniocalcin-1 in vascular cell function. *Dr. thesis, UCL (University Coll. London).* **2013**.

444. He, L.F.; Wang, T.T.; Gao, Q.Y.; Zhao, G.F.; Huang, Y.H.; Yu, L.K.; Hou, Y.Y. Stanniocalcin-1 promotes tumor angiogenesis through up-regulation of VEGF in gastric cancer cells. *J. Biomed. Sci.* **2011**, *18*, 39, doi:10.1186/1423-0127-18-39.

445. Zlot, C.; Ingle, G.; Hongo, J.; Yang, S.; Sheng, Z.; Schwall, R.; Paoni, N.; Wang, F.; Peale, F. V.; Gerritsen, M.E. Stanniocalcin 1 Is an Autocrine Modulator of Endothelial Angiogenic Responses to Hepatocyte Growth Factor. *J. Biol. Chem.* **2003**, *278*, 47654–47659, doi:10.1074/jbc.M301353200.

446. Kanellis, J.; Bick, R.; Garcia, G.; Truong, L.; Tsao, C.C.; Etemadmoghadam, D.; Poindexter, B.; Feng, L.; Johnson, R.J.; Sheikh-Hamad, D. Stanniocalcin-1, an inhibitor of macrophage chemotaxis and chemokinesis. *Am. J. Physiol. - Ren. Physiol.* **2004**, *286*, doi:10.1152/ajprenal.00138.2003.

447. Holmes, D.I.R.; Zachary, I.C. Vascular endothelial growth factor regulates Stanniocalcin-1 expression via Neuropilin-1-dependent regulation of KDR and synergism with fibroblast growth Factor-2. *Cell. Signal.* **2008**, *20*, 569–579, doi:10.1016/j.cellsig.2007.11.009.

448. Yeung, H.Y.; Lai, K.P.; Chan, H.Y.; Mak, N.K.; Wagner, G.F.; Wong, C.K.C. Hypoxia-inducible factor-1-mediated activation of stanniocalcin-1 in human cancer cells. *Endocrinology* **2005**, *146*, 4951–4960, doi:10.1210/en.2005-0365.

449. Basini, G.; Bussolati, S.; Santini, S.E.; Grasselli, F. Stanniocalcin, a potential ovarian angiogenesis regulator, does not affect endothelial cell apoptosis. *Ann N Y Acad Sci* **2009**, 1171:94-9, doi: 10.1111/j.1749-6632.2009.04680.x.

450. Klopfleisch, R.; Gruber, A.D. Derlin-1 and Stanniocalcin-1 are Differentially Regulated in Metastasizing Canine Mammary Adenocarcinomas. *J. Comp. Pathol.* **2009**, *141*, 113–120, doi:10.1016/j.jcpa.2008.09.010.

451. Shiffman, M.A.; Mirrafati, S. Fat transfer techniques: The effect of harvest and transfer methods on adipocyte viability and review of the literature. *Dermatologic Surg.* **2001**, *27*, 819–826, doi:10.1046/j.1524-4725.2001.01062.x.

452. Billings, E.; May, J.W. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. *Plast. Reconstr. Surg.* **1989**, *83*, 368–381, doi:10.1097/00006534-198902000-00033.

453. Arshad, Z.; Karmen, L.; Choudhary, R.; Smith, J.A.; Branford, O.A.; Brindley, D.A.; Pettitt, D.; Davies, B.M. Cell assisted lipotransfer in breast augmentation and reconstruction: A systematic review of safety, efficacy, use of patient reported outcomes and study quality. *JPRAS Open* **2016**, *10*, 5–20, doi:10.1016/j.jpra.2016.08.004.

454. Lasso, J.M.; Poletti, D.; Scola, B.; Gómez-Vilda, P.; García-Martín, A.I.; Eugenia Fernández-Santos, M. Clinical Study Injection Laryngoplasty Using Autologous Fat Enriched with Adipose-Derived Regenerative Stem Cells: A Safe Therapeutic Option for the Functional Reconstruction of the Glottal Gap after Unilateral Vocal Fold Paralysis. *Stem Cells Int*. **2018**, 2018:8917913, doi:10.1155/2018/8917913.

455. Bunnell, B.A.; Flaat, M.; Gagliardi, C.; Patel, B.; Ripoll, C. Adipose-derived stem cells: isolation, expansion and differentiation. *Methods* **2008**, 45(2):115-20, doi: 10.1016/j.ymeth.2008.03.006.

456. Blanchette-Mackie, E.J.; Dwyer, N.K.; Barber, T.; Coxey, R.A., Takeda, T.; Rondinone, C.M.; Theodorakis, J.L.; Greenberg, A.S.; Londos, C. Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J Lipid Res.***1995**, *36*(6):1211-26.

457. Arshad, Z.; Karmen, L.; Choudhary, R.; Smith, J.A.; Branford, O.A.; Brindley, D.A.; Pettitt, D.; Davies, B.M. Cell assisted lipotransfer in breast augmentation and reconstruction: A systematic review of safety, efficacy, use of patient reported outcomes and study quality. *JPRAS Open* **2016**, *10*:5-20, doi:10.1016/j.jpra.2016.08.004.

458. Huttala, O.; Sarkanen, J.R.; Mannerström, M.; Toimela, T.; Heinonen, T.; Ylikomi, T. Development of novel human in vitro vascularized adipose tissue model with functional macrophages. *Cytotechnology* **2020**, *72*, 665–683, doi:10.1007/s10616-020-00407-6.

459. Aubin, K.; Safoine, M.; Proulx, M.; Audet-Casgrain, M.-A.; Côté, J.-F.; Têtu, F.-A.; Roy, A.; Fradette, J. Characterization of In Vitro Engineered Human Adipose Tissues: Relevant Adipokine Secretion and Impact of TNF-α. *PLoS One* **2015**, *10*, (9): e0137612 doi:10.1371/journal.pone.0137612.

460. Toda, S.; Uchihashi, K.; Aoki, S.; Sonoda, E.; Yamasaki, F.; Piao, M.; Ootani, A.; Yonemitsu, N.; Sugihara, H. Adipose tissue-organotypic culture system as a promising model for studying adipose tissue biology and regeneration. *Organogenesis* **2009**, *5*, 50–56, 10.4161/org.5.2.8347.

461. Sonoda, E.; Aoki, S.; Uchihashi, K.; Soejima, H.; Kanaji, S.; Izuhara, K.; Satoh, S.; Fujitani, N.; Sugihara, H.; Toda, S. A new organotypic culture of adipose tissue fragments maintains viable mature adipocytes for a long term, together with development of immature adipocytes and mesenchymal stem cell-like cells. *Endocrinology* **2008**, *149*, 4794–4798, doi:10.1210/en.2008-0525.

462. Emont, M.P.; Yu, H.; Jun, H.; Hong, X.; Maganti, N.; Stegemann, J.P.; Wu, J. Using a 3D culture system to differentiate visceral adipocytes in vitro. *Endocrinology* **2015**, *156*, 4761–4768, doi:10.1210/en.2015-1567.

463. Baran, C.N.; Çelebioğlu, S.; Şensöz, Ö.; Ulusoy, G.; Civelek, B.; Ortak, T. The behavior of fat grafts in recipient areas with enhanced vascularity. *Plast. Reconstr. Surg.* **2002**, *109*, 1646–1651, doi:10.1097/00006534-200204150-00023.

464. Brucker, M.; Sati, S.; Spangenberger, A.; Weinzweig, J. Long-term fate of transplanted autologous fat in a novel rabbit facial model? *Plast. Reconstr. Surg.* **2008**, *122*, 749–754, doi:10.1097/PRS.0b013e3181815a41.

465. Coban, Y.K.; Ciralik, H. The effects of increased ischemic times on adipose tissue: A histopathologic study using the epigastric flap model in rats. *Aesthetic Plast. Surg.* **2007**, *31*, 570–573, doi:10.1007/s00266-006-0196-3.

466. Zhong, X.; Yan, W.; He, X.; Ni, Y. Improved fat graft viability by delayed fat flap with ischaemic pretreatment. *J. Plast. Reconstr. Aesthetic Surg.* **2009**, *62*, 526–531, doi:10.1016/j.bjps.2007.11.020.

467. Ko, M.S.; Jung, J.Y.; Shin, I.S.; Choi, E.W.; Kim, J.H.; Kang, S.K.; Ra, J.C. Effects of expanded human adipose tissue-derived mesenchymal stem cells on the Viability of cryopreserved fat grafts in the Nude Mouse. *Int. J. Med. Sci.* **2011**, *8*, 231–238, doi:10.7150/ijms.8.231.

468. Li, K.; Li, F.; Li, J.; Wang, H.; Zheng, X.; Long, J.; Guo, W.; Tian, W. Increased survival of human free fat grafts with varying densities of human adipose-derived stem cells and platelet-rich plasma. *J. Tissue Eng. Regen. Med.* **2017**, *11*, 209–219, doi:10.1002/term.1903.

469. Topcu, A.; Aydin, O.E.; Ünlü, M.; Barutcu, A.; Atabey, A. Increasing the viability of fat grafts by vascular endothelial growth factor. *Arch. Facial Plast. Surg.* **2012**, *14*, 270–276, doi:10.1001/archfacial.2011.1633.

470. Zhang, Y.; Xiao, L.L.; Li, J.X.; Liu, H.W.; Li, S.H.; Wu, Y.Y.; Liao, X.; Rao, C.Q. Improved fat transplantation survival by using the conditioned medium of vascular endothelial growth factor transfected human adipose-derived stem cells. *Kaohsiung J. Med. Sci.* **2017**, *33*, 379–384, doi:10.1016/j.kjms.2017.05.009.

471. Yu, F.; Witman, N.; Yan, D.; Zhang, S.; Zhou, M.; Yan, Y.; Yao, Q.; Ding, F.; Yan, B.; Wang, H.; et al. Human adipose-derived stem cells enriched with VEGF-modified mRNA promote angiogenesis and long-term graft survival in a fat graft transplantation model. *Stem Cell Res. Ther.* **2020**, *11*, 490, doi:10.1186/s13287-020-02008-8.

472. Wang, Y.; Wu, Y. Assessment of the clinical efficacy of cell-assisted lipotransfer and conventional fat graft: A meta-analysis based on case-control studies. *J. Orthop. Surg. Res.* **2017**, *12*, 155, doi:10.1186/s13018-017-0645-5.

473. Contreras, G.A.; Thelen, K.; Schmidt, S.E.; Strieder-Barboza, C.; Preseault, C.L.; Raphael, W.; Kiupel, M.; Caron, J.; Lock, A.L. Adipose tissue remodeling in late-lactation dairy cows during feed-restriction-induced negative energy balance. *J. Dairy Sci.* **2016**, *99*, 10009–10021, doi:10.3168/jds.2016-11552.

474. Kakudo, N.; Tanaka, Y.; Morimoto, N.; Ogawa, T.; Kushida, S.; Hara, T.; Kusumoto, K. Adipose-derived regenerative cell (ADRC)-enriched fat grafting: Optimal cell concentration and effects on grafted fat characteristics. *J. Transl. Med.* **2013**, *11*, doi:10.1186/1479-5876-11-254.

List of Figures

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List of Abbreviations

°C	Degree Celsius
2D	Two-dimensional
3D	Three-dimensional
A2P	Ascorbate-2-phosphate
ANG	Angiogenin
Ang-1/2	Angiopoietin 1/2
ANOVA	Analysis of variance
aP2	Adipocyte protein 2/ fatty acid binding protein 4 (FABP4)
ASC	Adipose-derived stromal/stem cell(s)
AT	Adipose tissue
BAT	Brown adipose tissue
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BM-MSC	Bone marrow-derived stromal/stem cell(s)
BMP	Bone morphogenetic protein
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
BSA	Bovine serum albumin
CAL	Cell-assisted lipotransfer
Calcein-AM	Calcein acetoxymethyl ester
CD	Cluster of differentiation
cDNA	Coding deoxyribonucleic acid
C/EBPα,β,δ	CCAAT-enhancer-binding protein alpha, beta, delta
CETP	Cholesteryl ester transfer protein
cm	Centimetre
CO_2	Carbon dioxide
COL	Collagen
CXCL	C-X-C motif chemokine ligand
d	Day
DAPI	4′,6-diamidino-2-phenylindole
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle´s Medium
DMEM/F-12	Dulbecco's Modified Eagle´s Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPBS	Dulbecco´s phosphate buffered saline

E2	17-β Estradiol
EC	Endothelial cell(s)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor-1 alpha
e.g.	Exempli gratia (Latin “for example”)
EGF	Epidermal growth factor
eGM	Endothelial growth medium
ELISA	Enzyme-linked immunosorbent assay
EthD-III	Ethidium bromide homodimer III
EV	Extracellular vesicle(s)
FACS	Fluorescence activated cell sorting/scanning; flow cytometry
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF 2	Fibroblast growth factor 2
g	Gram
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES-1	Hairy and enhancer of split 1
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1 alpha
HUVEC	Human umbilical vein endothelial cell(s)
IBMX	3-isobutyl-1-methylxanthine
i.e.	id est (Latin “that is”)
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin(s)
IR	Insulin resistance
KGF	Keratinocyte growth factor
KLF	Kruepple-like factor(s)
L	Liter
M	Molar
MCP-1	Monocyte chemoattractant protein-1
mg	Milligram
$MgCl_2$	Magnesium chloride

miRNA	Micro ribonucleic acid
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MMP	Matrix metalloproteinase(s)
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal/stem cell(s)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
MV	Microvesicle(s)
µg	Microgram
µm	Micrometre
µM	Micromolar
NaCl	Sodium chloride
ng	Nanogram
NGF	Nerve growth factor
nm	Nanometre
ORO	Oil Red O
O_2	Oxygen
PBM-2	Preadipocyte basal medium 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
Pref-1	Preadipocyte factor-1
PSP	Phenol red
qRT-PCR	Quantitative real-time polymerase chain reaction
rhSTC-1	Recombinant human STC-1
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
Rpm	Rounds per minute
RT	Room temperature
SD	Standard deviation
SDF-1	Stem cell-derived factor 1
STC-1	Stanniocalcin-1
SVF	Stromal-vascular fraction

TG	Triglyceride(s)
TGF-α,β	Transforming growth factor alpha, beta
TIMP	Tissue inhibitor of metalloproteinase(s)
TNF-α	Tumor-necrosis factor-alpha
U	Unit
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WMS	Whole mount staining
w/o	Without
x g	Times gravity

Affidavit

I hereby confirm that my thesis entitled *"Role of Adipose-Derived Stromal/Stem Cells in Cell-Assisted Lipotransfer – Characterization of their Secretory Capacity under Ischemia-Like Stress Conditions and Establishment of a 3D Adipose Tissue-ASC Co-Culture"* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not been submitted as part of another examination process neither in identical nor in similar form.

Place, date

Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation *„Bedeutung von mesenchymalen Stammzellen aus dem Fettgewebe für den zellassistierten Lipotransfer – Charakterisierung der Sekretionskapazität unter Ischämie-artigen Stressbedingungen und Etablierung einer 3D Fettgewebe-ASC-Kokultur"* eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Explanations

I, J. Bachmann, have done the main work on this study. I planned it, conducted the experiments, evaluated, and interpreted the data and wrote the manuscript. P. Bauer-Kreisel contributed to the study design, methodology and data interpretation and revised the manuscript together with me. E. Ehlert assisted with data collection for the study. M. Becker contributed to the data interpretation and proofreading of the manuscript. K. Radeloff and C. Otto were also involved in the review and revision of the manuscript. T. Blunk is the principal investigator and was involved in the study design, data interpretation and writing of the manuscript and did the proofreading.

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Curriculum Vitae