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Evaluation and Optimization of Mesenchymal Stromal Cell based therapies

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1 Introduction

Due to their regenerative properties and potential clinical usage, stromal cell biology is a field of increasing interest. Although several studies and reviews have described stromal cells as the future of regenerative medicine, no unified definitions exist of their characteristics and properties (1-4). However, one common element of every definition is that a stromal cell has the potential to differentiate into tissue and simultaneously to self-renew (1, 5). This characteristic is called asymmetric division, leading to one progenitor cell and one daughter stromal cell (6). Some reviews suggest that this asymmetric division can be seen not on an individual cellular level but on a population level (7). This means that external cues lead either to division into progenitor cells or to self-renewal.

Many other definitions are more extensive and further include the existence in a quiescent form and the ability to restore and form all different cell types that are essential for the tissue that contains them (8, 9) as unique abilities for stromal cells. Therefore, if using the appropriate extraction mechanism, almost every tissue in the human body can serve as a reliable source for stromal cells.

Although stromal cells can be harvested from various tissues, they initially have to be divided by age of origin, into ESCs and adult-derived stromal cells. The induced pluripotent stromal cell (iPSC) is an exception that shows functional characteristics of stromal cells due to reprogramming factors. As the name suggests, the pluripotency among these cells is induced by external cues (10) provided by transduction (11).

The differentiation of stromal cells is mainly induced by external influences. This includes an exactly organised signalling orchestration between progenitor cells, surrounding cells and stromal cells. This is called the niche or microenvironment of stromal cells. The pathways that lead to differentiation have initially been investigated with hematopoietic stromal cells (HSCs).

HSCs create progenitor cells stochastically (7). Secreted factors do not directly influence the division ability of stromal cells, but lead to the survival of progenitor populations (7). In contrast, neural crest stromal cells show a proliferation and

differentiation pattern that is highly regulated by secreted external factors such as transforming growth factor (TGF) beta (7). Additionally, mechanical triggers and direct cell–cell or cell–extracellular matrix (ECM) interactions show influences on stromal cells. The ECM has been proven to significantly change differentiation pattern of stromal cells by enhancing differentiation into specific lineages (12). This characteristic is now commonly used in regenerative medicine. By synthetically mimicking certain ECM structures, it is possible to stimulate lineage-specific differentiation, thereby reconstructing specific tissues only by providing initial cues (13).

Although stromal cells can differentiate into specialised cells, they show a predefined sequence of interim stages – the so-called progenitor cells. Very important in an in vivo setting, these progenitor cells can only form terminally differentiated cells and cannot transform back to stromal cells. Several in vitro mechanisms have been discovered to reverse differentiation and to re-induce pluripotency.

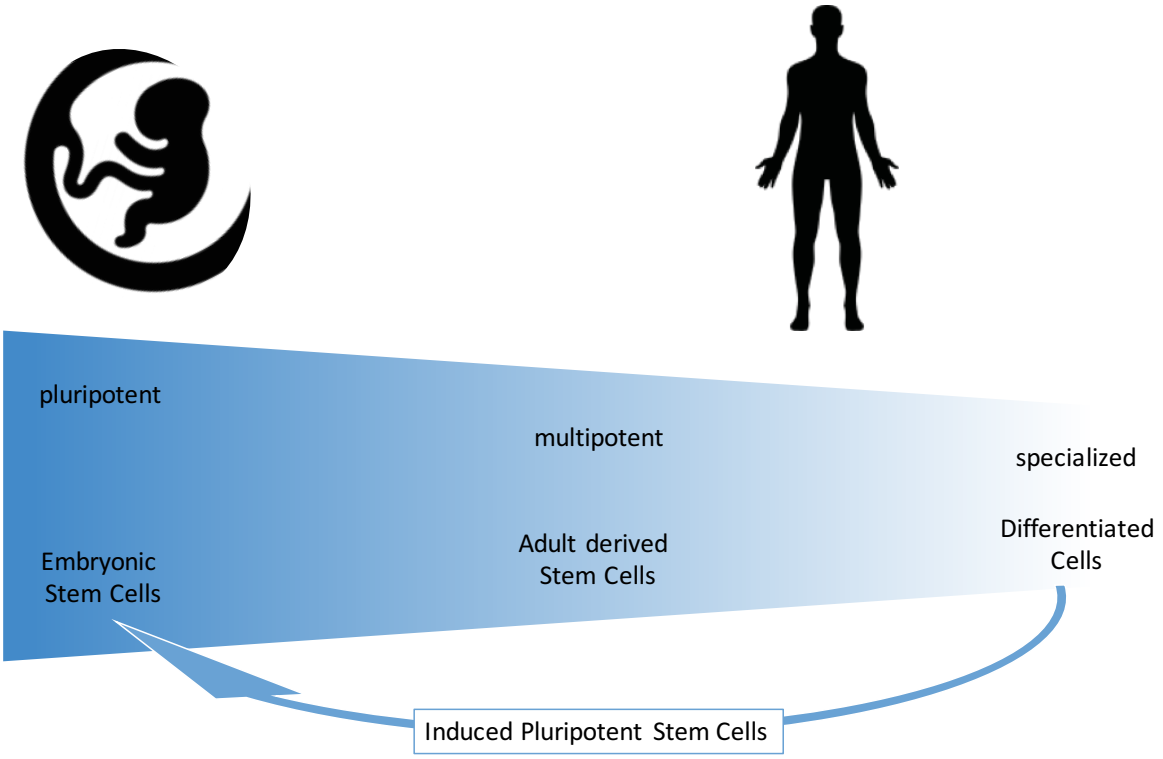


Figure 1. Potency of stromal cells is highly dependent on their age of origin. While embryonic stromal cells can be seen as pluripotent, they become multipotent in adults. By further specialisation and differentiation, this stromal cell becomes the actual differentiated cell. Pluripotency can be re-induced by external stimuli and thereby cells can be set into a previous stage.

1.1 Adult-Derived Stromal Cells

Compared to both stromal cell types described above, adult-derived stromal cells are more specialised. Due to their ability to generate several, but not all, tissues, they are called multipotent. These stromal cells can be derived from almost every tissue of the human body. Depending on their origin and function, they are named MSCs, HSCs or neural stromal cells. Due to their quiescent status and their non-specialised appearance in tissues, adult-derived stromal cells are difficult to detect. While most organs underlie continuous reorganisation and renewal, stromal cells remain non-dividing. Once tissue has to be regenerated, they are activated by several stress-responsive pathways and start differentiating.

Although self-renewal capacity is one major aspect of stromal cells, this self-renewal seems to be limited by several factors. Aging and several diseases, with diabetes the most prevalent, have shown to significantly diminish adipose-derived MSC (ASC) function and regenerative capacity. Thereby some but not all subpopulations are depleted by the continuous aging process (14).

Determining specific stromal cells is mainly based on their surface marker profile, and the cluster of differentiation (CD) markers play an essential role. Specific cells have been found to show specific marker profiles. During the differentiation process, this marker profile changes (15, 16).

Although experiments have been carried out for over 30 years, there is still an ongoing discussion about the optimal surface marker selection to specifically target the cells of interest. For example, while many groups define MSCs only by using a selection based on three CD markers, others suggest an identification of at least six CD markers to verify MSC status (14, 17).

Other studies include plastic adherence as ‘specific stromal cell markers’ (18). Given all facts, appropriate selection and isolation of cells is broadly disputed, but one of the most important topics in stromal cell biology. Using a too-wide selection pattern can destroy work by including or targeting wrong cells. Too-specific isolation can eliminate specific subpopulations that might be important for findings.

To sum up, all stromal cells are not equal. Many essential aspects such as the donor tissue, isolation method and defined marker profile can determine the success of research and significantly change the cells of interest.

1.1.1 Mesenchymal Stromal Cells

MSCs today are the most important cell type for regenerative medicine. They can fully restore a range of tissues, including fat, bone marrow (=BM), muscle and liver among the most important. Although all of these tissues contain an appropriate number of stromal cells, mainly BM-derived (BM-MSCs) or ASCs are used for experimental approaches.

For specifically isolating MSCs, selection based on surface markers or on plastic adherence are reliable methods suitable for different approaches (18). Plastic adherence provides the possibility to culture the cells under specific conditions. It does not require specific equipment and thereby can be performed by trypsinisation and washing steps. Explant cultures are one of the initial and simplest methods of cell isolation and cell culture (see Chapter 1.1.3). By simply adding small, thin pieces of tissue to a cell-culture plate, plastic-adherent cells such as MSCs grow out of the tissue. This approach was the one initially used and is still performed today (19). An alternative is the pre-experimental digestion of the extracellular matrix (=ECM). By using collagenase, the ECM is broken and included cells are released. This cellular pellet can then be cultivated. By performing several washing-steps the days after seeding, non–plastic adherent cells can be removed.

When exact cell-profiling is aimed and cellular transcription patterns need to be explored, these approaches might be misleading. Every cultivation step leads to changes in the transcriptional pattern and thereby to changes in profiling. Therefore, transcriptional analysis has to be performed as soon as possible after harvesting.

Surface marker–based selection can be carried out either by fluorescence-activated cell sorting (FACS) or by magnetic-activated cell sorting (MACS).

1.1.1.1 *Regenerative Potential*

The regenerative potential of MSCs is undoubted. They can be either locally or systemically distributed, dependent on the mechanism and place of action.

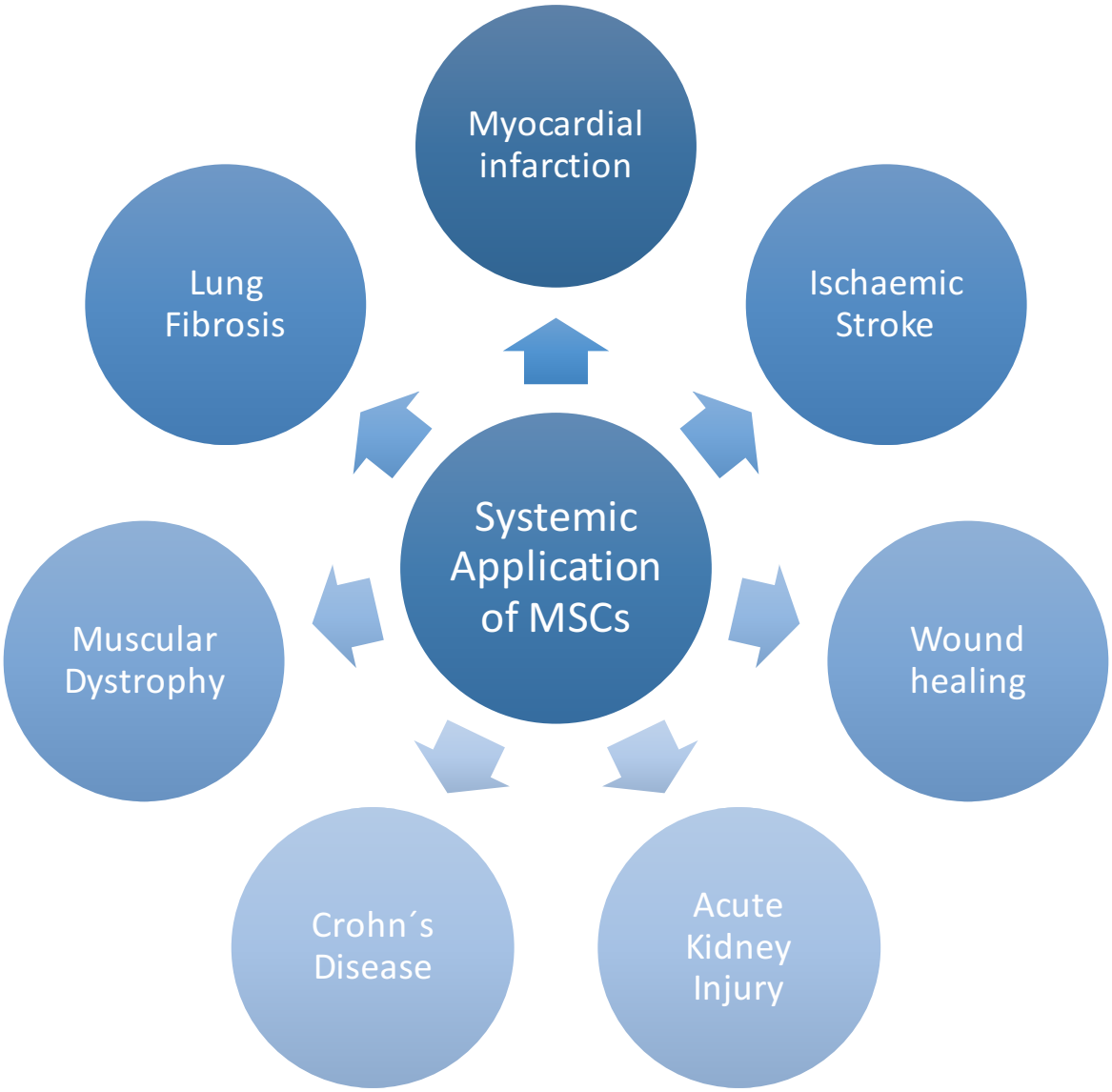


Figure 2. Systemic application of MSCs can serve cell-based therapy for several diseases. (20-25)

1.1.1.1.1 Systemic Applications

The systemic use of MSCs relies on their secretion of regenerative and anti-inflammatory cytokines, their ability to differentiate into almost every tissue possible, and their ability to automatically migrate towards injured tissue. These abilities make MSC-based therapies a promising and effective regenerative tool.

1.1.1.1.2 Homing Ability of Mesenchymal Stromal cells

Due to their abilities to differentiate into multiple types of tissues and to secrete regenerative cytokines, MSCs are used to enhance wound healing and restore cartilage, muscle and soft tissue. Besides these well-known characteristics, their 'homing' potential can be used to partially restore function after ischaemic myocardial infarction, lung fibrosis, muscular dystrophy or neurological strokes (21-25). Homing refers to the migration of MSCs towards areas where their regenerative potential is needed (22). Host MSCs may be activated and mobilised into circulation after injury (26, 27).

Because these cells are highly likely to change their marker profile and are circulating only for several minutes, locating and harvesting MSCs from peripheral blood is difficult (28, 29). Crucial for homing of MSCs to myocardial ischaemic areas is mainly, but not only, activation of key cytokines and surface markers, most importantly SDF-1, its corresponding CXC-chemokine receptor 4 (CXCR-4), and VLA-4 (30, 31). Others, such as the stromal cell factor-receptor (SCF-Kit) and MCP-1 have been proven to induce MSC migration to the central nervous system (32). Several studies suggest that migration of MSCs to traumatised tissue is induced by a specific pattern of these homing factors.

The molecular mechanism of this phenomenon has been described in recent years. As soon as cells undergo necrosis, factors such as the high mobility group box 1 (HMGB-1), SDF-1 and tumour necrosis factor-alpha (TNF-α) are secreted. These proteins bind to specific 'homing receptors' on circulating progenitor cells, inducing a kinase complex and activating nuclear factor kappa B (NF-κB). This leads to nuclear

internalisation of NF-κB, resulting in transcription of genes that are pivotal for homing (33, 34). Adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) are expressed on endothelial cells. Integrins on the surface of MSCs, in particular integrin-α4 and β1, recognise these molecules, leading to MSC–endothelial cell adhesion (34).

When cultivated and expanded in vitro, MSCs downregulate expression of some of these factors (35). Re-inducing some of these markers and thereby enhancing their homing capacity has been the focus of many studies in the last decades (35-38) Viral transfection of CXCR-4 has been the most promising approach up to now (39). Several studies have shown beneficial effects of modified MSC application, not only on ischaemic strokes (40, 41) or myocardial infarctions (42) but also on wound healing (43, 44) and acute kidney injury (45); Figure 7). The injury-related homing ability of MSCs leads to significant reduction of infarction area and provides a promising cell-based treatment method.

1.1.1.1.3 Immunosuppression of Mesenchymal Stromal cells

MSCs have been shown to lack immunogenicity, providing the possibility of allogenic instead of autologous transplantation, as well as immunosuppressive potential (46). This immunosuppressive potential is a combination of several different interactions between MSCs and immune cells. Studies have shown that MSCs suppress the proliferation of cytotoxic T-lymphocytes (47, 48). MSCs can further interfere with the maturation and differentiation of dendritic cells and thereby inhibit antigen presentation (49). Additionally, MSCs interact with B-cells and regulate the production, activation and secretion of specific immunoglobulins, as well as the ability of B-cells to differentiate into plasma cells (50). By either interleukin-2 (IL-2) or IL-17 regulated pathways, MSCs inhibit the proliferation of natural killer cells and thereby limit their cytotoxic potential (51, 52). These anti-inflammatory processes and interactions can be potentially used to treat severe autoimmune diseases such as Crohn's disease or graft versus host reaction in transplant patients (20).

1.1.1.2 *Local Application*

The local application of MSCs is regularly used and is mainly based on the secretion of regenerative and anti-inflammatory key cytokines. These factors lead to the formation of new tissue and enhance cell division and angiogenesis (53-55). Asymmetric division of applied MSCs can additionally form fully functional tissue including all required cell types (56). This provides many novel therapy modalities, especially for the reconstruction of soft tissue (57), bone (58), cartilage (59) and cornea (60).

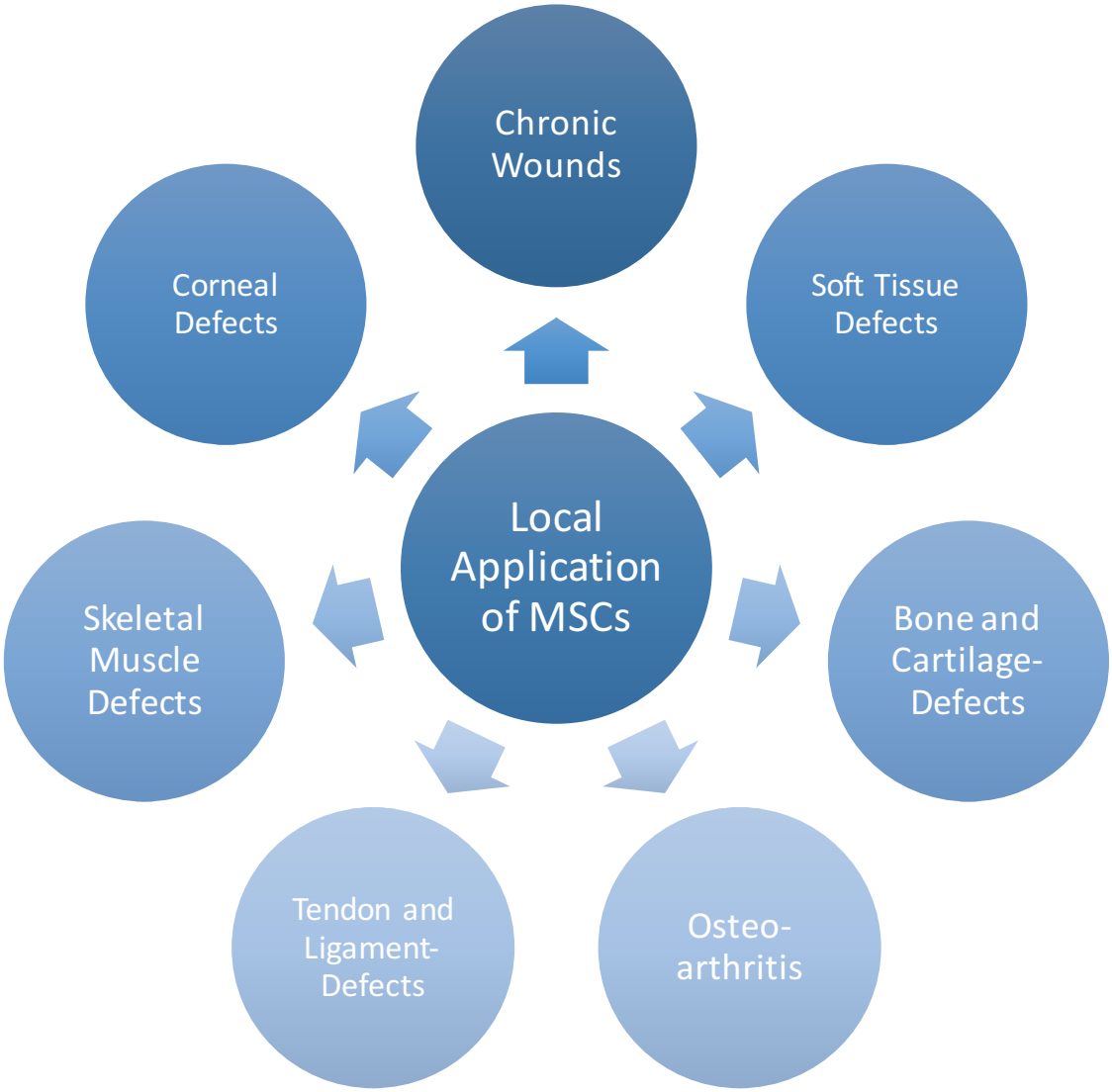


Figure 3. Several possibilities exist for local clinical application of MSCs. (57-60)

Chronic wounds are a major personal and economic burden. Beside severe restrictions in the quality of life of patients, non-healing wounds lead to annual costs of approximately US$25 billion (61). Effective but also cheap approaches are therefore needed. Conventional methods to treat chronic wounds are limited. Currently, they include general measures such as good diabetic and blood pressure control, special dressings, appropriate surgical care, radical debridement and vacuum-assisted therapy (62). Although all of these approaches are beneficial for many patients, many wounds cannot be treated sufficiently. Therefore, clinical trials are being carried out to develop new treatment modalities. All of these studies such as bioactive scaffolds that not only cover the wound but stimulate formation of granulation tissue, interfering RNA (i-RNA) that can lead to silencing or enhancing specific pathways or recombinant factors that promote specific wound healing pathways, have not shown great success up to now and are highlight the need for novel and effective tools to treat chronic wounds.

MSCs have the potential to fill this gap and serve as an autologous cell-based therapy to enhance wound healing (4). As we have previously stated, ‘growth factors released from stromal cells stimulate local cell proliferation and migration, increased angiogenesis, organised ECM production, and antimicrobial activity’ (63). Together with the differentiation capacity of MSCs, these abilities are essential for tissue regeneration. Additionally, MSCs have been shown to modulate the immune system and recruit progenitor cells, leading to improved wound closure time and reduced scar formation (64, 65).

Unlike all other approaches, MSC-based therapy is a holistic approach that might survive the step into clinical practice. The autologous origin of MSCs is essential for its usage, but might represent a major limitation and a significant translational challenge.

1.1.1.3 Sources and Harvesting

1.1.1.3.1 Bone Marrow–Derived Stromal Cells

Harvesting murine BM and isolation of MSCs from it has long been problematic. Unlike in other species, simple plastic adherence is not a valuable marker for stemness (66). Many other cell types, such as immune cells, appear in cultures. Therefore, specific techniques to specifically target MSCs have been invented.

A specific pattern of medium changes and trypsinisation steps can purify the cell culture and eliminate immune cells and debris (67). Alternatively, immunodepletion can eliminate cell types that present non-desired surface markers (68), given that this method has the potential to interact with the cell cycle of non-depleted cells and alter their transcription and proliferation pattern.

Recognition of surface antigens and thereby sorting cells underlies the already acknowledged problem of identifying and verifying a broadly accepted marker profile (68, 69). Again, it has to be concluded that all approaches provide great possibilities, but the appropriate method must be chosen in accordance with the whole experimental plan.

To isolate BM from humans (h-BMSCs), an aspiration has to be performed. Unlike lipoaspiration, this procedure has no potential benefit for the patient, but is predictable and standardised with a low complication rate. While in the neonate, BM can be found in every bone, in the mature human only tubular bones such as the femur or the iliac crest can serve as donor sites (70). Over the lifespan, BM is reduced by 50% and replaced by adipose tissue. BM harvesting is an easy and safe procedure that is indicated not only for experimental purposes or to harvest h-BMSCs but also for diagnosing and verifying haematological diseases (71).
In clinical practice, BM aspiration is usually performed by puncturing the posterior iliac crest. In obese or immobile patients this is sometimes challenging; for these patients,

the sternum is a possible alternative (72). Local anaesthesia is most likely sufficient (73). By puncturing the bone with a special needle, BM can be harvested and then used for histological examination in case of leukaemia, experimental setups or donation purposes.

1.1.1.4 Adipose-Derived Stromal cells

1.1.1.4.1 Problems in harvesting murine ASCs

Adipose tissue is by far the most likely used tissue for stromal cell harvesting. Because the procedure is easy and harmless, and can be performed using local or general anaesthesia, these cells are a unique cell population that has successfully overcome the substantial gap in clinical practice.

Isolation for clinical purposes cannot be compared to isolation for experimental purposes. While experimental settings require specifically targeted cell populations, a clinical on-hand application has to be easier to handle and completely sterile, and can include several populations. Therefore, several techniques that are beneficial for preclinical trials are not translatable to clinical practice. For example, FACS is a valuable method in experimental non-clinical trials. However, due to sterility and hygiene standards, complex interfaces and commercialisation hurdles, translation is still not possible.

Many in vitro and in vivo experiments are based on murine ASCs. These differ from human ASCs in their proliferation capacity and their stability when being cultivated (74). Another approach is the use of human stromal cells in an immunocompromised mouse model. Thereby cellular function displays human characteristics, but nevertheless, specific immunospecific reactions cannot be investigated. Therefore, direct translation of experimental findings to humans is difficult. The perfect model still has to be found.

Additionally, the harvesting procedure needs to be different for murine models and humans. Because mice have limited adipose tissue, liposuction cannot be performed.

This harvesting step has the potential to change the profile of cells. For experimental purposes, mice with genetically identical profiles have to be used. Harvesting is performed by dissecting dead mice and carefully isolating fat deposits. Most commonly, parascapular, inguinal and omental fat depots are used. Although this is the standard, we have successfully shown that this approach is potentially misleading (75).We harvested adipose tissue from the visceral and subcutaneous compartments of C57 BL mice. By using a previously described method, we analysed 96 genes that play an essential role in tissue remodelling, neovascularisation and regeneration (76). Additionally, we used a standardised murine model that effectively simulates human wound healing to investigate wound healing capacity in an in-vivo model.

1.1.1.4.2 Differences in harvesting Human-Derived Adipose Stromal cells

When human ASCs are used for either experimental or translational regenerative purposes, standardisation of every harvesting step is critical. Human ASCs can be harvested by performing abdominoplasty or liposuction. While abdominoplasty is a standardised procedure, based on mechanical isolation, which does not really harm the ASC, liposuction is more complex and depends on a range of factors, equipment and timepoints.

To obtain ASCs for experimental purposes, fat has to be digested using enzymes such as collagenases as well as several centrifugation steps. This guarantees separation of the fatty, watery and cellular layers. The cellular pellet that can be obtained by this procedure is called the stromal vascular fraction (SVF) and contains ASCs, fibroblasts, endothelial cells, monocytes, macrophages, erythrocytes and haematopoietic progenitor cells (77, 78). To separate ASCs from other cells, separation based on either plastic adherence or surface markers can be performed.

Figure 4. Process of isolation of adipose-derived mesenchymal stromal cells (ASCs). Initially, fat tissue is obtained by performing liposuction or abdominoplasty. By digesting extracellular structures and breaking or dissolving adipose tissue, a cellular pellet is obtained. This pellet is called the stromal vascular fraction (SVF).

Today, liposuction is easy to perform, has almost no side effects and has a wide range of possible applications. Cellular mechanisms and characteristics have played only a limited role in these trends and developments, while clinical aspects have been of major importance.

All new inventions are improved to minimise the length of surgery and the local side effects such as bleeding, hematoma formation or nerve injuries (79, 80).Essential for the regenerative use of fat is maximising the regenerative potential of lipoaspirate by improving the effective number of ASCs. This cell population affects the viability of surrounding cells (yield) by producing pro-angiogenetic and anti-apoptotic proteins. Additionally, hypoxic signals and cell stress lead to differentiation of ASCs into adipose tissue.

Cellular viability and preclinical outcome of liposuction depend on three main factors: the appropriate tumescent solution, the right cannula diameter and suction pressure, and the liposuction device. Simple steps such as changes in the composition of tumescent solution, cannula diameter or suction pressure change the ASC viability and regenerative potential.

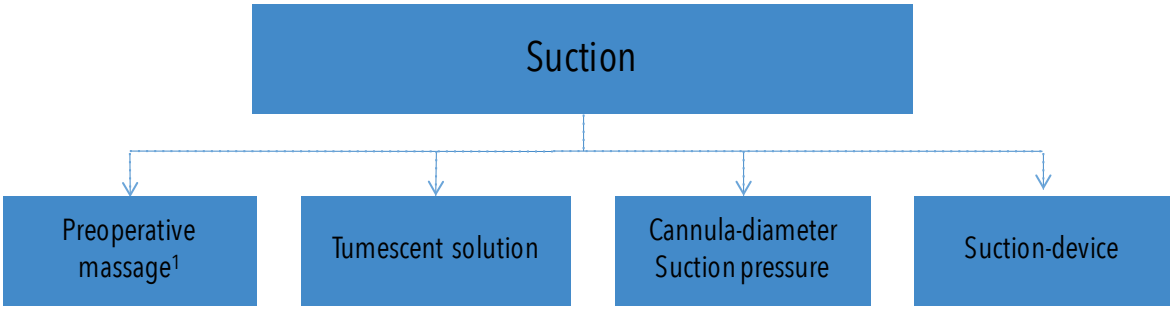


Figure 5. Factors in liposuction that can interfere with the outcome of studies. When performing liposuction, a tumescent solution is initially injected. Additives such as lidocaine or epinephrine are added to the tumescent solution. This reduces postoperative pain but can enhance the apoptosis rate of ASCs and within the SVF (81-84) (85, 86). 1Preoperative massage has only historical interest and is rarely performed today (87). The appropriate cannula diameter must be chosen and the suction pressure adapted. High suction pressure and a high cannula diameter lead to faster liposuction but might also increase the risk for postoperative asymmetries in skin structure. The appropriate suction device can enhance viability and regenerative potential of harvested ASCs.

1.1.1.4.2.1 Suction Device

Due to technical developments within the field of liposuction, many devices, that are based on different techniques are currently available on the market. While suction-assisted liposuction (SAL) is a valuable method to obtain viable ASCs and adipocytes, newer methods such as ultrasound-assisted 'vibration amplification of sound energy at resonance' (VASER) liposuction aim to positively affect skin structure and offer the possibility of high definition (88).

While SAL was the first method and remains the gold standard, novel techniques attempt to break extracellular structures by alternative methods. For example, power-assisted liposuction (PAL) uses vibrations to release adipocytes from their cellular compound (89). Ultrasound has been re-invented as a method to destroy fibrous strains, and ultrasound-assisted liposuction (UAL) has been developed (90).

These three techniques are currently the most popular. In 2011, 51.4% of liposuctions in the USA were performed using a SAL device, 23% with PAL and 20.9% with UAL (91). This suggests a shift towards novel suction devices. Other techniques such as water-assisted liposuction (WAL), laser-assisted liposuction (LAL), radiofrequency-assisted liposuction (RFAL) and tissue liquefaction have been adopted in clinics but are rarely used (92).

The effects of harvesting techniques on ASC survival, cytokine secretion and the clinical long-term retention of fat grafts have only been partially investigated up to now. Therefore, it is essential to compare every novel technique with both gold-standard suction-harvested and mechanically isolated ASCs to provide guidance for clinical grafting.

Harvesting methods do not only rely on different mechanisms to break the ECM; they further interact with the tissue-based ASCs. For example, mechanical stimuli can lead to differentiation of ASCs and enhanced secretion of specific factors such as α-SMA (93, 94). Low-intensity ultrasound waves have been shown to enhance adipogenesis of ASCs and might positively affect their chondrogenic differentiation capacity (95, 96). Low-level laser radiation can enhance the viability and proliferation of ASCs (97). Several stimuli can effectively separate cells from the ECM, but simultaneously interact with cells, and thereby have the potential to influence viability and regenerative capacity. Therefore, continuously comparing novel techniques is essential.

1.1.1.4.2.1.1 Suction-Assisted Liposuction

The gold standard of liposuction, SAL has been compared to tissue excision regarding regenerative potential. Although research has found SAL-harvested cell suspension showed fewer ASCs than abdominoplasty samples, their viability and adipogenic differentiation capacity was comparable, leading to no differences in an in vivo wound healing assay (98). Abdominoplasty and SAL served as control groups for several studies aiming to investigate the function and potential of novel harvesting techniques (99, 100).

1.1.1.4.2.1.2 Ultrasound-Assisted Liposuction

Although UAL is a frequently used liposuction technique, its regenerative potential has been under-investigated. UAL relies on the conversion of electronic energy into waves that lead to thermal and mechanical forces and thereby to destruction of the ECM and comminution of fat. It was developed and popularised by Zocchi as ‘ultrasonic liposculpturing’ in 1992 (101). Initial infiltration of tumescent solution leads to

homogeneous and more effective forwarding of waves (102). The non-acoustic cyclic waves generated by the UAL device lead to negative and positive pressure on cells and thereby, dependent on the cycle, to compression and rarefaction of cells. Due to their high molecular cohesion, dense tissues such as bone, cartilage and muscle are not affected by low ultrasound waves (101).

UAL has been modified over time, leading to three generations of devices. These generations can be distinguished by the frequency of the waves and by their cannula design. While initial approaches such as the SMEI device used a frequency of 17–20 kHz, LYSONIX 2000 (LYSONIX Inc., Carpentera, CA, USA), a second-generation UAL device, used 22.5-kHz waves, and third-generation VASER (Solta Medical, Hayward, CA, USA) uses 36 kHz. Additionally, the diameter of probes has steadily reduced, from 6-mm to 2.2-mm cannulas in the third generation. Smaller cannulas decrease the risk of uneven bumps and therefore optimise outcomes.

Many studies have described a positive effect of low-level ultrasound with a frequency of <100 kHz on the regeneration of bone, tendon and retinal cells (103-107). High-intensity focused ultrasound (HIFU) has been successfully used to selectively destroy cancer. Waves with a frequency of 1.5 MHz are lead to coagulative necrosis in a predefined selective zone (102, 108). These findings show that the effect of ultrasound-based therapies is significantly determined by their frequency. Different frequencies lead to different molecular mechanisms, stimulating cells in the case of low-intensity ultrasound, but can lead to selective necrosis in the case of HIFU.

The regenerative potential of second-generation UAL–harvested ASCs has been described as inferior to SAL or resection samples (109). However, this conclusion has been drawn based on in vitro studies investigating the quantity of ASCs within the probes as well as their proliferation capacity. A lack of in vivo studies and therefore inability to assess the regenerative and therapeutic potential is a major limitation.

The third generation has not been evaluated before. Therefore, we compared a novel harvesting method, the third generation of UAL, with second-generation UAL samples (99).

1.2 Translational Challenges and Concerns

Although stromal cells have recently been seen as the future of regenerative medicine, negative aspects of autologous stromal cell therapies have also appeared and are hindering clinical translation for many applications (4).

1.2.1 Carcinogenic Potential

The fear that the application of stromal cells might be associated with enhanced carcinogenicity can significantly diminish their use. This issue has been widely discussed in relation to breast reconstruction. Reconstruction of breast tissue that has been removed because of cancer can be seen as critical. Due to their regenerative mechanisms, MSCs could potentially lead to breast cancer recurrence (110). MSCs have also been reported to home to specific types of cancer. This homing might be explained by upregulation of some pathways that are causal for stress-responsive homing of MSCs (see Chapter 2.1.5; (111, 112). Migration of MSCs toward cancer might lead to promotion of metastasis, increased invasiveness and poor clinical prognosis (113, 114).

The homing ability of MSCs might negatively affect cancer prognosis, but could also provide many possibilities. By ex vivo modification of MSCs and then re-applying them to the bloodstream, they can act as cell-based anti-cancer therapeutics (115, 116). It should be mentioned that there is currently no clinical evidence that fat grafting can cause breast cancer relapse (117-119), but mechanistic studies based on co-culture and transwell assays show an enhancing paracrine effect of ASCs on breast cancer cells (118, 120).

In vitro expansion of MSCs can also lead to chromosomal aberrations and therefore the potential to grow independently and autonomous of external signalling – characteristics of tumorous tissue (121, 122). However, again, this cancerogenic potential has only been proven in vitro. Further studies based on more complex assays are urgently needed to assess the safety of clinical use of externally expanded MSCs (46).

1.2.2 Aging

While the utility of MSCs for age-related morbidities such as chronic wounds is undoubted, aging can also be seen as the main restrictor for MSC function. During the lifespan, influences such as radiation, cell stress and accumulation of reactive oxygen species result in loss of telomeres, DNA modification, and thereby aging of MSCs.
Several studies have focused on age-related changes to MSCs. While the osteogenic differentiation potential has been found to be reduced in aged MSCs, the effect of aging on adipogenic and chondrogenic differentiation capacity is disputed (123-125). The migration ability of aged MSCs is significantly reduced when compared to young ones (126). Remarkably, not only MSCs harvested from older donors but also MSCs cultivated for many passages show these migration characteristics. Further studies have shown decreased homing marker and receptor expression, suggesting reduced homing ability of aged MSCs (126). The effect of aging on the immunomodulatory ability of MSCs has not yet been investigated (125).
Although most of these age-related mechanisms have been described in vitro and was verified in an in vivo setting, translation into clinical practice remains difficult (125). For example, the use of MSCs has proven effective for myocardial infarction. Aged MSCs have shown limited outcomes in a rat model (127). Clinical data comparing MSCs of older and younger donors are missing but essential for patients.
We harvested BM-MSCs from younger (24 ± 8.3 years) and older donors (73 ± 3.7 years) and performed a microfluidic-based single-cell gene expression analysis. We included 96 genes essential for the main pillars of wound healing – tissue regeneration,

migration, stemness and proliferation – and found significant changes between groups. Additionally, we used a diabetic wound healing model to show the diminished age-related wound healing capacity of MSCs. We showed a change in subpopulations that was clearly connected to the age of the donor and concluded that these changes in subpopulation dynamics might radically limit the usage of MSC-based therapies in older patients (128). These results confirmed the previous findings of Duscher et al. (129), who used adipose-derived murine stromal cells and also found a selective age-related depletion of pro-vasculogenic cells within the MSCs.

Although MSCs have been proven to positively affect a broad range of diseases, these diseases are most likely more prevalent in the older population. Therefore, the loss of regenerative function of MSCs associated with selective age-related depletion of regenerative subpopulations, in combination with the need for autologous donation, can radically limit the clinical use of MSC-based approaches.

1.2.3 Comorbidities

Besides aging, several comorbidities have been shown to diminish the functionality of MSCs. The most prevalent and severe, diabetes, is characterised by permanently elevated glucose levels. These glucose levels lead to non-enzymatic glycation of proteins and formation of advanced glycation end products (AGEs;(130). These AGEs bind to a specific receptor, the RAGE. A specific cascade induces the production of reactive oxygen species. Acting via this pathway, AGEs can be seen as promotors of several conditions such as microvascular diseases, Alzheimer disease and rheumatoid arthritis (131).

Several studies have suggested a diminished potential of MSCs harvested from diabetic donors. Cianfarani et al. showed a significant reduction of stromal cells and limited proliferation and migration capacity, as well as reduced applicability for autologous wound therapy (132). These effects have further been investigated in a murine wound healing model. Other studies have investigated these effects on a single-cell level, concluding that they might be caused by pro-vasculogenic alteration of specific subpopulations (133).

Diabetes combined with the accumulation of AGEs leads to diminished regenerative function of MSCs and thereby limited clinical applicability. This should be kept in mind when translating novel therapies from bench to bedside. Success or failure depends not only on the approach itself but more importantly on the patients included.

1.3 Enhancing MSC Functionality for Soft Tissue Reconstruction

MSCs, whether derived from BM or adipose tissue, represent a promising approach for many diseases and morbidities. Although their effectiveness has been proven for multiple approaches, clinical translation is limited by severe comorbidities. Therefore, methods to enhance their regenerative potential are urgently needed to drive translation. To do so, preconditioning cells with certain molecules or under specific conditions can be performed. Additionally, carrier substances or scaffolds can be used to optimise cell surroundings and thereby prolong the MSC lifespan, or specific subpopulations can be selected to only use cells with the highest regenerative potential.

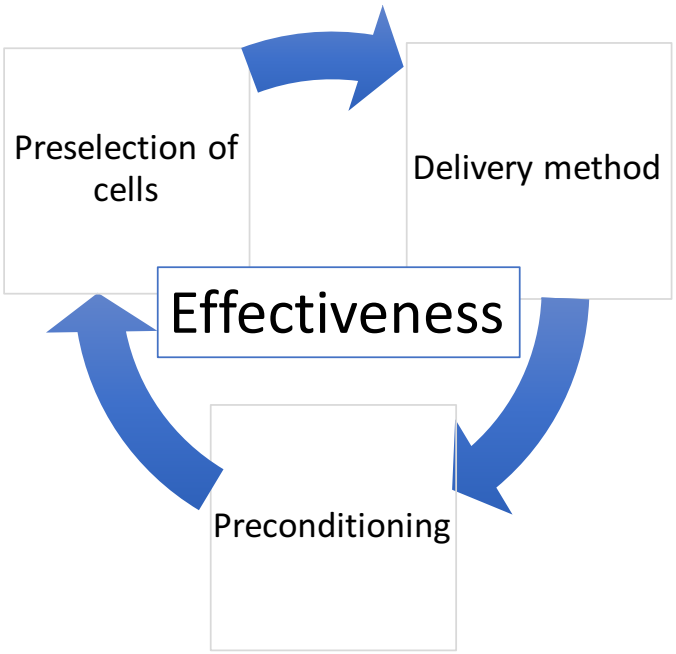


Figure 6. Methods to enhance the effectiveness of cell-based therapies. Preselection of cells offers the possibility to distinguish between specific subpopulations, that are more effective for tissue regeneration. By modification of the delivery method, the wound environment can be modified and by preconditioning of cells, specific pathways, that are essentiall for soft tissue reconstruction can be upregulated.

1.3.1 Preconditioning

Preconditioning of cells during expansion by using several stimuli to enhance their efficacy has been broadly investigated within regenerative medicine. While several studies have used conditions such as anoxia or hypoxia, others have tried to stimulate pathways by using specific factors or drugs. Waves such as ultrasound can also be used to enhance differentiation capacity towards specific lineages.

One study used hypoxia (oxygen levels of 0.5%, 1% and 3%) to cultivate MSCs. Proangiogenic and survival markers such as hypoxia-inducible factor-1 (HIF-1) alpha, VEGF, CXCR-4 and c-Met were shown to be enhanced in hypoxic-cultivated MSCs (134). Additionally, study of these effects in rat and mouse models led to the suggestion that hypoxia preconditioning enhances the homing capacity and efficacy of MSCs for treatment of myocardial infarction, cerebral stroke and brain injuries (135-139). Cultivation of MSCs under hypoxic conditions can also lead to determination of their lineage differentiation. Volkmer et al. showed enhanced osteogenic differentiation capacity of MSCs when cultivated with 2% oxygen (140).

Preconditioning of cells can also be performed by using specific molecules. Lipopolysaccharides have shown to interact with the toll-like receptor (TLR-4) pathway and thereby to have a cardioprotective effect (141). TGF alpha has further been shown to be crucial for the secretion of pro-regenerative cytokines, with VEGF the most important. Preconditioning with this factor also has the potential to improve MSC efficacy in tissue regeneration (142). Others factors such as insulin-like growth factor-1 (IGF-1) and fibroblast growth factor-1 (FGF-1) have further been shown to positively affect MSC function (143). Other molecules block the prolyl hydroxylase domain (PHD) protein, thereby stimulating the HIF-1 alpha pathway (144). This is an efficient and elegant way of mimicking hypoxia. Iron chelators, such as deferoxamine and deferiprone, which are currently also in clinical use, can also enhance the HIF-1 alpha pathway by inhibiting PHD (145). While these molecules can be used to enhance wound healing or for aesthetic purposes such as skin rejuvenation, their effectiveness for preconditioning cells is currently being investigated by our group (145, 146).

Ultrasound can be used not only for harvesting purposes but also to enhance the chondrogenic differentiation potential of these cells. This approach, using low-level ultrasound waves, might not only be beneficial for preconditioning but also allow post-injective modification of MSCs, an approach that is urgently needed in the case of cartilage repair (147-149)

1.3.2 Preselection of Cells

Along with the success of MSC-based therapies, limitations such as aging and diabetes have appeared. By investigating these limitations in depth, it has become obvious that not all diabetic or aged MSCs lack regenerative ability, but only some subpopulations are partially depleted (128, 129, 133, 150).
MSCs that show a higher potential for regeneration (such as higher expression of regenerative pathways or higher secretion of pro-regenerative cytokines) can be identified and matched with certain surface markers. By sorting pre-operatively for these markers, only cells that are capable of regeneration can be used for MSC-based therapies.
Although preselection of cells would be highly effective and promising, technology is still lacking (151). Because there is currently no adequate, effective and sterile mechanism for clinical cell sorting available, preselection of cells remains experimental. While MACS is already in clinical use, but limited to single markers, no real high-throughput system allows taking advantage of the findings of Duscher et al. (129, 133) Solving this issue has the potential to enhance the efficacy of MSC-based cell therapies.

1.3.3 Modification of Delivery Method

When being delivered, MSCs likely face a harsh niche, characterised by inflammation and immune cells, particularly in chronic wounds. Because they are reachable and associated with enormous economic costs, they have become an outstanding example of scaffold development. Therefore, different carrier substances have been developed

to improve cell surroundings, enhance ingrowth or viability of ASCs, or restore their regenerative potential and thereby overcome translational hurdles.

It should be mentioned that these scaffolds do not only serve as carrier vehicles. By mimicking cell surroundings and the ECM, they have the potential to significantly change differentiation, migration capacity and intracellular mechanisms (152-154). Therefore, the term 'delivery method' seems hardly sufficient.

Gelatine-based hydrogels combined with ASCs have been shown to positively affect wound healing ability in vitro and in vivo (155). Remarkably, this was further proven in a large animal study.

Other carrier substances for MSCs are based on silk fibroin and chitosan (156). Before use, these scaffolds have to be seeded with MSCs ex vivo. Although initial approaches have shown promising results, ex vivo cultivation and insecurity about the potential changes of MSC characteristics might have hindered clinical translation.

Similarly, polycaprolactone, a biomaterial used for manufacturing resorbable threads, can be electrospun to create a nanoporous scaffold ideal for cell adhesion and delivery (157). Although this delivery vehicle is broadly applicable, its use in bone (158) and cartilage formation (159) has been investigated in particular.

Alginate has also been successfully proven as a suitable material for soft tissue reconstruction and tissue replacement (160). Its use in bone formation (161), cartilage reconstruction (162) and regeneration of neural cells (163) has been proven extensively. Wound healing might be a novel approach suitable for the characteristics of alginate.

Collagen was one of the first scaffolds tested for cell delivery. Collagen (mainly types I and III) is a main building block of the ECM. Synthetically manufactured collagen sheets are used in clinical practice for many purposes. Collagen is biodegradable, biocompatible and readily available, and can be crosslinked with multiple polymers, making it the ideal substitute for regeneration of tissue (164). It has been popularised as an artificial skin substitute (165) to treat thermal injuries and prevent scarring (166). Furthermore, collagen has been commercialised and can be used as a nerve conduit after peripheral nerve injury (167). Additional studies have shown beneficial effects on

MSC-based chondrogenesis (168). Collagen sheets can easily be seeded with cells, whether fibroblasts (169), keratinocytes (170) or MSCs (171). Again, the need for external expansion of cells and seeding of scaffolds represents a major translational limitation.

Fibrin, a molecule essential for homeostasis in the human body, can also be used as a carrier vehicle. Fibrin is already in widespread use in the clinics as a haemostatic agent (172, 173). Its properties make fibrin an indispensable drug for surgeons. Due to its molecular structure, fibrin is usually used as a gel, which can be easily mixed with cells of every kind. It is currently used to deliver fibroblasts in combination with growth factors (174). ASCs have also been mixed with fibrin (175). Zimmerlin et al. investigated the effect of fibrin on ASC in vitro by performing tubule formation assays, demonstrating its neoangiogenic capacity (175). However, the lack of in vivo and translational data are limitations.

We therefore used a commercially available fibrin glue in combination with human-derived ASCs to drive translation. We investigated the regenerative potential in vitro and its efficacy to close diabetic wounds in a murine model (176).

2 Summary

Adipose derived Stromal cells represent a novel and effective method within the field of regenerative medicine. They can be used to reconstruct soft tissue (57), bone (58), cartilage (59) or to accelerate wound healing. Nevertheless, preclinical studies show great variability regarding to the harvesting process. While some studies use murine ASCs in immunopotent models, others are using human ASCs, obtained by liposuction in immunocompromised mouse models. The harvesting process and slight changes, such as differences in donor region or changes in liposuction device could have significant impact on experimental outcome.
Therefore, we investigated changes in the harvesting process and their effects on ASC function. Additionally we used a Fibrin based scaffold, that has the potential to improve cell-viability and enhance efficacy of ASCs.

2.1 Single-Cell Gene Expression Analysis and Evaluation of the Therapeutic Function of Murine Adipose-Derived Stromal Cells (ASCs) from the Subcutaneous and Visceral Compartment

Many in vitro and in vivo experiments are based on murine ASCs. Due to the inability to perform liposuction in mice, harvesting procedure needs to be different for murine models. Most commonly, parascapular, inguinal and omental fat depots are used. While in clinical routine only subcutaneous fat is used, visceral origin might change transcriptional profile as well as experimental characteristics of fat cells and ASCs.

Therefore, we investigated differences between the subcutaneous and visceral fat compartments, screening for the 96 most important genes for tissue regeneration and using an in-vivo murine wound healing model (75).

We showed different genetic patterns and significant differences in RNA profiles of subcutaneous murine ASCs (S-ASCs) and visceral ASCs (V-ASCs) by using an already established method to screen the 96 most relevant genes for tissue regeneration and wound healing. When trying to prove these phenomena on a protein

level in vivo, we found no differences in the regenerative potential of S-ASCs and V-ASCs. Therefore, we concluded that although S-ASCs and V-ASCs follow different transcriptional patterns, they both have the ability to lead to effective wound closure (75).

Although, ASCs obtained from both origins showed similar efficacy to accelerate wound healing, underlaying pathways are different.

2.2 Differences in the regenerative potential of ASCs, derived by different liposuction methods

While a liposuction is easy to perform, has almost no side effects and has a wide range of possible applications, cellular mechanisms and characteristics of harvesting methods have played only a limited role. Harvesting methods do not only rely on different mechanisms to break the ECM - they interact with ASCs and have the potential to change their genetic fingerprint and regenerative potential.

UAL represents an upcoming technique with a market-share of 20.9% (91). Nevertheless, its regenerative potential has been under-investigated up to now. Therefore, we compared the third generation of UAL, with second-generation UAL samples and the gold-standard (99). Additionally, we compared the genetic profiles of third-generation UAL (VASER) and abdominoplasty samples regarding their regenerative capacity (177).

We measured viability and adipogenic and osteogenic differentiation capacity, and found comparable viability and adipogenic differentiation capacity, but enhanced osteogenic differentiation ability in VASER-assisted liposuction samples. Additionally, we used a murine in vivo wound-healing model to investigate the regenerative potential of ASCs. Both second- and third generation-harvested ASCs led to faster wound healing compared to non-treated wounds. We found no significant differences in wound healing kinetics between second- and third generation-harvested UAL samples. When performing histological analysis of closed wounds, CD31 levels, representing neovascularisation, were significantly enhanced in both UAL-harvested samples.

When comparing UAL (VASER), SAL and abdominoplasty samples regarding their cytokine profile, we found no differences in HGF, b-FGF, SDF-1 and VEGF, but significantly enhanced MCP-1 levels in UAL samples, a crucial factor for wound healing and scar formation.

We found significant differences of ASCs obtained by different harvesting methods. Nevertheless, these findings still have to be proven in a clinical setting.

2.3 Optimizing the delivery method of ASCs

When being delivered, MSCs likely face a harsh niche, characterised by inflammation and immune cells, particularly in chronic wounds. Several carrier substances have been developed to improve cell surroundings, to enhance ingrowth or viability of ASCs or to enhance their effectiveness. Many scaffolds have shown success in preclinical trials but are hindered from clinical translation (156, 160, 164).
We therefore used a commercially available fibrin glue in combination with human-derived ASCs to drive translation. We investigated the regenerative potential in vitro and its efficacy to close diabetic wounds in a diabetic murine model (176).
While conventional proteome profiling kits failed, we found significantly enhanced secretion of the key-cytokines SDF-1 alpha, b-FGF and MMP2. When testing in-vivo, Fibrin glue prolongs ASC-survival, enhances diabetic wound healing kinetics and neovascularisation.

Fibrin Glue has the potential to act as carrier substance, that can counteract the harsh wound environment and simultaneously enhance ASC function. Being already in clinical use it might overcome translational hurdles.

3 Results

3.1 Single-Cell Gene Expression Analysis and Evaluation of the Therapeutic Function of Murine Adipose-Derived Stromal Cells (ASCs) from the Subcutaneous and Visceral Compartment (75)

3.1.1 S-ASCs and V-ASCs show a different regenerative genetic pattern

We initially harvested ASCs from different regions to compare visceral and subcutaneous (V-ASCs and S-ASCs) on a genetic level. Analysis was carried out using a microfluidic based single cell analysis, screening for the 96 genes, that are important for tissue regeneration, cell migration and proliferation. This microfluidic based analysis has initially been described in Stanford (178). By using 96.96 dynamic arrays and loading each well with only one cell, we had the chance to screen for up to 96 genes based on qRT-PCR technology. Nowadays single cell sequencers have displaced these dynamic arrays.

Figure 7 shows q-PCR cycles for individual genes, each dot representing a single cell. Decreased m-RNA content leads to increased cell cycles. Following previous studies (178), 40 cycles were determined as cycle- threshold.

Gene expression is shown in Figure 8 and 9. Hereby every row represents a gene and every column a single cell. Yellow fields show, that a gene is 32 times higher expressed in a cell, then the median, blue a 32 times lower expression then the median.

At the single cell level, we found significant changes in the genetic fingerprint between S-ASC and V-ASC. Especially tissue remodeling related genes like COL3a1 (Collagen 3alpha1), MMP3 (Matrix-Metalloproteinase 3), CCL 2 (C-C Motif Chemokine Ligand 2 or also called MCP-1) or neoangiogenesis related genes like Csf 1 (Colony stimulating factor 1) or VEGF-A were significantly enhanced in S-ASCs. Genes like VwF (Van Willebrandt Factor), FGF 8 (Fibroblast Growth Factor 8) or MMP 10 were not detectable within 40 amplification cycles.

Then single cells were clustered based on their genetic profile. Within the cells, different subpopulations, characterized by specific genetic profiles were detected. Therefore, a Fuzzy c-means Clustering method that has been described previously (178) was used. This method allows cells to share different clusters. We were able to distinguish between four cellular

subpopulation clusters. Clusters are shown in Figure 9. While S-ASC are dominant in Cluster 1 and 2, V-ASCs are predominantly present in Cluster 3 and 4. 53% of S-ASCs can be clustered into the first subpopulation, 36% into the second, 3% into the third and 8% into the fourth. While no V-ASC is present in Cluster one, 9% can be found in Cluster 2, 49% in Cluster 3 and 42% in Cluster 4.

Highly expressed genes were identified as key genes for each cluster. These genes were used to identify genetic pathways by using Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA, http://www.qiagen.com/ingenuity) (Figure 10). Ingenuity Pathway Analysis represents an Internet based platform combining more than 59.000 datasets from RNA-sequencing, RNA-seq, small RNA-seq, microarrays including miRNA and SNP, metabolomics, proteomics, and small scale experiments and is a scientifically valuable platform for pathway analysis.

Cluster 1 is mainly defined by genes that are pivotal for ECM production and remodeling as well as immune cell recruitment. MMP-3, COL1a2, Cxcl12 (CXC-Motiv-Chemokin 12= SDF-1) and COL3a1 are significantly increased in Cluster one (all with a p value <0.001).

Cluster 2 can be defined by DPP-4 (=Dipeptidylpeptidase- 4; p<0.001), CD-55 (= Complement Decay Accelerating Factor; p<0.001), IGF-1 (Insulin like Growth Factor-1; p<0.001), TIMP- 1 (Tissue Inhibitor of Matrix-Metalloproteinase; p<0.001). Overexpression of these genes leads to T-cell recruitment (DPP-4), complement activation (CD-55), tissue remodeling and homeostasis (IGF-1) and promotion of cell proliferation and an anti-apoptotic effect (TIMP).

IGF-1, DPP-4, Thy-1 (Thymocyte differentiation Antigen-1) and Tek (Thyrosine kinase, endothelial) represent key genes for Cluster 3 (p<0.001). Transcription of these genes promotes pathways that are involved in T-cell mediates immune response (DPP-4), Tissue remodeling (IGF-1), cell adhesion (Thy-1) and endothelial cell survival and angiogenesis (Tek).

Cluster 4 is mainly defined by MMP-3 (p<0.001). Other genes, like Col1A2, CCL-2 (=MCP-1) or FGF-7 are only slightly upregulated in Cluster 4 (p<0.1).

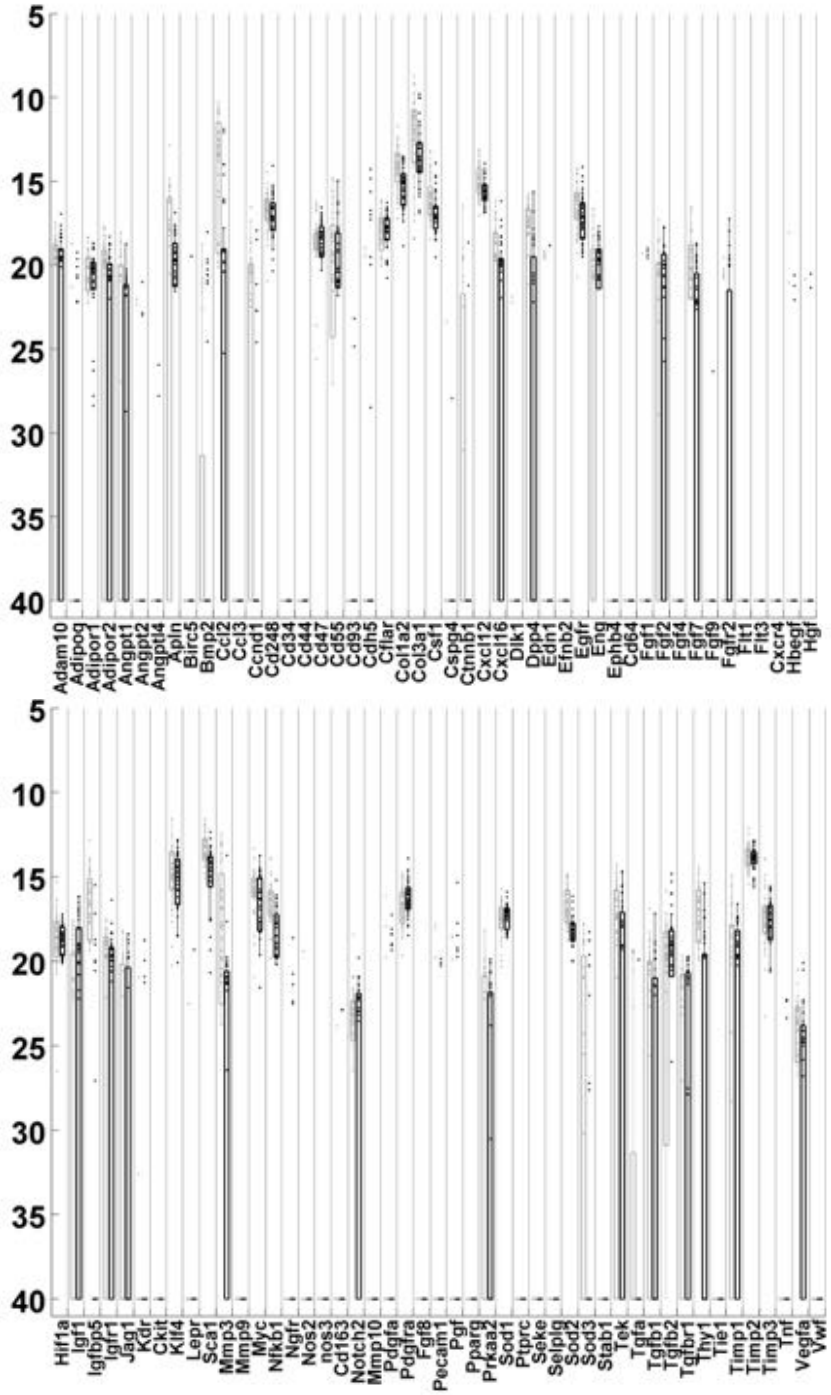


Figure 7 qRT-PCR cycles for each gene. Every cell is represented by a dot. 40 cycles were taken as cycle threshold- genes with an amplification number >40 failed to achieve detectable levels of amplification; the Y-axis shows the replication cycles, the X-axis represents different genes. The higher the cycle number, the lower the m-RNA content of each cell.

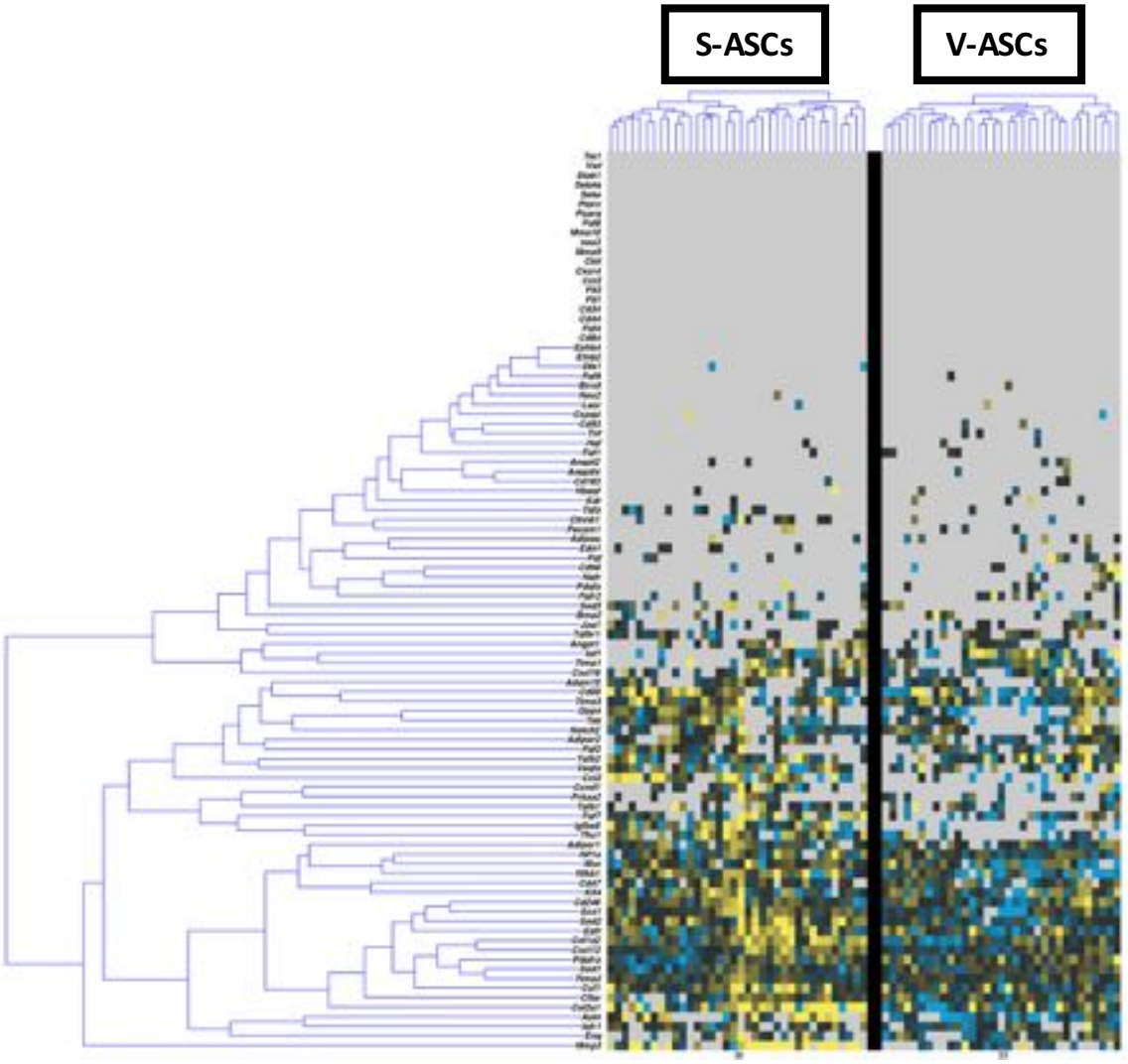


Figure 8 Gene expression plot, presented as fold change from the median. Yellow represents a high expression 32-fold above the median, blue a low expression (32 times lower than the median). Clustering was performed hierarchical. Clustering algorithm was adapted, so four main subpopulations were created. These are shown in Figure 9

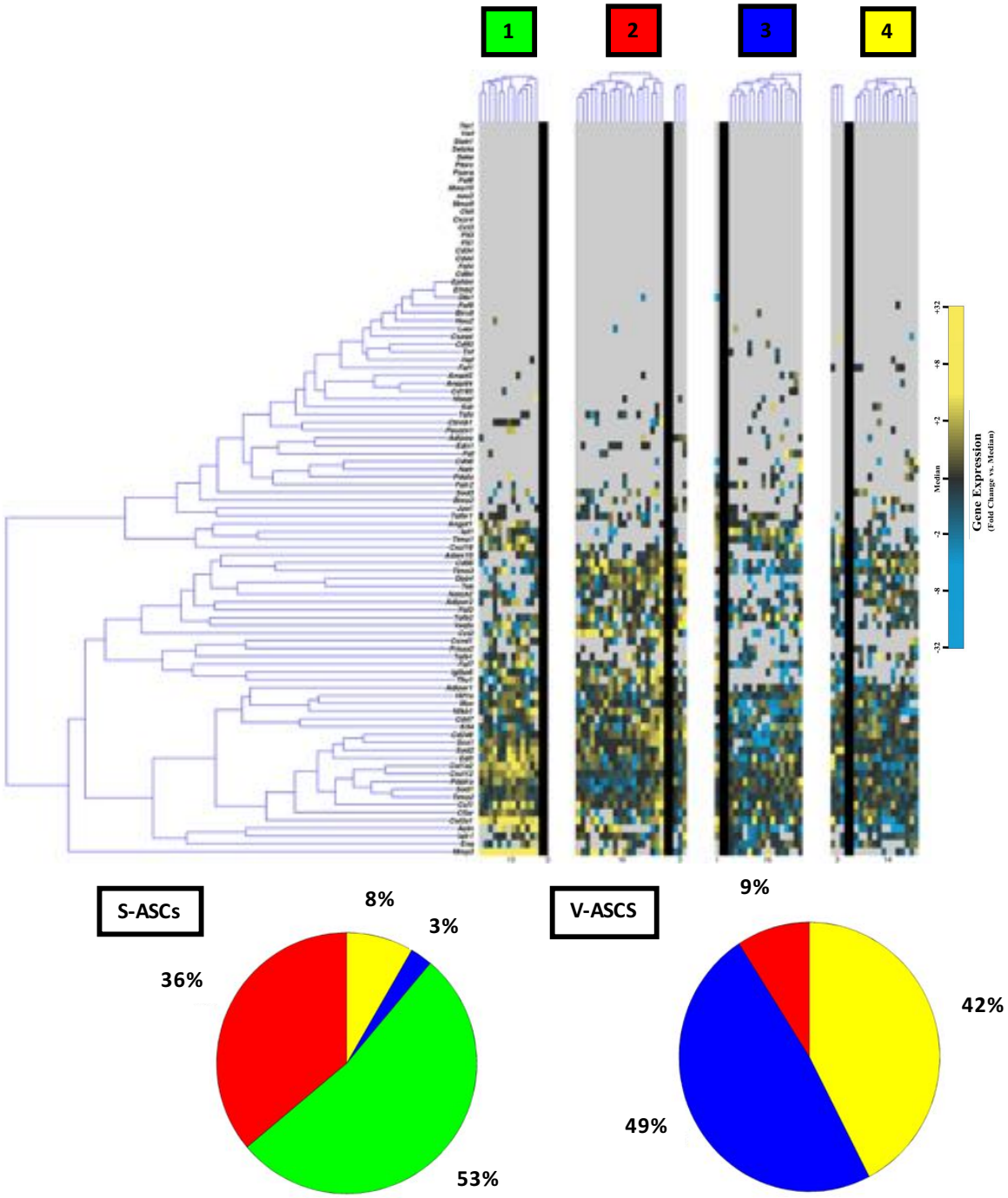
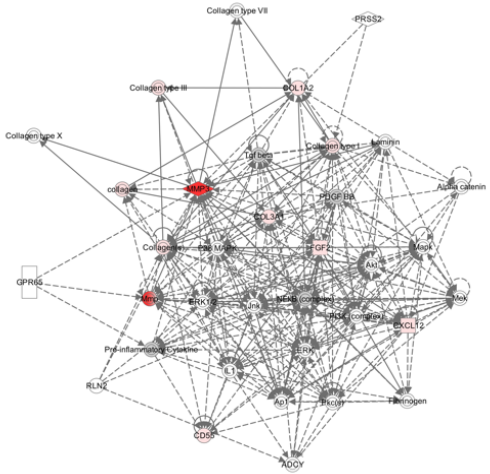


Figure 9 Cellular clustering was performed following their transcriptional profile and using a Fuzzy-C means clustering method. Based on the genetic expression we formed four subpopulations based on their key-genes. While S-ASCs are only present in Cluster one and are dominant in Cluster 2, V-ASCs characterize Cluster 3 and 4 (data shown in the pie-chart below; green= Cluster 1, red= Cluster2, blue = Cluster 3 and yellow = Cluster 4)

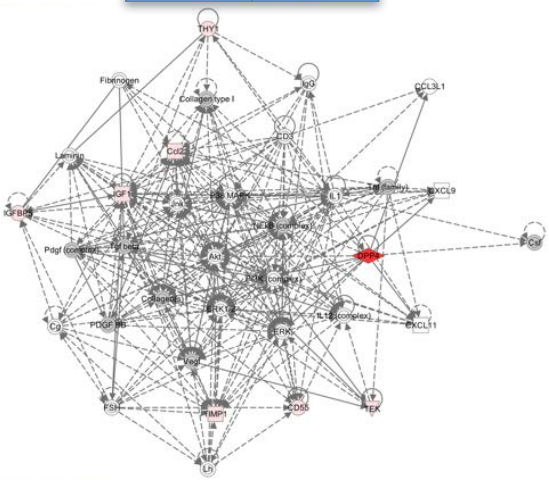
A

Selected Cluster 1 Defining Genes	
Mmp3	$p < 10^{-7}$
Col1a2	$p < 10^{-4}$
Cxcl12	$p < 10^{-3}$
Col3a1	$p < 10^{-3}$



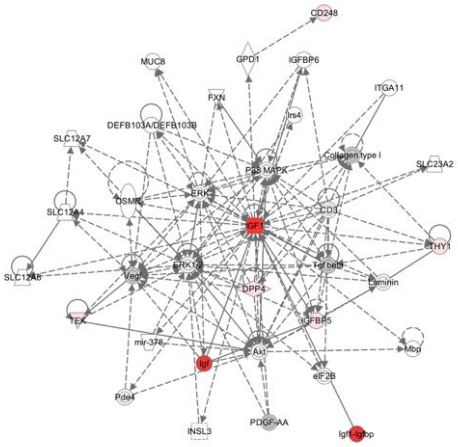
B

Selected Cluster 2 Defining Genes	
Dpp4	$p < 10^{-7}$
Cd55	$p < 10^{-6}$
Igf1	$p < 10^{-5}$
Timp1	$p < 10^{-5}$



C

Selected Cluster 3 Defining Genes	
Igf1	$p < 10^{-4}$
Dpp4	$p < 10^{-3}$
Thy1	$p < 10^{-3}$
Tek	$p < 10^{-3}$



D

Selected Cluster 4 Defining Genes	
Mmp3	$p < 10^{-3}$
Col1a2	$p < 10^{-1}$
Ccl2	$p < 10^{-1}$
Fgf7	$p < 10^{-1}$

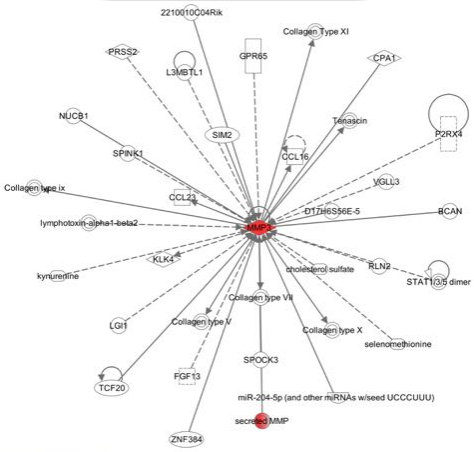


Figure 10 Cluster Characterization. Key Genes and p-values are listed for each cluster separately. Pathways were created by using Ingenuity Pathway Analysis. Key genes are highlighted in red. While Cluster 1-3 are characterized by several key-genes, Cluster 4 is mainly influenced by MMP-3.

3.1.2 S-ASCs and V-ASCs show similar characteristics in murine wound healing

While differences in the genetic fingerprint of murine S-ASCs and V-ASCs have been found, we wanted to investigate capacity for tissue regeneration in vivo. Therefore, we used a previously described and well established murine wound healing model.
Many other groups are using a simple excisional model without splinting the wound (179). As a result, due to the skin muscle (Panniculus cannosus muscle) rodents are minimizing the wound area by simple muscle contraction. Therefore, it is essential to splint the wound and to ensure, that wound closure is achieved by granulation, similar to wound healing in humans.
Additionally, a hydrogel, that allows constant interaction seeded with an equal number of cells per wound was used. This allows constant photographic evaluation of wound area. Wounds were evaluated till complete wound closure.
Full wound closure was achieved in average on day 10.4 in V-ASC group and on day 10.4 in the S-ASC group and on day 11.9 in control group. While both, V-ASCs and S-ASCs significantly stimulated wound healing, when compared to control group ($p<0.05$), we were not able to find any significant differences between ASC treated groups (Figure 11).

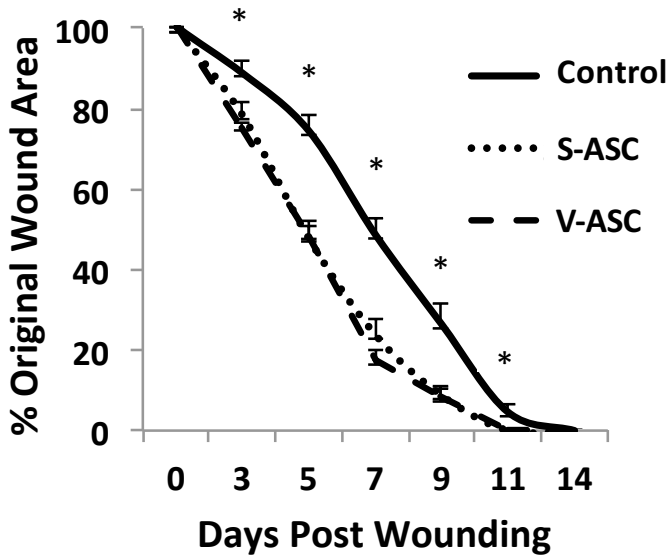
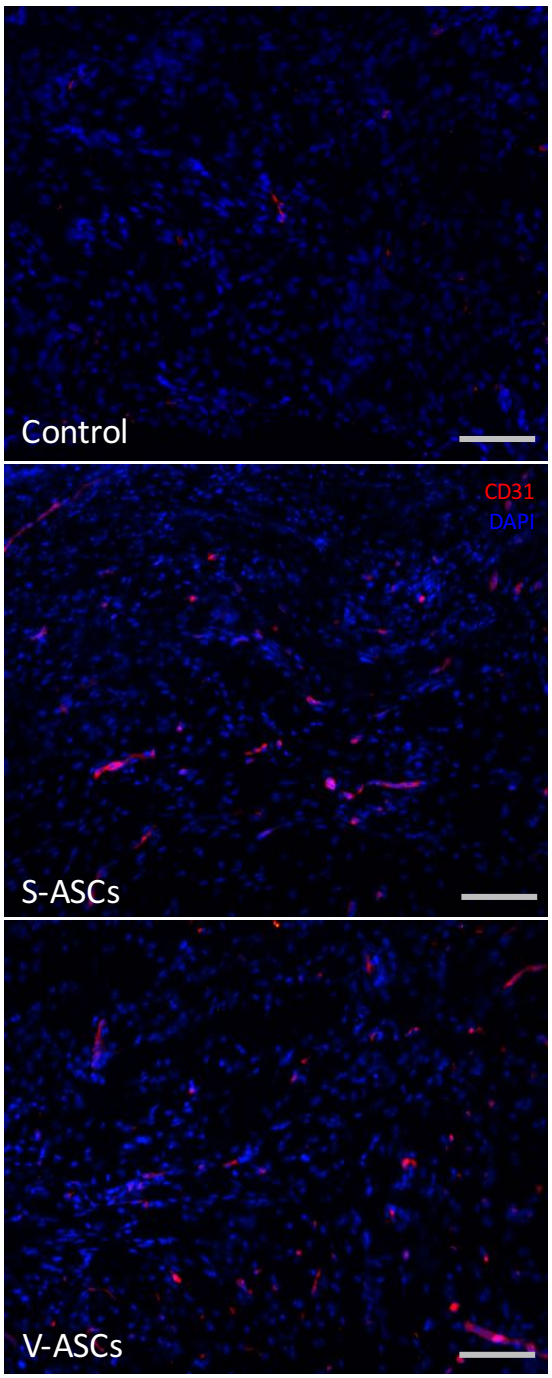


Figure 11 Wound healing kinetics of S-ASC, V-ASC and control group (n=5 wounds per group); Wound area (Y-axis) is given as % of the initial wound; the x-Axis represents the days after treatment. N= 10, Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups

3.1.3 S-ASCs and V-ASCs enhance neoangiogenesis



When full wound closure was achieved, wounds were excised and CD31 expression was immunohistologically evaluated.

While other groups stain specific factors such as VEGF, CD31 is a definite endothelial cell marker, representing neoangiogenesis. CD31 is a surface protein, that is mainly expressed on hematopoetic cells.

We found significantly higher levels of CD31 ($p<0.05$) in both ASC-treated groups, in comparison with control group. Potentially explaining findings of wound healing kinetics, there was no statistical difference between S-ASC and V-ASC group regarding to CD31 expression.

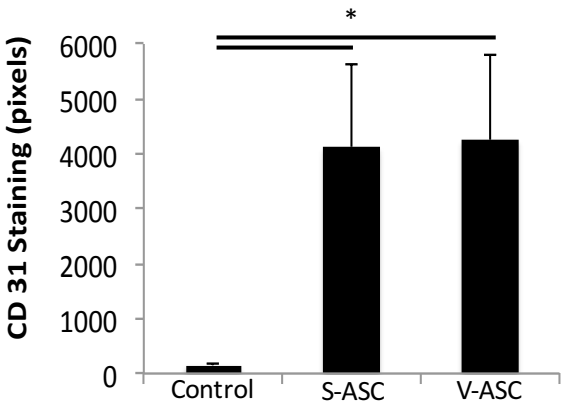


Figure 12 Immunohistological evaluation of S-ASC, V-ASC treated wounds and wounds of control group. Scale = 25 µm; CD31 is quantified by using Image-J. The Y-axis represents the number of positive pixels (arbitrary units); S-ASCs and V-ASCs showed significantly enhanced levels of CD31 when comparing to control group, but no differences between groups were found. n=10; Scale bar= 25 µm; Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups

3.2 Differences in the regenerative potential of ASCs, derived by different liposuction methods

3.2.1 SAL and UAL lead to no significant change in cytokine expression when comparing to Abdominoplasty Samples

To assess changes between ASCs harvested by different liposuction methods and to minimize person depended varieties, we performed Ultrasound assisted, Suction assisted and Abdominoplasty in each patient. Due to publications, that showed that cultivation steps have the potential to change expression of genes within ASCs, we only used freshly isolated primary cells for experimental approaches (180, 181).
After clinical isolation of SVF, we used FACS to isolate ASCs (CD45-/CD31-/CD34+) Within the Abdominoplasty group we found 64% of cells with this surface marker profile, in SAL group 44% and in UAL group 42% matched marker characteristics.
Then cytokine expression analysis for key regenerative cytokines (HGF, FGF-2, MCP-1, SDF-1 and VEGF) was performed using RT-q-PCR (Figure 13). No significant difference in cytokine expression except for MCP-1 ($p<0.05$) was detectable.

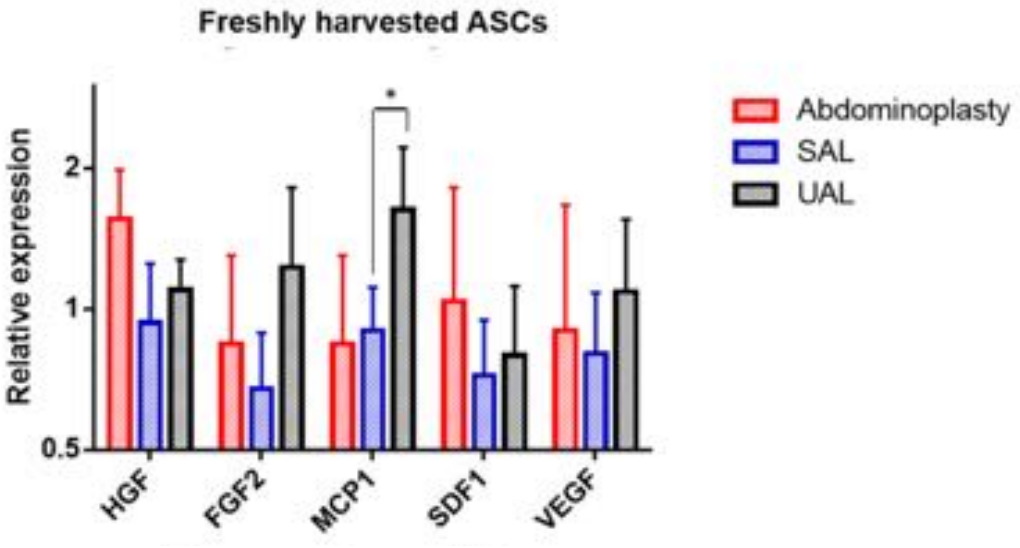


Figure 13 Cytokine expression in ASCs isolated from Abdominoplasty samples (red), SAL samples (blue) and UAL samples (black). The Y-axis represents the relative expression of cytokines (arbitrary units); significant enhanced expression of MCP-1 was found in UAL harvested ASCs. Data are given as mean ±SEM (Standard Error of the Mean; n=3); a Student´s T-test was used for comparison of groups

3.2.2 2nd and 3rd generation of UAL show similar cellular composition

Then we addressed possible varieties between different UAL methods. We harvested SVF from VASER assisted and LYSONIX assisted Liposuction (both UAL assisted Liposuction methods) as described below. Freshly isolated SVF was taken to FACS analysis and ASC population was isolated by CD45-, CD31- and CD34+ marker profile. When comparing ASC cell populations in VASER and LYSONIX we found no statistical difference in ASC frequency. While 44.6% of cells in VASER group were ASCs, 43% of LYSONIX group matched characteristics (Figure 14).

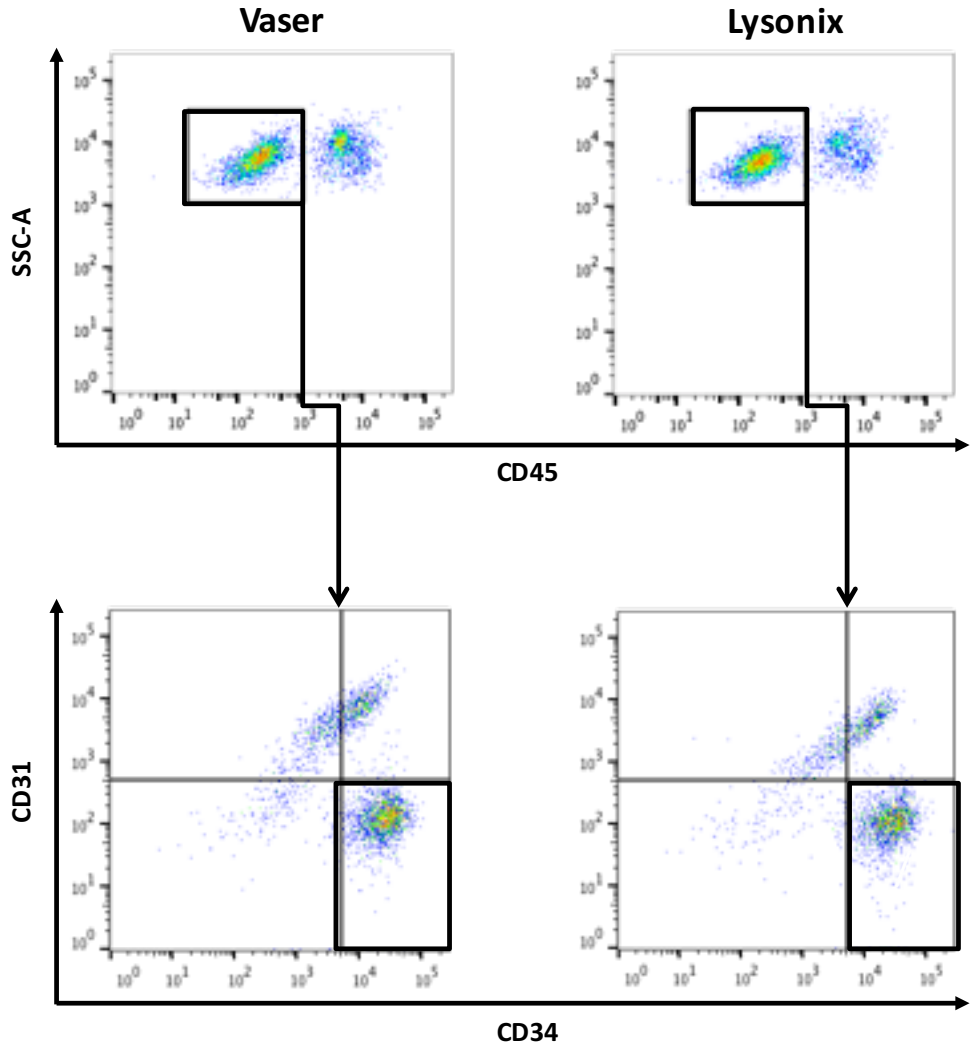


Figure 14 FACS analysis of SVF, harvested from three young female patients. ASC cell fraction was defined as CD45-, CD31- and CD34+; SSC-A = Side Scatter-A; CD= Cluster of Differentiation; percentages are presented in Figure 15

Viability was then measured using an MTT assay. Again, ASC viability in VASER and LYSONIX group was comparable.

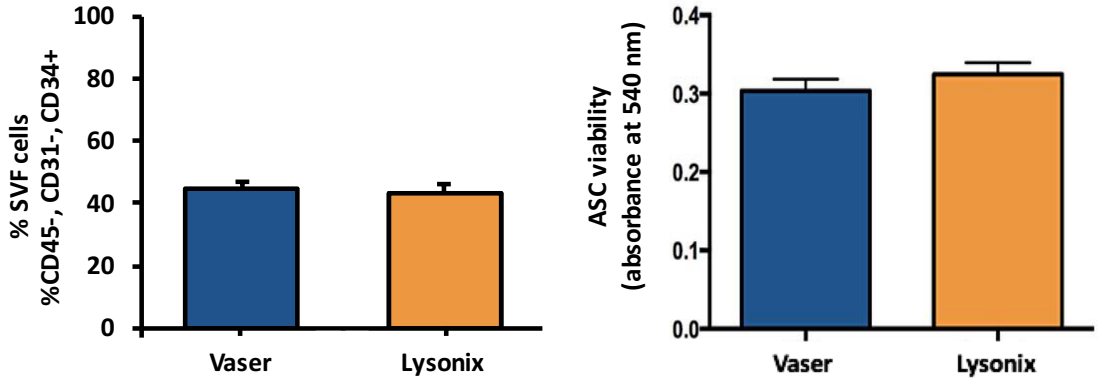


Figure 15 Comparison of ASCs (CD45-/CD31-/CD34+) cells between VASER and LYSONIX group. The Y-axis represents the relative number of ASCs (in percent). ASC viability in VASER and LYSONIX group was measured as absorbance at a wavelength of 540nm (Y-axis). No statistical difference was found in cell composition and in ASC viability. Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups

3.2.3 Comparison of the osteogenic differentiation potential of ASCs harvested by VASER and LYSONIX

For comparison of the osteogenic differentiation capacity, we cultivated cells as shown in the Material and Methods Section. Histological pictures and quantification are provided in Figure 16 – calcium deposits are hereby stained with Alizarin Red. We found no significant difference in calcification, but the trend towards VASER-harvested ASCs.

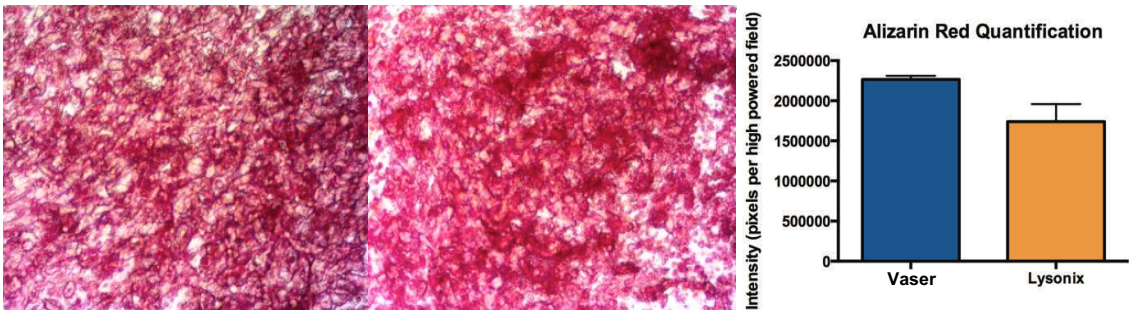


Figure 16 Histological picture of VASER (left) and LYSONIX (right) ASCs, at cultivation day 14 in osteogenic differentiation medium. Cells are stained with Alizarin Red; Quantification is performed by using Image-J, measuring Pixels per high powered

field (Y-axis). No significant differences were found, but the trend towards higher calcification in VASER-samples. Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups

Cell Lysates were harvested on day 0, 7 and 14 after start of cultivation in osteogenic differentiation medium. To assess osteogenic differentiation capacity, we performed a RT-q-PCR for RUNX-2, OPN and OCN. We found significantly enhanced levels of RUNX 2 on day 7 and 14 of osteogenic differentiation ($p<0.05$). OCN was significantly increased on day 14 ($p<0.05$), while OPN showed significantly lower levels on day 0 ($p<0.05$).

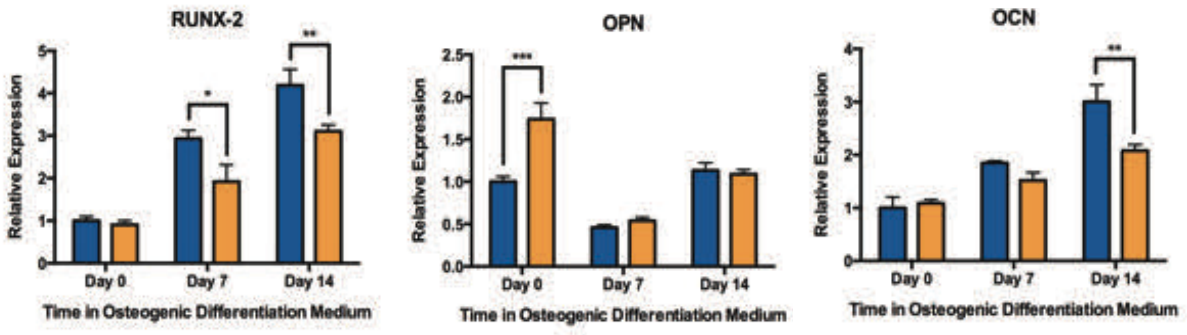


Figure 17 RT-q-PCR for RUNX-2, OPN and OCN at three timepoints of in-vitro osteogenic differentiation (day 0, 7 and 14). Y-axis represents the relative expression. VASER-harvested ASCs showed significantly enhanced levels of RUNX-2 on day 7 and 14 as well as significantly enhanced levels of OCN on day 14. Levels of OPN were significantly increased in LYSONIX samples on day 0. Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups

To sum up, we found the trend towards enhanced osteogenic differentiation in VASER group, underlined by significantly increased markers for osteogenesis.

3.2.4 Comparison of the adipogenic differentiation potential of ASCs harvested by VASER and LYSONIX

For comparison of adipogenic differentiation potential we used specific medium leading to stimulation of adipogenic pathways and formation of adipocytes. Oil-Red-O solution was used to stain lipid vesicles. Comparison of VASER and LYSONIX group was performed by quantification of staining intensity and by harvesting RNA on day 0 and 7 and measuring RNA expression by RT-q-PCR for three adipogenic markers (peroxisome proliferator- activated receptor γ (PPAR-γ), fatty acid binding protein 4 (FABP4) and lipoprotein lipase (LPL)).

Immunohistochemistry for lipid formation showed no significant difference between VASER and LYSONIX group. While only RNA of PPAR-gamma was found on day 0, RNA expression analysis on day 7 showed significantly enhanced levels of FABP-4 ($p<0.001$) and LPL ($p<0.01$) in VASER group.

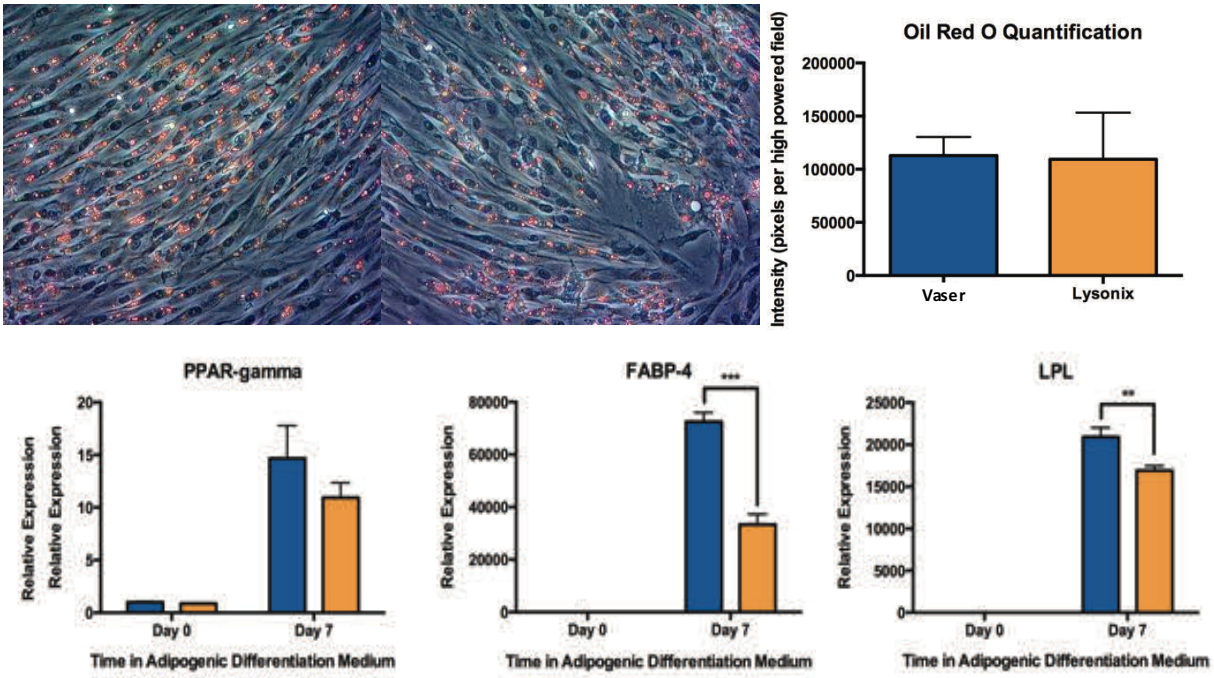


Figure 18 Adipogenic differentiation of VASER and LYSONIX harvested ASCs, Immunohistological staining (Oil-Red-O) was performed and quantified. No statistical difference between VASER and LYSONIX group was found. When comparing the adipogenic differentiation markers PPAR-gamma, FABP-4 and LPL, significantly enhanced levels of FABP-4 ($p<0.001$) and LPL ($p<0.01$) were found on day 7 of cultivation. Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups.

3.2.5 In-vivo wound healing kinetics of VASER, LYSONIX and control group

We then used ASCs to treat wounds in an immunocompromised murine wound healing model, that has been excessively described before (98, 182-184). The only way to test human stromal cells in a murine model is to utilize an immunocompromised mouse model. Thereby cellular function displays human characteristics, but nevertheless, specific immunospecific reactions cannot be investigated. In contrast, many in vitro and in vivo experiments are based on murine ASCs. These differ from human ASCs in their proliferation capacity and their stability when being cultivated (74). Therefore, it

has to be concluded, that translation of experimental findings to humans is difficult. Neither usage of murine cells in an immunpotent model, nor usage of human cells in an immunocompromised model have the power to exactly mimic the clinical setting. Photography was taken on days 0, 3, 5, 7, 9, 11, 13 and 15. On day 15 wound closure was achieved in every wound. While mean closing time was 11 days in VASER group, LYSONIX group showed complete wound closure in average after 11.2 days and control group after an average of 14.1 days.

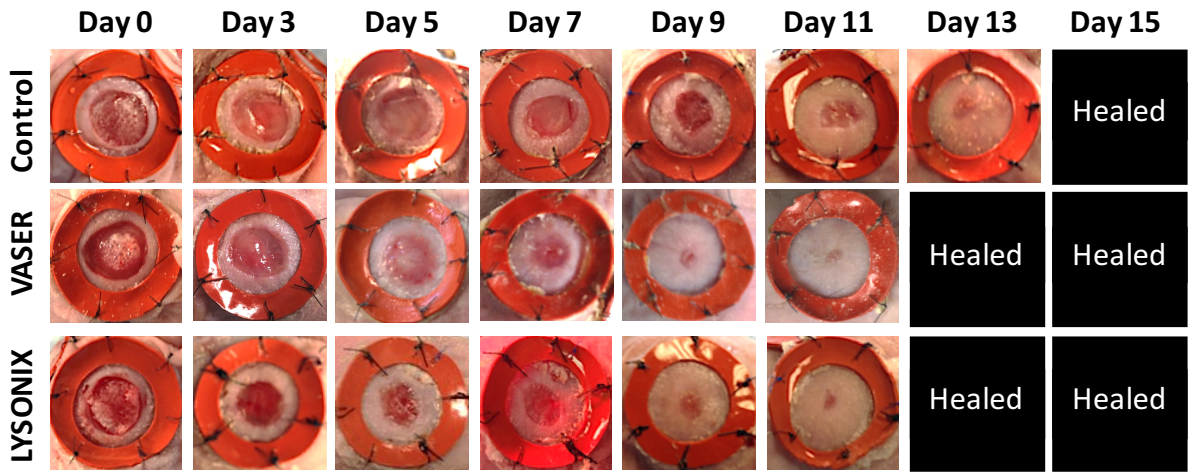


Figure 19 Wounds were created on the dorsum of immunocompromised mice and randomized in 3 groups (n=8 per group)). Mean closure time was 11 days in VASER group, 11.2 days in LYSONIX group and 14.1 days in Control group, being significantly prolonged. Photography was taken on day 0,3, 5, 7, 11, 13 and 15.

We then quantified wound area on every day by using Image J based analysis of each picture. Both, VASER and LYSONIX assisted ASCs led to significantly enhanced wound healing when comparing to non-treated wounds ($p<0.01$). It is remarkable, that significant differences between study groups and control group were observable from day 3 on. No differences in wound healing kinetics and in complete wound closure time between UAL groups were found.

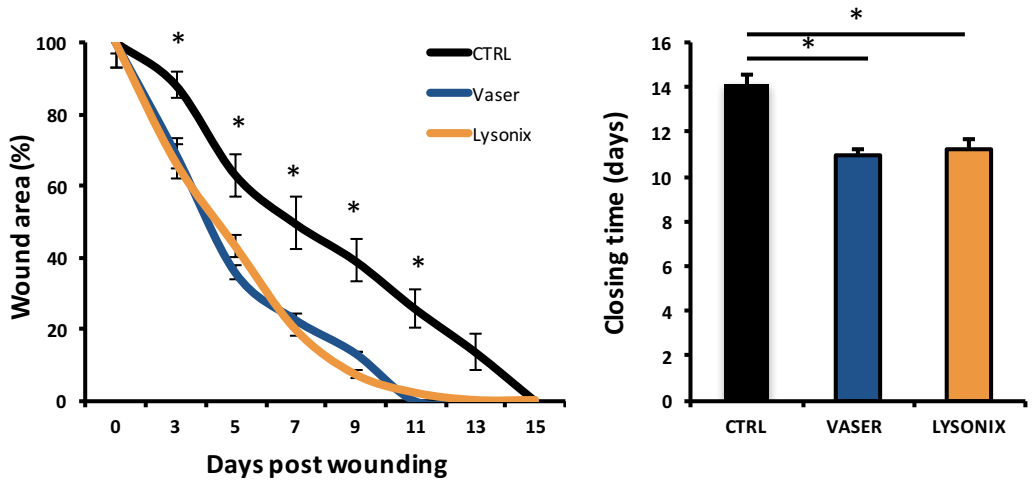
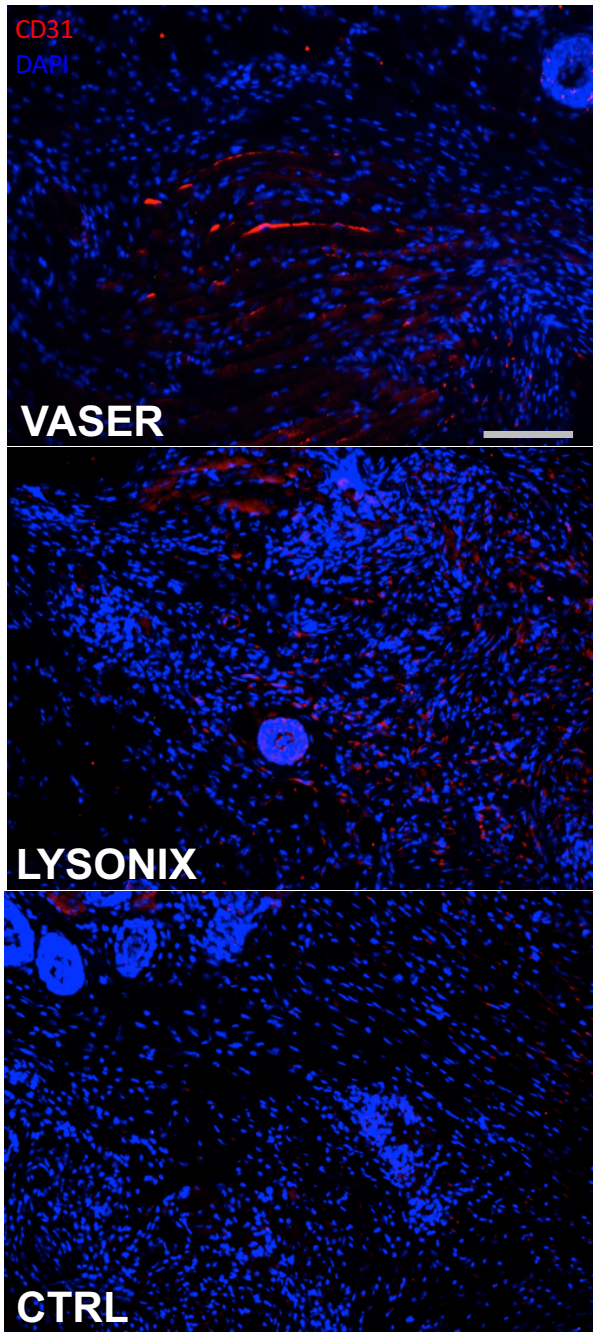


Figure 20 Wound healing kinetics of VASER, LYSONIX and Control group (CTRL). Complete wound closure was achieved on day 11, 11.2 and 14.1 retrospectively. Data are given as mean ±SEM (Standard Error of the Mean). While significant differences were found when comparing both UAL groups to the control group ($p<0.01$), no differences in wound healing kinetics in between the UAL groups were visible (n=8). a Student´s T-test was used for comparison of groups

3.2.6 Analysis of neovascularisation in healed wounds



When complete wound closure was achieved, mice were anaesthetized, killed and wounds were surgically excised and prepared for immunohistological staining as described in the Material and Methods section. The endothelial cell marker- CD31 and DAPI, staining the nuclei, were used and signalling was quantified by measuring the pixel positive area using Image-J.

Both, VASER and LYSONIX groups showed similar vascularity, being significantly enhanced to control group ($p<0.05$). These findings could potentially explain significantly shorter wound closure time in ASC treated groups.

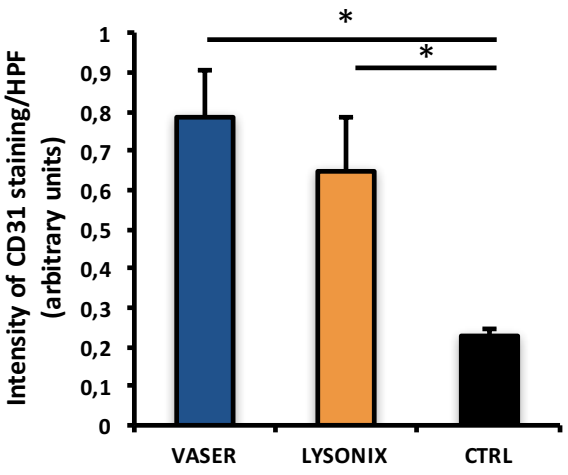


Figure 21 Immunohistological Staining of VASER, LYSONIX and Control (CTRL) wounds. CD31 was used as primary antibody, DAPI was used to stein nuclei. Quantification was performed by measuring the pixel positive area of CD31 (Y-axis). Significant differences between UAL groups and control group were found (n=8, p<0.05). Scale bar= 100 µm; Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups

3.3 Optimizing the delivery method of ASCs

3.3.1 Fibrin Modulates ASC Cytokine Secretion

After seeding ASCs with and without Fibrin Glue and Fibrin glue alone (negative control; n=3 per group) for 7 days, we used a Proteome Profiler Human Pluripotent Stromal Cell Array Kit. This kit provides a screening method, that allows simultaneous detection of 15 stromal cell related proteins. Proteins and their characteristics are described in Table 1.

Marker	*Characteristics*
Oct-3/4	• Octamer binding transcription factor 3/4 • Essential for embryonal development (185) • Germ line specific (186)
Nanog	• Homeodomain protein • Only expressed in pluripotent cells • Drives self-renewal of Stromal cells(187)
SOX2	• Sex determining region Y (SRY) - Box 2 • Transcriptional modulator of nanog (187) • Essential for linage determination (188)
E-Cadherin	• Cell adhesion molecule • Essential for reprogramming (189)
Alpha-Fetoprotein (AFP)	• Marker for primate ESCs (190) • Essential for liver development and hepatocarcinogenesis (191)
GATA-4	• GATA-binding protein 4 • Essential for cardiogenesis (192)
HNF-3 beta/FoxA2	• Hepatocyte nuclear factor-3 beta / forkhead box protein A2 • Driver of organogenesis (192) • Endoderm marker (193)

PDX-1/IPF1	• Pancreatic and duodenal homeobox 1 / insulin promoter factor 1 • Essential for self-renewal (194) • Highest expression in dental stromal (195)
SOX17	• SRY - Box 17 • Endoderm marker (196)
Otx2	• Orthodenticle homeobox 2 • Crucial for neurogenesis (197) • Midbrain marker (198)
TP63/TP73L	• Transformation-related protein 63 • Ectodermal linage marker (194)
Goosecoid (GSC)	• Crucial for cell migration (199) • Involved in tumour metastasis (199)
Snail	• Zinc finger protein • Promotor of endothelial–mesenchymal transition (200)
VEGF R2/KDR/Flk-1	• VEGF receptor 2 / kinase insert domain receptor / foetal liver linase-1 • Promotor of MAPK, PI3K/AKT, Scr and Ras pathways (201) • Crucial for osteogenic differentiation (202)
HCG	• Human chorionic gonadotropin • Placental marker in ESCs (203)

Table 1 Screened markers and their characteristics

We initially mechanically extracted cells from the Fibrin scaffold. Then cells were lysed and processed according to manufacturer's instructions.
Despite multiple repetition of experiment and an exposure time of up to 300 seconds, we were not able to detect any fluorescence signal except the reference spots (Figure 22). Thereby, quantification of dots was impossible. Interaction of Fibrin glue with the assay as well as failure in mechanical extraction could explain this failure.

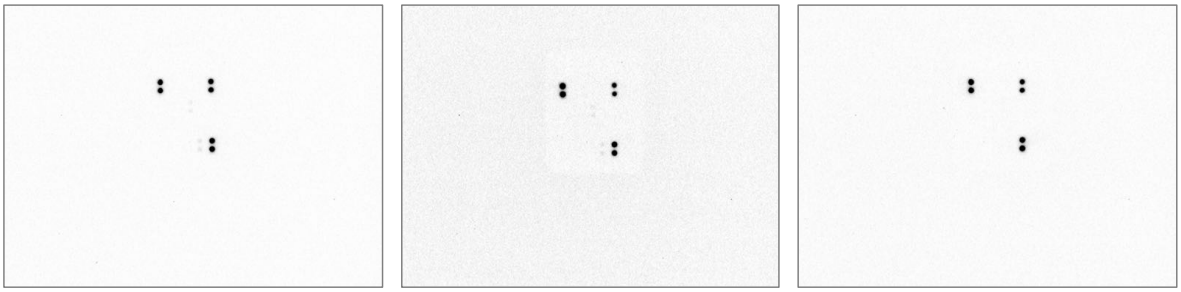


Figure 22. Proteome Profiler Human Pluripotent Stromal cell Array Kit. No signal could be found except reference spots.

Due to failure of protein screening, we used ELISA for the previously described regenerative cytokines SDF-1 alpha, b-FGF, VEGF-A and MMP-2 (177) to analyse effect of fibrin glue on ASCs (Figure 23 and 24). The secretion of b-FGF ($p<0.01$) and SDF-1 ($p<0.05$) was significantly increased in the Fibrin+ ASC group. We found no protein levels in the negative controls.

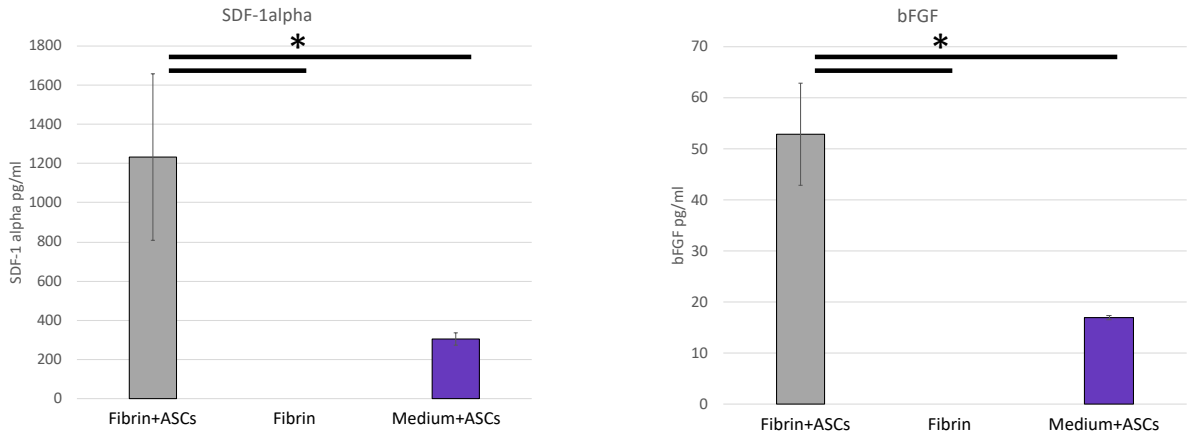


Figure 23. Cytokine levels of SDF-1 alpha and b-FGF in the Fibrin+ ASC group was significantly increased when comparing to control groups, Data are given as mean (pg /ml) ±SEM (Standard Error of the Mean). N=3; a Student´s T-test was used for comparison of groups

While MMP-2, an enzyme pivotal for angiogenesis and keratinocyte activation, showed significantly lower levels in the Fibrin+ASC group ($p<0.01$) no differences in VEGF-A expression between Fibrin+ASC and cultivated ASC group were found. Again, no protein secretion was found in Fibrin group.

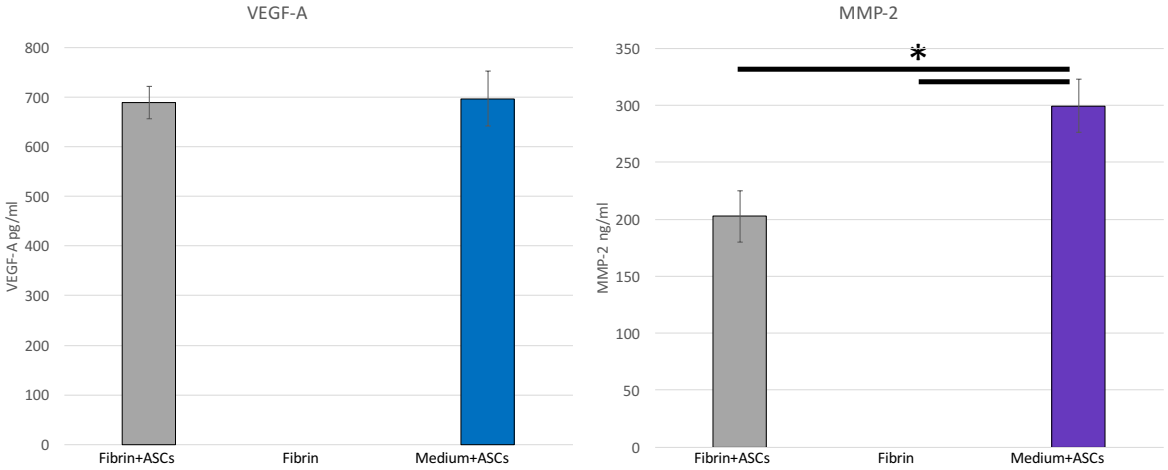


Figure 24. Cytokine levels of MMP-2 (ng/ml) and VEGF-A (pg/ml). We found significantly diminished levels of MMP-2 in the Fibrin+ASC group (p<0.01). No protein secretion was found in the Fibrin only group. VEGF-A levels showed no differences in expression between Fibrin+ASC and Medium+ASC group. Data are given as mean (pg /ml) ±SEM (Standard Error of the Mean). N=3; a Student´s T-test was used for comparison of groups

3.3.2 In-Vivo ASC Survival Analysis in a murine wound healing model

By using an in-vivo imaging system (IVIS) we were able to specifically track cells after delivery. Therfore, luciferase positive mice, that were genetically equal, served as donor for ASC harvesting. Cells were then isolated and delivered as described before. To mimic the harsh wound environment, wounds were created on the dorsum of diabetic mice. Under short time anaesthesia, and injection of D-luciferin, Spectrum Photographies were taken. Luminescence signalling was quantified by luminescence intensity.

On day 0, 3, 5, 7 and 9 we found significantly increased luminescence levels in Fibrin +ASC treated wounds, when comparing to wounds that were injected with PBS and ASCs ($p<0.01$). While luminescence signal was detectable for an average of 11 days, no signalling was detectable in the injected group from day 9 on, leading to the conclusion, that Fibrin glue provides a cellular niche, that significantly prolongs ASC survival and enhances their viability when being delivered.

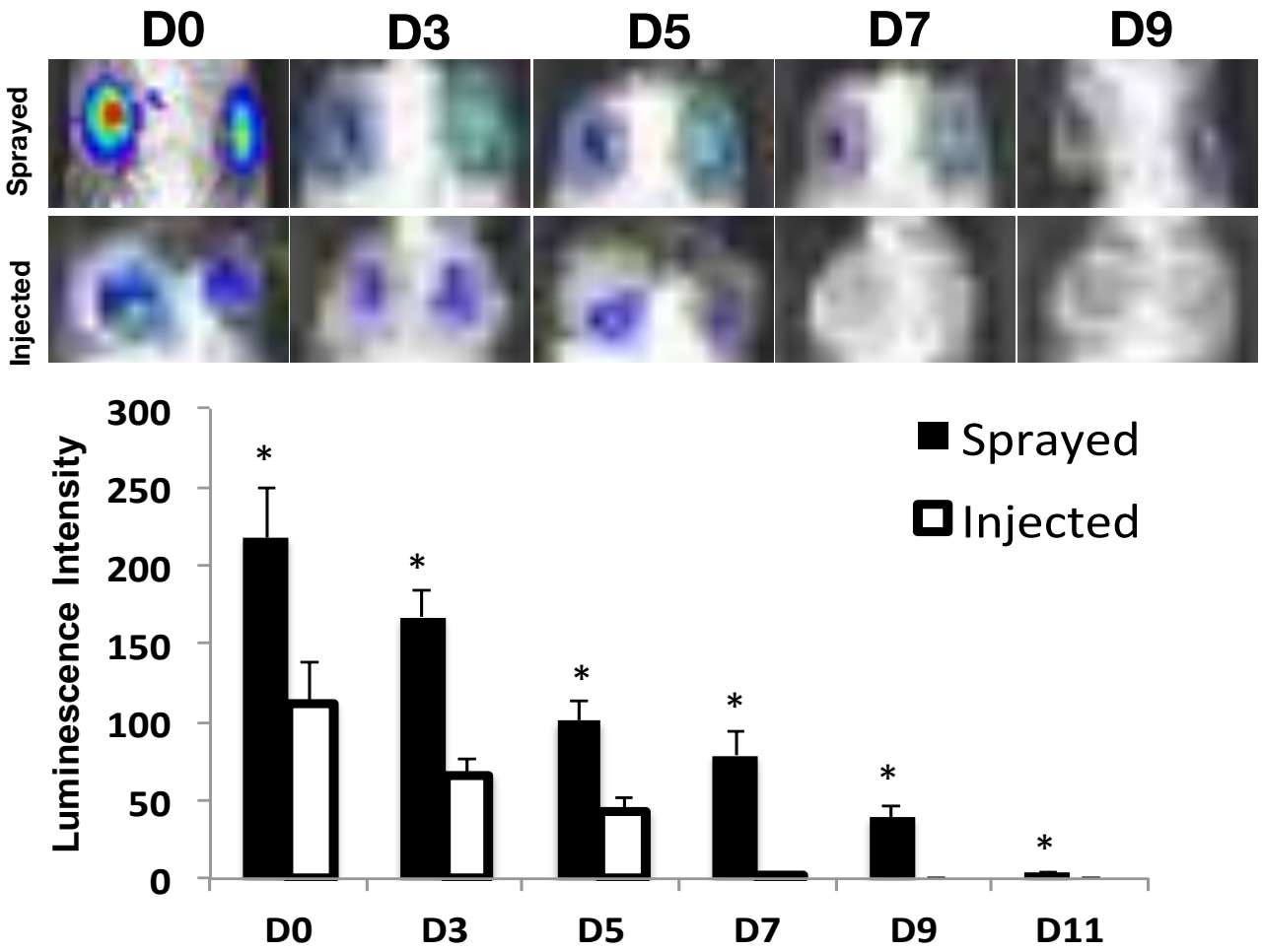


Figure 25. Fibrin Glue significantly luminescence intensity when being delivered to diabetic wounds. Luciferase positive mice were stimulated by intraperitoneal injection of D-luciferin. Flourescence signal was detected on day 0, 3, 5, 7 and 9 and luminescence intensity was calculated. Luminescence was anhanced on each timepoint and significantly prolonged (11 days vs. 7 days; p<0.01) in Fibrin Glue+ASC group; Data are given as mean ±SEM (Standard Error of the Mean), n=8; a Student´s T-test was used for comparison of groups

3.3.3 Fibrin-Delivered ASCs Significantly Enhance Wound Healing

Similar to previous studies, wounds of diabetic mice were photographically evaluated on specific timepoints (day 0, 4, 8, 12, 16, 18 and 20) till complete wound closure was observed in all mice. Then quantification was performed, and wound healing kinetics was calculated by Image-J based measurement of total wound area.

Wounds in the Fibrin+ASC group were closed after an average of 16 days, wounds that were treated with PBS+ASCs in average after 18 days and non-treated wounds

after 20 days. Complete wound healing was obtained significantly faster in Fibrin group than in the others ($p<0.05$). Wounds in ASC group showed enhanced wound healing, when being compared to control group.

When comparing wound healing kinetics to those described above, the diabetic model showed prolonged wound healing in comparison to immunocompromised mice treated with human ASCs (Figure 11; 10.4 -11.9 days) and immunopotent mice (Figure 19; 11-14.1 days), differences in duration till complete wound closure are obvious and underlines the substantial burden of diabetic wounds.

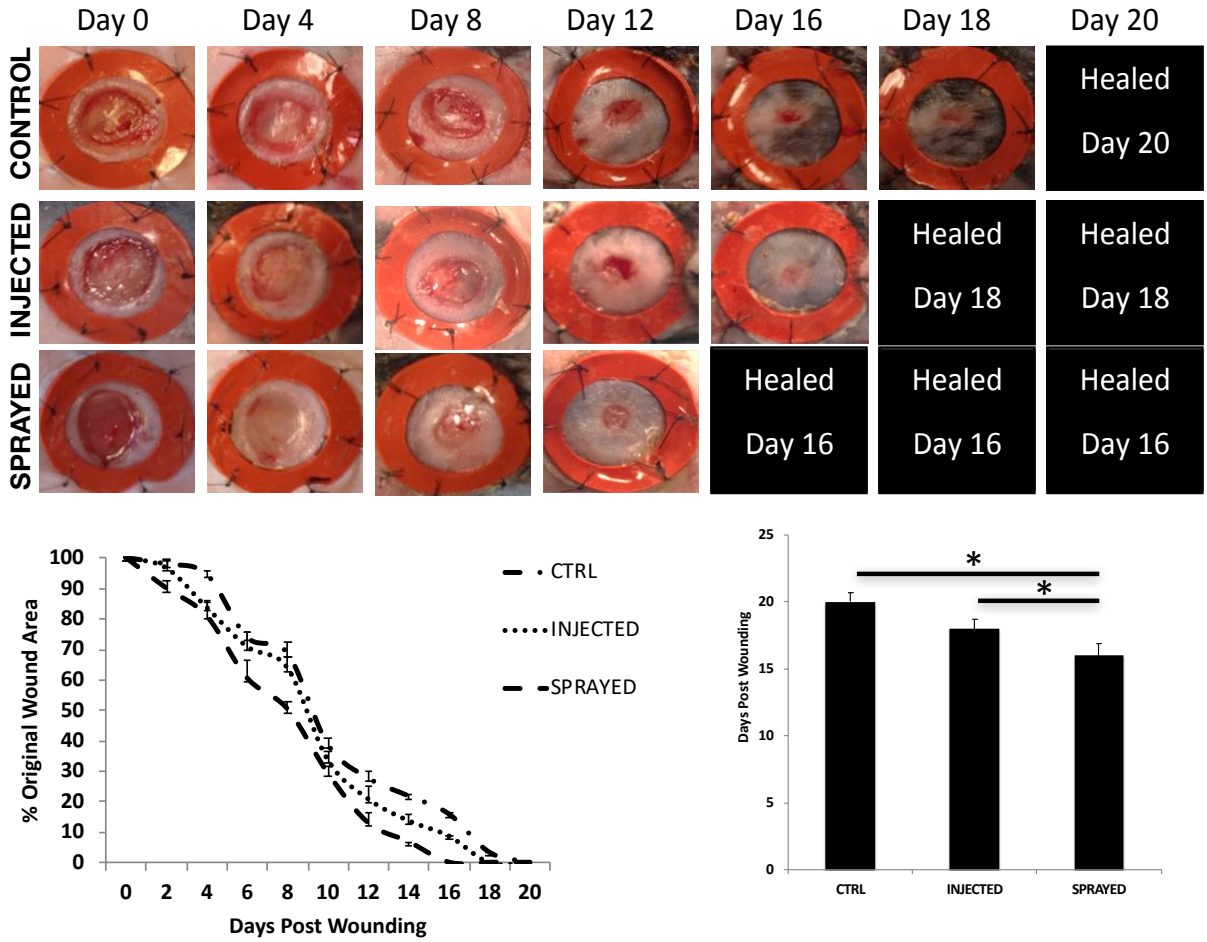


Figure 26. Photos of excisional diabetic wounds of Fibrin group (sprayed), PBS+ASC (injected) and control group. Standardized pictures were taken on day 0, 4, 8, 12, 16, 18 and 20. While control group showed complete wound closure after an average of 20 days, with 18 days injected wounds were closed significantly faster than control group. Fibrin group showed complete wound closure after 16 days- significantly enhanced compared to both other groups. Data are given as mean ±SEM (Standard Error of the Mean), n=8; a Student´s T-test was used for comparison of groups

3.3.4 Immunohistological evaluation of closed wounds

After complete wound closure mice, wounds were stained with DAPI and CD-31 as described below. CD31, a neovascularization marker, was quantified. Pixel Positive Area was measured (HPF= high powered field) and was given as percentage. Luminescence signal in PBS-ASC treated wounds was 4.27%, wounds in the Fibrin+ ASC group showed 7.41% luminescence signalling. We found significant difference between both groups ($p<0.05$) going along with observed wound healing kinetics.

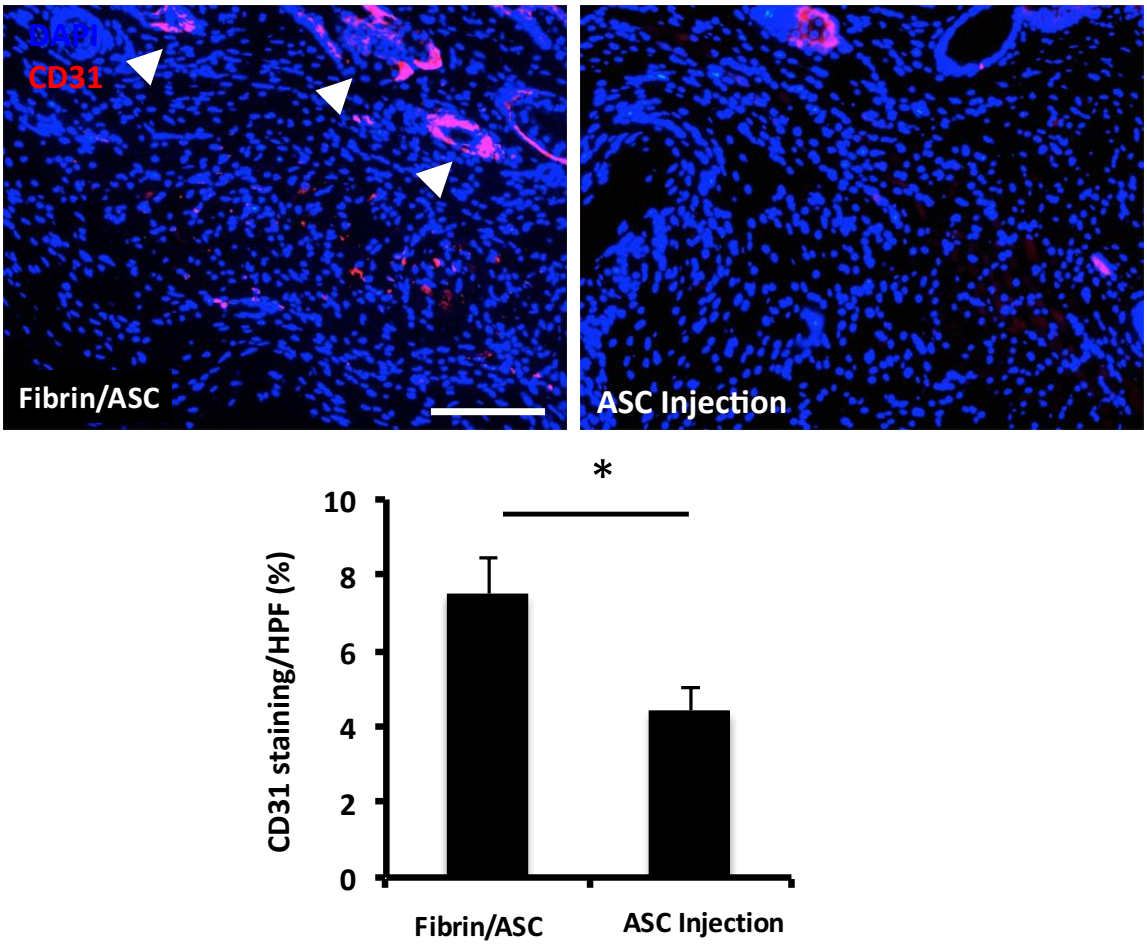


Figure 27 Immunohistological microscopic pictures of Fibrin+ASC od ASC+PBS injected group. Quantification of PPA led to significantly enhanced CD31 levels in Fibrin+ASC group (7.41% and 4.27%; p<0.05); Scale bar= 100 µm; Data are given as mean ±SEM (Standard Error of the Mean); a Student´s T-test was used for comparison of groups

4 Discussion

4.1 Clinical Relevance

MSCs are a unique cell type, crucial for tissue regeneration. Besides the already approved use as an anti-GvHD agent, their regenerative ability is in focus of modern medicine. Due to their secretion of key cytokines and their ability to differentiate into multiple different cells, MSCs have the potential to fully restore functionality and integrity of tissues. Interaction with immune cells is the key to their anti-inflammatory effect.

MSC-based therapies are therefore the focus of many medical disciplines, including ophthalmology (204), cardiology (37, 42, 127, 136, 139, 142, 205), immunology (43, 47) and plastic surgery (65, 175, 206-220). Some of these approaches, such as for allogenic BM transplantation for GvHD, are already in clinical use and represent main pillars and gold standards (221, 222). Others are on their way to be translated into clinical practice (207), and some are currently of experimental interest but show promising results (24, 42, 49, 142, 223).

Within the field of plastic surgery, MSCs are mainly used as an additive for autologous lipotransfer to improve outcomes and to enhance wound healing. Both approaches are on their way to clinical translation but are facing severe challenges.

The use of MSCs for autologous lipotransfer for breast reconstruction is broadly disputed, due to possible malignancy that might accompany the regenerative function of MSC (110, 114, 117, 224). Although initial results have not confirmed malignancy or possible cancer recurrence after lipotransfer (110), many plastic surgeons still avoid this technique in cancer or post-cancer patients.

In wound therapy, the clinical use of MSCs is still limited due to variable efficacy caused by differences in harvesting process and patient dependent factors.

4.2 Translational Aspects

Harvesting-specific and Patient-specific factors significantly contribute to the unpredictability of clinical success. Additionally, translation of experimental findings into clinical practise is difficult.

Many preclinical trials utilize murine models and harvest ASCs from all different tissues (omental, parascapular fat tissue, inguinal fat pads) (225-227). Therefore, in most studies no attention was given on the source of ASC. To address the question, if the donor region has an effect on ASC and their applicability, we isolated visceral and subcutaneous ASCs and compared their genetic expression profile based on a single cell analysis and regarding their ability to close wounds. We used an assay, that is based on single cell analysis by using a 96.96 well plate (Fluidigm, USA, South San Francisco, CA) and q-PCR amplification.

When comparing to novel techniques, that are based on modern sequencing (228), this method seems to be outdated. While focus of biotechnological companies has in particular been laid on driving development of sequencing, this field has been radically changed within the last years. New sequencing machines, the easiness of their use and broad availability have made old strategies, such as 96.96 well assays obsolete (229).

In contrast to these novel techniques, that have the possibility to sequence complete cellular RNA within minutes, our assay was limited on 96 genes and 96 single cells. Therefore, we focussed on genes, essential for tissue regeneration and ECM-production.

Usage of novel mechanisms such as single cell sequencing, might have explained found differences between RNA analysis and wound healing kinetics.

We tested gene expression and the regenerative capacity in an in-vivo model. Surprisingly, although V-ASC and S-ASC showed a complete different genetic fingerprint, their wound healing ability can be seen as equal. This leads to the

suggestion, that both groups are stimulating different pathways, but their regenerative capacity is similar.
Additionally, these results highlight, that simple gene expression analysis must always be verified by further protein testing or by in-vivo studies.
While RNA analysis such as PCR can only measure the total amount of transcribed RNA within cells, no statement about efficacy on a protein level or in-vivo efficacy can be made.
Gene expression can be modified at the post-transcriptional level by polyadenylation, capping and different splicing mechanisms (230, 231). RNA- interference by endogenous miRNAs (micro-RNAs) can neutralize transcribed RNA (232). Due to all of these possibilities, high levels of RNA do not always lead to high levels of protein secretion or enhanced efficacy in vivo. Results, that are based on PCR or other gene expression analysis must be always verified by protein testing or in-vivo testing. Lacking of verification on a protein level can be seen a huge limitation of one of our studies "Vergleich des Regenerativen Zytokinprofils von Adipose Derived Stromal Cells (ASCs) Gewonnen Mittels Abdominoplastik, Suction Assisted Liposuction (SAL) und Ultrasound Assisted Liposuction (UAL)" (177).

As described in Chapter 2.1.3, slight differences in harvesting and processing have a great influence on differentiation, viability and cytokine secretion of ASCs (98-100, 233). Within the last years many novel techniques have been developed to facilitate the liposuction process, but no particular attention was given on ASCs and their regenerative potential. All methods advertise to reduce postoperative pain, reduce ecchymosis and enhance skin structure, but not much is known about their effect on ASCs. We compared ASCs, harvested by UAL and SAL- frequently used Liposuction methods, regarding their regenerative capacity. By showing differences regarding osteogenic and adipogenic differentiation, but no significant differences in wound healing capacity, we were able to conclude, that VASER assisted Liposuction, that uses higher frequency ultrasound waves than LYSONIX assisted Liposuction leads to benefits- especially for osteogenic differentiation. Therefore, when ASCs are used in

a clinical setting to reconstruct bone tissue, VASER harvested ASCs represent a reliable source and might optimize outcome. Nevertheless, these results have to be proven in a clinical setting.
These results underline previously published studies, that report that enhanced differentiation marker levels are a result of mechanical manipulation of cells (234) . While Keck et al showed increasement of adipogenic differentiation markers when being isolated by Power assisted Liposuction, Grottkau et al reported enhanced osteogenesis in mechanically stimulated ASCs (234, 235).

In addition to varieties in Liposuction, differences in the wound bed and healing capacity of patients as well as ASC function and viability are dictated by age, gender and comorbidities such as diabetes (129, 150, 211, 212, 236-240). Almost all patients with chronic wounds are older and most have diabetes. Therefore, using MSCs in an effective manner within this cohort of patients becomes tremendously difficult. Although addressing these comorbidities would be the ideal solution, enhancing the niche in which these cells reside is an alternative and elegant strategy. Many delivery vehicles already exist, but despite promising results both in vitro and in vivo, to date, use of alginate (160), silk fibroin and chitosan (156), or collagen (171) for ASC delivery has not been translated to clinical practice (241-243). This may, in large part, be because most of these carrier vehicles have been designed specifically for research proposes and are not freely available in clinical settings.
To overcome these problems, we used fibrin glue, a carrier system that is already FDA-approved and in broad clinical use. It is biocompatible, biodegradable and currently the most effective tissue adhesive available (244). Although its ability as a cell carrier is rarely described, its clinical safety has been proven extensively and it is readily available in most hospitals (172, 173, 245-248). Furthermore, fibrin glue is often used to improve skin graft adherence, particularly in the setting of large burns, and could enhance outcomes in these patients.
For adequate homeostasis, two factors are needed: fibrinogen and thrombin. Mixing fibrinogen and thrombin results in the formation of porous fibrin strains. In the human

body, factor XIII (fibrin stabilising factor) leads to crosslinking of fibrin strains and thereby to clot formation (249). Modern fibrin sealants most likely mimic this process, and furthermore contain aprotinin, a molecule that prolongs the haemostatic effects of fibrin by inhibiting fibrinolysis (250).

Fibrin was first described as essential for wound healing in 1908 (251). In the following decades, fibrin-soaked gazes were applied to enhance wound healing. In the 1930s, technology led to the possibility of extracting purified protein, and purified thrombin has since been available. Thrombin and fibrinogen have both been extracted from human blood and were first used allogeneically (244). Many patients treated in this early phase contracted viral hepatitis. Developments in technology made isolation and concentration of factors easier and cheaper. Additionally, viruses within the fibrin were inactivated, avoiding infections. In the 1970s, Matras (252) used a novel fibrin glue for nerve repair in rabbits, followed by the first commercially available fibrin sealant in 1982 in Europe (244). Remarkably, although initial reports and studies showed promising results regarding operative outcomes, fibrin glue was mainly used in Europe while its usage in the USA was restricted by the FDA until 1998 (244, 253). A two-chamber mechanism including thrombin and fibrin was later popularised. Tissucol/Tisseel (Baxter, Illinois, USA) emerged as the most used (248), and other systems that extract autologous fibrin-like products such as the CryoSeal FS (Thermogenesis, Rancho Cordova, CA; (254) or the Vivostat system (Vivolution A/S, Birkerod, Denmark) are now rarely used. For standardisation and clinical applicability we decided to use the Tisseel Fibrin Sealant as the delivery vehicle for MSCs in this study, avoiding regulatory issues related to the vehicle as well as individual differences in protein concentration.

Fibrin sealant represents a promising tool that can easily overcome all hurdles for clinical translation. This delivery system could be beneficial for almost all fields of medicine. Treating large burns, regenerating cardiac tissues or enhancing healing of chronic wounds by simply harvesting MSCs, mixing them with fibrin and applying them to the patient is an easy, safe and effective way to use MSCs without manipulating or changing their profile, but enhancing their function.

4.3 Comparative Aspects

Fibrin has already been used as a delivery vehicle for cells. When using a two-chamber system, fibrinogen and thrombin are blended and form a network of single strains that is ideal for cell adhesion. Mogford et al. recognised this potential, and mixed fibrin glue with fibroblasts and loaded it with growth factors (174). They investigated the effect of this novel application on excisional wounds in a rabbit model, finding the fastest healing in wounds treated with fibrin glue in combination with fibroblasts and PDGF-BB. The combination of fibrin glue and fibroblasts further showed accelerated wound healing compared to fibrin alone, but significantly slower than the combination with PDGF-BB. Growth factors in combination with fibrin glue can therefore be used to enhance efficacy of cellular delivery, but clinical translation of these approaches will be difficult. Currently, Regranex (Ortho-McNeil, Raritan, NJ, USA) is the only growth factor that is FDA-approved (63). Regranex gel has shown beneficial effects on wound healing compared to placebo (255). Nevertheless, in 2008 the FDA reacted to follow-up studies that reported fivefold increased mortality due to cancer in patients who used more than three tubes of Regranex. The FDA applied a black-box warning for Regranex in patients with known malignancy (63, 256). Therefore, clinical use of growth factors, even only applied locally as a gel, can have severe side effects, is seen with caution and would certainly limit translation. We therefore avoided growth factors in our setting, although they most likely would have enhanced the outcome.

Zimmerlin et al. mixed ASCs with fibrin (175). They initially harvested ASCs by performing liposuction and used a predefined marker profile for FACS-based cell sorting, then used Tisseel glue for seeding ASCs. Although this was a well-performed study that proves the principle of cell delivery by fibrin carrier substances, many limitations exist. The study included only one group, making it impossible to compare the efficacy of delivery to standardised methods such as seeding without a carrier substance. Although Zimmerlin et al. mentioned that the fibrin glue they used ‘resulted in a well-organised mesh that supported growth and differentiation of SVF cells’ (175), no statement can be made about the efficacy of this approach. Additionally, the

assessment of adipogenic differentiation, immunohistological staining for specific markers (van Willebrand Factor, CD-31, CD-34, CD-146 and alpha-smooth muscle actin) and tubule formation was again without a control group. Although the study aimed for 'obtaining [FDA] approval for combination therapies (a cellular product plus a device)' (175), the models chosen were hardly suitable for translation.

We therefore wanted to further drive clinical translation and focused on an in vivo model that represented the clinical problem. Similar to Zimmerlin et al., we used the Tisseel sealant to avoid translational problems, but we investigated its effect on ASCs on a protein level in vitro and proved its efficacy in a diabetic wound healing model in vivo (176). While previous studies only concluded that fibrin glue is suitable for delivery of ASCs and that 'they may participate in granulation, regeneration, angiogenesis and anti-inflammatory response when applied in a wound-healing environment' (175), we proved these hypotheses. Furthermore, we were able to show a beneficial effect of fibrin on ASC survival and a fibrin-induced stimulation of regenerative cytokines – mechanisms that are crucial for wound healing.

Falanga et al. further cultured MSCs and delivered them with fibrin sealant to chronic and therapy-resistant wounds (257). They also used the Tisseel fibrin glue and investigated efficacy in a murine wound healing model and in a clinical setting. Initially, they harvested MSCs (as described in Chapter 2.1.3) by puncturing the iliac crest and aspirating BM. Because many studies underline significant differences between BM-MSCs and ASCs, our study is hardly comparable (258-261). Nevertheless, Falanga et al. successfully used BM-MSCs for tissue reconstruction in a clinical setting. Additionally, they created full excisional thickness wounds on the tail of C57BL/6 and db/db mice and treated them either with fibrin and MSCs or fibrin alone. No splinting of the wound was used, to guarantee humanised wound healing, which relies on granulation rather than contraction. They tracked viability by fluorescence labelling of MSCs. We modified this protocol and used luciferase-positive mice for harvesting of cells. An IVIS allowed life tracking of implanted cells and imaging of distribution and migration patterns (Figure 15). In contrast to Falanga et al., we used injected cells as a control group. Thereby, we could distinguish between fibrin-induced and MSC-

induced effects. We were able to show prolonged viability of fibrin-delivered MSCs, suggesting significant improvement of niche (262).

Falanga et al. found that fibrin alone could not enhance wound closure, but fibrin in combination with MSCs could. This effect was even enhanced in the diabetic model. Therefore, we investigated this phenomenon in depth. Additionally, their clinical trial showed no adverse effects, suggesting that MSC delivery by fibrin sealant is a safe and reliable procedure. It has to be mentioned that significance was only detectable when applying an adequate number of cells (at least 10^6 cells/cm^2). Therefore, our experimental setting was adapted, and we used that cell number for in vitro studies. The study of Falanga et al. represents a keystone for clinical translation of this approach. Nevertheless, performing a BM aspiration is an invasive procedure. Harvesting MSCs by simple liposuction, which has almost no adverse effects and is regularly performed, seems easier and might be more beneficial for patients.

4.4 Underlying Mechanisms

The regenerative potential of ASCs is mainly based on two synergistic effects:

- Asymmetric Division
- Secretion of cytokines

Thereby, ASCs can influence surrounding cells, attract specific cell types and regenerate mesenchymal tissue.

Adipogenic and osteogenic differentiation capacity- key effectors of asymmetric division can be tested by cultivation of ASCs in specific medium and histological analysis for calcificatzion (when testing osteogenesis) or lipid-formation (when testing adipogenesis).
For secretion of cytokines, either RNA-based profiling or ELISA-based profiling for were used.

4.4.1 Adipogenic and Osteogenic Differentiation

By using specific medium, that contains factors leading to differentiation of ASCs such as dexamethasone or indomethacin (regarding adipogenic differentiation) or ascorbate and beta-glycerophosphate (regarding osteogenic differentiation) cells are led towards specific pathways.
Although staining after 7 days with Oil-Red-O in adipogenic differentiation group and after 14 days by using a Alizarin Red as calcification-specific marker represented a quantitative endpoint, we additionally screened for PPAR-γ, FABP4 and LPL in adipogenic group and for RUNX 2, OPN and OCN by using RT-qPCR.

Markers were chosen according to their time dependent importance in tissue formation. While PPAR-gamma stimulates initial lipid uptake, FABP4 binds lipids within the cells and LPL leads to further lipid syntheses. Therefore, these markers can be seen as early, intermediate and late adipogenic markers. Similar RUNX 2 represents an early transcription factor, OPN is a gene being mainly expressed in early osteogenesis (263) and OCN marks mature osteoblasts (264).

We found significant enhanced adipogenic and osteogenic marker expression in VASER harvested ASCs when comparing to LYSONIX group. Nevertheless, no differences in Oil-Red-O Staining of Alizarin Red staining was found, strengthening the suggestion, that although changes between osteogenic and adipogenic marker profile were found, these are not significant enough to lead to changes in mineralization of lipid formation or to changes in wound healing kinetics. Again, it has to be notified, that q-RT-PCR is based on genetic expression profile of specific markers and underlies posttranscriptional modifications and possible silencing mechanisms. Therefore, histological staining, that displays a result of all cytokines and protein analysis are closer to reality than genetic analysis.

4.4.2 Upregulation of Key Cytokines

The secretion of cytokines, that are pivotal for immunoregulation, tissue homeostasis, regeneration, represents a main mechanism of ASCs. While paracrine factors can enhance tissue formation, stimulate and activate surrounding cells and lead to neovascularisation, several cytokines can systemically attract immune cells.
We focussed on key factors for tissue regeneration, represented by SDF-1, b-FGF, MMP-2, VEGF-A, HGF, and MCP-1.

4.4.2.1 Stromal-Derived Factor-1

SDF-1 is a key regenerative factor of MSCs. Its necessity for effective wound closure has been described extensively (265-268). Several studies suggest a stress-related mechanism of SDF-1 secretion (33, 265). SDF-1 is recognised by the CXCR-4 receptor (41, 45) and leads to transcription of downregulated genes. CXCR-4 is expressed on HSCs, endothelial progenitor cells, osteogenic progenitor cells, MSCs, fibroblasts, macrophages and many other immune cells (30, 39-41, 43-45, 268-275). By SDF-1/CXCR-4 binding, cells are trafficked towards the SDF-1 gradient to the injured tissue. All of these cells are crucial for neovascularisation, tissue regeneration and the immune defence, underlining the necessity of SDF-1 in wound healing.
Some research groups have successfully developed specific systems, scaffolds and wound dressings that continuously deliver exogenous SDF-1 to the wound environment, aiming to enhance wound closure (276, 277). Due to all the characteristics of SDF-1, its involvement in wound healing and stress-responsive pathways, and even the ability of external SDF-1 to improve wound healing kinetics, it should be undoubted that increased SDF-1 represent a key mechanism for accelerated wound healing in vivo.

4.4.2.2 Basic Fibroblast Growth Factor

B-FGF is another factor crucial for organogenesis, embryonal development and tissue repair (278). It interacts with the fibroblast growth factor receptor (FGFR), a tyrosine kinase receptor that activates intracellular pathways such as signal transducers and activators of transcription and RAS–mitogen-activated protein kinase (MAPK) mechanisms that are upregulated in tissue repair, but also in cancer development (278, 279). The necessity of FGF and its receptor is shown by achondroplasia, a disease that has its origins in an autosomal dominant mutation in FGFR-3 and therefore insufficient formation of cartilage. Achondroplasia results in disproportional dwarfism with short arms and legs (278). B-FGF is a cytokine released in response to cellular stress and tissue damage, and one of the first factors identified to play a crucial rule in tissue homeostasis and neovascularisation (280). In 1986, scientists purified b-FGF from bovine pituitary glands and successfully used it as a wound-healing agent (281). B-FGF has been shown to stimulate fibroblast proliferation, collagen production and migration, and the proliferation rate of endothelial cells (282, 283). These effects are crucial in the initial phase of wound healing (284). Due to all these abilities, recombinant b-FGF has been popularised as wound healing agent and extensively used as additive to scaffolds and hydrogels for enhancing tissue repair (285-289). In contrast, neither gelatine (288), chitosan (289), nor collagen and cellulose (290) loaded with b-FGF have entered clinical practice.

4.4.2.3 Matrix Metalloproteinase- 2

MMP-2 is a hypoxia-responsive cytokine responsible for matrix degradation (291). It serves as an antagonist and inhibitor of SDF-1 function (292). Although some reviews have described the stimulation of keratinocyte migration by MMP-2, other murine as well as human trials have shown a significant beneficial effect of selective MMP-2 inhibition on wound closure time (293, 294). Additionally, high MMP-2 levels in human wound fluid have been proven to be associated with the occurrence of non-healing wounds (295).

MMP-2 therefore seems to serve as a wound healing inhibiting factor. Decreased MMP-2 secretion of ASCs has not been described but might represent a possible mechanism for wound healing acceleration.

4.4.2.4 VEGF-A

VEGF-A is the key factor for neovascularisation and therefore tissue regeneration. It mediates angiogenesis, especially during the proliferative phase of wound healing (284). VEGF-A is also essential for chemotaxis and endothelial cell migration- a factor that can be histologically evaluated by quantification of CD31 positive cells (endothelial cells) (296).

The great potential of VEGF is represented by the effort to use recombinant VEGF as wound healing agent. In preclinical trials injected r-VEGF has led to acceleration of wound closure in diabetic mice (297), but interestingly, also non-treated wounds on the contralateral site of animals receiving VEGF therapy showed accelerated wound healing. These findings suggested an additional systemic effect of local VEGF treatment. The possibility of interacting with tumour growth and therefore potential malignancy has hindered its clinical translation. Additionally, only one experimental clinical trial has compared VEGF treatment with placebo, showing no significant benefit of VEGF (298).

Nevertheless, local VEGF levels are pivotal for neovascularization and thereby essential for wound closure.

4.4.2.5 Hepatocyte Growth Factor

HGF or also called Scattering Factor (SF) represents a potent growth factor, essential for embryonic organ development, for myogenesis, cell trophism and matrix formation (299, 300). HGF is not only secreted by stromal cells, it has also the potential to recruit progenitor and stromal cells and thereby induces a positive feedback mechanism (300).

HGF binds to specific thyrosine kinases such as MET, leads to their dimerization and thereby transcription of genes, that are essential for survival and motility of cells and tissue growth (301). Due to its efficacy HGF has become a main target for anti-cancer therapeutics (299, 301-303). Several studies suggest promotion of tumor metastasis by HGF (304-306).

4.4.2.6 Monocyte Chemoattractant Protein 1

MCP 1 represents the main molecule for recruiting Macrophages and regulator for T-lymphocyte differentiation. Pro-inflammatory cytokines such as IL-4, TNF-alpha are affecting MCP-1 secretion.
By binding to its G-protein coupled chemokine receptor CCR-2 it leads to dimerization, internalization and transcription of genes of the NF-Kappa-B pathway. Many recombinant and non-recombinant molecules like Glucocorticoids or Curcumin are non-selective MCP-1 inhibitors and thereby regulators of immune-reaction. Several studies have shown involvement of MCP-1 in inflammatory diseases, artherosclerosis or cancer progression (307-309).

We found significant changes in genetic expression of MCP-1 in Ultrasound assisted Liposuction samples, when comparing to SAL and abdominoplasty harvested ASCs. Interestingly MCP-1 has further shown to influence scar formation. Wong et al reported Fokal Adhesion Kinase mediated MCP-1 secretion to trigger the endogenous fibrotic response (310). They used a hypertrophic scar model in MCP-1 knockout mice and found 70% less scar formation than in control group. Again, we did not verify findings in an in-vivo model, although the described hypertrophic scar model would have been beneficial.

ASCs delivered in fibrin glue have the potential to enhance wound healing by significant upregulation of pro-regenerative cytokines and simultaneous downregulation of pivotal inhibitors of wound healing (Figure 14). Fibrin glue therefore

represents a scaffold-like delivery vehicle that can counteract the harsh wound milieu and showed upregulation of MMP-2, SDF-1 and b-FGF. Additionally, we strongly agree with previous studies that direct injection of cells into tissue or topical application can significantly decrease cellular function, either by mechanical forces or by limited cell adhesion (175). When considered in tandem with existing studies that have shown its clinical benefits, our mechanistic study demonstrates a significant enhancement of ASC wound healing capacity when provided with a fibrin glue niche. The fibrin glue significantly enhanced secretion of SDF-1 and b-FGF, regenerative cytokines that enhance wound healing by improving recruitment of circulating cells, supporting fibroblast function and promoting neovascularisation (285, 311, 312).

4.4.2.7 Prolonging Cell Survival

Many studies have previously described that the harsh wound environment, with diminished blood and nutritional supply, rapidly depletes injected ASCs (4, 218, 313, 314). Wound beds of older and diabetic patients are especially characterised by hypoxia, poor blood supply and bacterial contamination (315, 316). Clinically, all of these factors lead to difficulties in wound management and most likely ineffectiveness of cell-based therapies (4, 63, 176, 313). Therefore, delivery systems that enhance the cell niche and thereby the cell survival are essential. These delivery systems most likely are scaffolds in vitro seeded with cells, a method that is seen as critical regarding its translational potential. The two-chamber fibrin sealant has been shown to form scaffold-like porous structures excellent for adequate cell adhesion, and without any translational hurdles (174, 175, 247, 317).

To investigate cell characteristics and survival within this fibrin glue in an in vivo model, we used an IVIS system, luciferase-marked ASCs and a murine wound healing model with two wounds created on the dorsum of each mouse (Figure 15). Because the subdermal panniculus carnosus muscle would contraction and cause wound closure, we splinted the wound by suturing the wound margins to silicone rings. This model has been previously described and is well established (133, 242, 318). This combination

allowed us to track cell viability over time in a humanised wound healing model, whereby wound healing is only achieved by granulation. By measuring the luminescence signal we observed prolonged ASC survival in the fibrin group (176).
In addition to improving the cytokine profile of ASCs, the fibrin glue maintained the benefit of these enhanced ASCs within the wound environment for an extended period. Collectively, these effects improved neovascularisation and accelerated wound closure.

4.4.2.8 Enhanced Expression of Endothelial Cell Markers

To drive clinical translation, we confirmed these findings in a well-established murine diabetic wound model (98-100, 133, 242, 318-321). By applying immunohistochemistry for CD-31 we showed significantly increased endothelial cells in fibrin and ASC–treated wound biopsies.
CD-31 is a marker strongly expressed on human endothelial and hematopoietic cells (322). CD-31 was previously known as platelet endothelial cell adhesion molecule-1 (PECAM-1) (323). Notably, this surface marker is not expressed on MSCs or ASCs (324). Therefore, CD-31 expression in tissue biopsies of our wound healing model can be explained by two main mechanisms.
The first explanation is differentiation of MSCs into endothelial cells (324). Several studies have suggested that MSCs cultured under specific conditions or with specific media (324, 325) have the potential to differentiate into fully functional endothelial cells. This has been proven in vitro and in vivo (326).
The second explanation is recruitment of hematopoietic progenitor cells. Paracrine secretion of specific cytokines is well known to lead to recruitment of endogenous immune and hematopoietic cells (215, 327). These cells lead to enhanced neovascularisation and blood supply in the wound bed and in the newly formed granulation tissue. In the in vitro model, we successfully showed significant differences in secretion of some of these factors.

Immunohistochemical observations of all healed wounds show a higher prevalence of endothelial cells within groups treated with ASCs. Therefore, for their regenerative capacity enhancing neovascularization further can be seen as key mechanism for ASC efficacy. This can be attributed to more effective differentiation capacity to endothelial cells and higher secretion of hematopoietic progenitor cell migration cytokines.

4.5 Outlook

ASCs have shown to be pivotal for tissue regeneration. The upregulation of certain transcriptional pathways resulting in enhanced secretion of specific cytokines was proven beneficial to this phenomenon.

Recombinant Erythropoetin (rhEPO), a drug that has been developed to stimulate erythopoesis, has shown to be beneficial in treatment of chronic diseases and to significantly improve outcome in critical ill patients (328, 329). Due to the fact, critical illness most likely goes along with anaemia, these effects are not really surprising.

Within the last years, other molecular effects, that are independent from erythopoesis have been discovered. Not only systemic effects based on enhanced proliferation of erythroid progenitor cells, but local mechanisms were reported to enhance wound healing capacity (330). Subcutaneos injection of rhEPO led to significantly improved neoangiogenesis, epithelial proliferation and maturation of the ECM in a murine burn model (331). Additionally topical application of rhEPO showed benefits in wound healing kinetics of diabetic rats (332). These effects might be explained by local phosphorylation of the JAK-2 (Janus Protein-Thyrosin Kinase-2). Via the JAK-STAT pathway his phosphorylation leads to transcription of genes essential for tissue formation and neoangiogenesis (333). Furthermore, rhEPO has the potential to activate stromal cells that are in a quiescent state (Chapter 1.1.7).

But not only local cues are responsible for the acceleration of wound healing by EPO, also systemic effects have been described. Specific EPO agonists can accelerate homing ability of circulating endothelial progenitor cells (334).

Although benefits of rhEPO in enhancing MSC based therapies are obvious, several studies link EPO and EPO signaling with enhanced tumor growth and potential oncogenesis (335, 336). Other studies do not link application of rhEPO to tumor growth (337) or even describe an induction of antitumor responses (338).

To sum up, EPO has the potential to either enhance or replace Stromal Cell based therapies, by stimulating and enhance effectiveness of endogenous self-healing mechanisms. Nevertheless, potential oncogenicity should be further investigated before actual clinical use.

5 Material and Methods

5.1 Single-Cell Gene Expression Analysis and Evaluation of the Therapeutic Function of Murine Adipose-Derived Stromal Cells (ASCs) from the Subcutaneous and Visceral Compartment (75)

5.1.1 Isolation of murine SVF

We used 6 mice (CL58 BL/6) to harvest adipose tissue from the inguinal tissue (subcutaneous fat) and from the omentum (visceral fat). Mice were initially anaesthetized in a cage, that was filled with isoflurane gas (5%). Then mice were killed by cervical dislocation.

An laparotomic incision was made by using a scalpel and a scissor. Quickly, inguinal fat pads and visceral fat was preparated, seperated and washed with PBS. Then fat was digested by using Collagenase I (Roche Applied Science, USA, Indianapolis, IN) in a waterbath (37°C). After centrifugation of fat at 1300 rpm (340g) for 10 minutes at 20°C the supernatant was aspirated. The remaining cell suspension was resuspended with PBS, washed and again centrifugated (1300rpm, 340g, 10min, 20°C).

5.1.2 Isolation of ASCs and RNA expression analysis

ASCs were isolated by using FACS. Initially red cell lysis buffer was utilized to remove blood cells. Then cell number of SVF-single cell suspension was determined by using a Neubauer counting chamber. Therefore, the chamber was filled with 10 µl of cell suspension, counted and then cell number per ml was calculated.

For FACS a cell number of 200.000-1.000.000 was used for each sample. After centrifugation at 300g, the supernatant was removed completely and the pellet resuspended in a FACS staining buffer (PBS, 3% calf serum).

Then FACS sorting was performed according to manufacturer's protocols. CD34+/ CD73+/ CD90+/ CD105+ and CD45−/ CD31− were used as marker profile to exclude

hematopoietic and endothelial cells. We used a Becton Dickinson FACSAria Flow Cytometer to sort cells into a 96-well plate filled with 6 µl of lysis buffer in each well (one cell per well). We then used a reverse transcription and low cycle preamplification (Cells Direct- Invitrogen, Thermo Fisher Scientific, USA, Waltham, MS) in combination with TaqMan primer sets (Applied Biosystems, USA, Foster City, CA) according to manufacturer's instruction. Then cDNA was loaded on 96.96 Dynamic arrays (Fluidigm, USA, South San Francisco, CA) and q-PCR amplification was performed using the TaqMan primer set.

5.1.3 Humanized Wound healing model

To mimic human wound healing, we utilized a predescribed model (339). Wounds were created on the dorsum of fifteen 12-week-old male C57 BL/6 mice. 6mm punches were used to create two full thickness wounds on the dorsum of each mouse. Wounds were splinted by using silicone rings, that were sutured onto the edges of the sore.

Then mice were sorted in three groups (n=5). Group A received S-ASC (2x10^5) loaded hydrogels, Group B V-ASC (2x10^5) loaded hydrogels and group C only hydrogel. Therefore, a collagen-pullulan hydrogel was used. For production of hydrogel 2g pullulan (Hayashibara Laboratories, Okayama, Japan), 2g sodium trimetaphosphate (SigmaAldrich, USA, St.Louis, MO), 2g KCl (SigmaAldrich, USA, St.Louis, MO) in H_2O and rat tail collagen I (SigmaAldrich, USA, St.Louis, MO) were mixed. 2mm thick films were created by compression, following dehydration in ethylalcohol (100%), dehydration for 12 hours and washing steps with PBS. By cutting these films, we crated scaffolds with a diameter of 6mm and loaded these scaffolds with previously isolated ASCs. Then incubation at 37°C in 5%CO_2 was performed. This method has been successfully established in a previous study (340) .
To prevent external influences and self-manipulation of the mice, wounds were covered with occlusive dressings (Tegaderm, 3M, USA, St. Paul, MN).

Digital Photos were taken on specific days (0,3,5, 7, 11 and 14) and evaluated by using Image J (NIH, USA, Bethesda, MD).

5.1.4 Immunohistochemistry

Immediately after full wound closure, the mouse was killed by cervical dislocation after previous isoflurane anaesthesia. Histological samples of the wound were taken by excision. Then samples were embedded in O.C.T- Optimal Cutting Temperature formulation (Sakura Finetek, Netherlands, Alphen aan den Rijn). We cut seven micron thin sections.

These sections were stained with CD31 as primary antibody and DAPI to mark nuclei. CD 31 represents an antibody for endothelial-cells, that represents a valuable neoangiogenesis marker. A rabbit polyclonal anti-CD 31 antibody (Ab28364, abcam, UK, Cambridge) was used as primary antibody. After diluting 1:100 it was incubated at 4° overnight.

After washing steps with PBS, the second antibody (AF547- Life Technologies, USA, Carlsbad, CA) Goat anti Rabbit was incubated for one hour at 37°. Then microscopy was performed.

Image J (NIH, USA, Bethesda, MD) was used to quantify luminescence-signal based on the pixel positive area per field.

5.2 Differences in the regenerative potential of ASCs, derived by different liposuction methods

5.2.1 Harvesting of human SVF

We harvested lipoaspirate of six women (32 - 50 years old) by performing liposuction or abdominoplasty. All donors were healthy without any co-morbidities.
Three women obtained liposuction by using VASER (3rd generation) ultrasound assisted liposuction on one side of the abdomen and LYSONIX (2nd generation) ultrasound assisted liposuction on the other.
In three women, initially liposuction was performed by using VASER liposuction, then by conventional SAL. Then abdominoplasty was performed. By minimizing the suction area and then harvesting abdominoplasty samples, we ensured, that cells were not influenced by previous liposuction steps. For standardization, in all liposuction procedures a suction pressure of 700mmHg and a cannula diameter of 5mm was used.

Lipoaspirates then were digested by using 0.075% collagenase II (1:1) in a waterbath (37°) for 30 minutes. Every 10 minutes, cell probes were inverted to ensure appropriate digestion. To inhibit collagenase activity, the rim was filled with culture medium (DMEM-Medium high Glucose (4,5g/L) with stable glutamin, PAA, Cells Direct-Invitrogen, Thermo Fisher Scientific, USA, Waltham, MS) and mixed carefully. Then centrifugation was performed (1500 rpm, 350g for 20 minutes and 4°C). After discarding the supernatante the remaining cell-pellet was resuspended with 5 ml of Dulbecco`s PBS (1x) without Calcium and Magnesium (SigmaAldrich, USA, St.Louis, MO), filtered through a 100 µm cell strainer (Falcon, BD Bioscience, USA, San Jose, CA) and centrifugated again (350g for 15 minutes). By using a red cell lysis buffer (SigmaAldrich, USA, St.Louis, MO) blood cells were eliminated. Centrifugation as suggested by the manufacturer (300g for 7 minutes) was performed.

To harvest SVF from abdominoplasty samples, initially skin was discarded. Subcutaneous fat was cut into small pieces of approximately 0.5x0.5x0.5mm each. Then several washing steps were performed. Digestion of the Extracellular matrix and isolation of SVF was performed identical to lipoaspirate samples.

5.2.2 Isolation of ASCs

Due to changes in transcriptional pattern during cultivation steps, pelleted SVF were directly taken to FACS. We used a BD FACSAria machine (BD Bioscience, USA, New Yersey, NY) and CD45−/CD31−/CD34+ as surface marker profile. FACS-sorting was performed as described in 5.1.2. (Isolation of ASCs).
All antibodies were mouse anti human from BD Bioscience (CD-31-PE, CD-45-PeCy7 and CD-34-APC). Propidium iodide was utilized for exclusion of dead cells.

5.2.3 Cell viability

Cell viability was measured by using a Vybrant MTT Cell Assay Kit (Invitrogen, Carlsbad, USA, CA). Basis of this test is metabolic activity of cells. Tetrazolium salt 3-[4,5-di-methylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) is the main ingredient of this test. This reagent is metabolized by active cells and thereby changes its colour from yellow to purple. All steps were performed as suggested by the manufacturer. Quantification was performed by measuring absorbance at a wavelength of 540nm.

5.2.4 Osteogenic differentiation

Osteogenic differentiation capacity was tested in vitro by using osteogenic differentiation medium. After seeding ASCs, that have been harvested as decribed above, in six- well plates, wells were filled with DMEM containing 10% FBS and Penicillin/Streptomycin 1%.

When wells reached 80% confluency we harvested total RNA in Trizol (ThermoFisher, USA, Waltham, MA), osteogenic differentiation medium (a mixture of DMEM plus GlutaMAX, 10% FBS, 1% Penicillin/Streptomycin, 100 µg/mL ascorbic acid and 10mMol β-glycerophosphate (SigmaAldrich, USA, St.Louis, MO) was applied to the cells. Wells were filled to the rim and medium was changed every 3 days.
RNA was harvested on day 7 and on day 14 after beginning of osteogenic stimulation. We evaluated gene expression profile of essential osteogenic factors (RUNX2- runt related transcription factor 2, a gene that mainly regulates osteoblast formation and proliferation (341); OCT- osteocalcin, a peptidhormone and specific marker for bone formation and OPN- osteopontin, a bone marker, that is present in calcified tissue and inhibits bone mineralization, and stimulates inflammation, cell adhesion and migration). Therefore, quantitative real time Polymerase Chain reaction (qRT-PCR) was performed by using Applied Biosystems Prism 7900HT Sequence Detection System.

After 14 days of cultivation, Alizarin Red staining was performed. Therefore, medium was aspirated and discarded. Then PBS was used to wash cells and then cells were fixed by using 3.7% Formaldehyde Solution for 5 minutes at room temperature.
Alizarin solution was freshly prepared by dissolving 0.5g Alizarin S in 100ml Ultrapurewater. Then wells were filled with Alizarin solution and incubated at room temperature for 30 seconds- 5 minutes.
After washing steps (Tab water) pictures were taken.

5.2.5 Adipogenic differentiation

Adipogenic differentiation capacity was tested in vitro by utilizing and seeding ASCS into six-well plates and filling the well to the rim with DMEM (10% FBS, 1% Penicillin/Streptomycin). When 80% of confluency was reached, culture medium was discarded and adipogenic differentiation medium was applied to the cells.
A suspension of DMEM plus GLutaMAX with 10%FBD, 1% penicillin and streptomycin, 10 µg/mL insulin, 1 µM dexamethasone (SigmaAldrich, USA, St.Louis, MO), 0.5 mM

methylxanthine (SigmaAldrich, USA, St.Louis, MO), and 200 µM indomethacin (SigmaAldrich, USA, St.Louis, MO) was used as adipogenic differentiation medium. Medium was filled uo to the rim.

Compareable to osteogenic differentiation, RNA was harvested prior cultivation and after 7 days. A qRT-PCR (Applied Biosystems Prism 7900HT Sequence Detection System) was used to evaluate gene expression levels of adipogenic key factors (peroxisome proliferator- activated receptor γ (PPAR-γ), fatty acid binding protein 4 (FABP4), and lipoprotein lipase (LPL).

Additionally Oil-Red-Staining (SigmaAldrich, USA, St.Louis, MO) was performed according to manufacturer´s guidelines. After removal of the medium and washing with PBS, formalin (10%) was added to the wells and incubated tor 30 minutes. Cells were washed twice with water and then 60% isopropanol was added to the cells. After removal of isopropanol, Oil Red-O solution was added and left for 10 minutes. After removal of Oil Red, Hematotoxylin was added and cells were incubated for 1 minute and then washed 5 times with tub water. Then microscopy was performed- lipids stained in red, nuclei in blue.

5.2.6 Animal model

Animal experiments were carried out in accordance with institutional animal guidelines. To test regenerative potential of ASCs in vivo, we obtained nude male CRL:CD-1-FOxn1^{nu} mice between 8 and 12 weeks of age from Charles River Laboratories (USA, Wilmington, MA). Wounds were created as described above (3.1.3.).

ASCs were loaded onto previously described Pullulan-Collagen hydrogels (3.1.3.). Then hydrogel sheets were placed in the wound beds and covered by occlusive dressings.

Therefore, mice were randomized into three different groups (n=8):

- Group 1 received treatment with hydrogel seeded with VASER harvested ASCs
- Group 2 received treatment with hydrogels seeded with LYSONIX harvested ASCs

- Group 3 received treatment with hydrogels alone

Photography was performed on day 0, 3, 5, 7, 9, 11, 13 and 15.

5.2.7 Immunohistochemistry

Healed wounds were exzcised and immunohistochemistry was performed as described in 3.1.4.

5.3 Optimizing the delivery method of ASCs

5.3.1 Expansion of Cells

ASCs from three different healthy donors were purchased from Lonza Group (Basel, Switzerland). To avoid any bias, all cells were collected from middle-aged donors and expanded under standard cell culture conditions in StemMACS expansion medium (Miltenyi Biotec, Bergisch Gladbach, Germany).

5.3.2 Experimental Set-Up

For experimental seeding, the ASCs were initially pooled and then counted with a Casy TT Counter (Omnilife Science, Bremen, Germany). Three different conditions were performed in triplicate (Figure 28). For the standard cell culture condition, 10^6 ASCs were resuspended in 8-mL StemMACS expansion medium and seeded in 10-cm dishes.

Cell culture in fibrin glue was performed with Tisseel fibrin glue (Baxter, Illinois, USA; Figure 11). Both components, fibrin and thrombin, were prediluted 1:4 in StemMACS expansion medium. 10^6 ASCs were resuspended in 4 mL of prediluted thrombin, transferred to a 10-cm dish and then mixed with 4 mL of prediluted fibrin.

As an additional control, fibrin glue without cells (prepared identically as described above) was included in the experimental set-up. All dishes were filled with 24 mL of

StemMACS expansion medium and cultivated for one week under standard cell culture conditions. Cell seeding was performed 7 days after cultivation. The supernatant was aspirated and centrifuged to obtain cell-free material for analysis. Cell-free supernatant was aliquoted and stored at -80 °C until analysis.

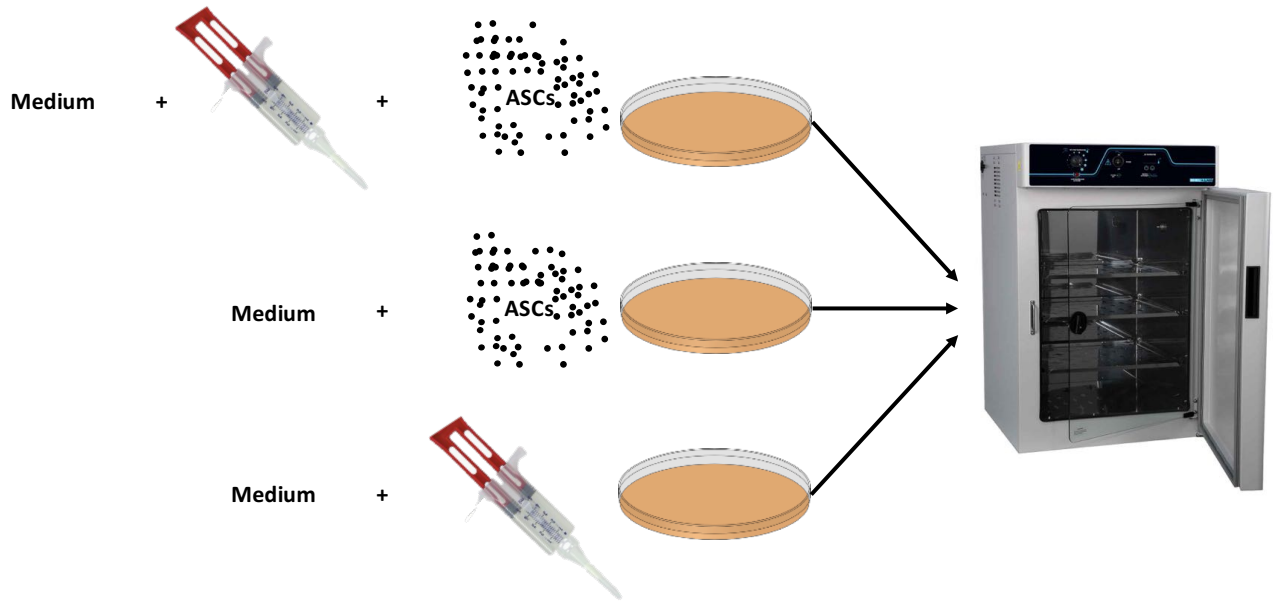


Figure 28 Experimental set-up. Three groups (fibrin, ASCs and medium; medium and ASCs alone; and medium and fibrin alone [negative control]) were seeded and incubated in 10-cm dishes for one week. Then analysis for protein secretion was performed.(n=3)

5.3.3 Protein Analysis

To examine protein expression, we initially performed a Proteome Profiler Human Pluripotent Stromal cell Array Kit (R&D Systems, Minnesota, USA) containing 15 markers for stemness as the following:
Cell lysis was performed using 200 µl of lysis buffer per well for 30 minutes, samples were vortexed for 5 minutes and immediately processed according to manufacturer instructions, using an exposure time of 34.2 seconds.
For analysis of specific protein secretion, supernatant were defrosted. Then, SDF-1 alpha, b-FGF/FGF2, VEGF and matrix metalloproteinase-2 (MMP-2) ELISAs (all Duoset, R&D Systems, Minnesota, USA) were performed according to manufacturer instructions. A BertholdTech Mithras Reader (Bad Wildbad, Baden Württemberg, GER) was used for quantification.

5.3.4 Animal Model

For animal experiments we used db/db mice and luciferase positive mice, ordered from Charles River Laboratories (Wilmington, MA, USA).
For IVIS experiment, luciferase positive mice were used as donor organisms for harvesting ASCs. Db/db mice serves as recipient organisms and wound healing models. Therefore, we used the previously described murine wound healing model.
For studying wound healing kinetics, mice were randomized in three groups (Figure 29):

- ASC+ Fibrin group
- ASC+ PBS group
- Control group

Digital photography was performed on days 0, 4, 8, 12, 16, 18 and 20 to evaluate wound healing kinetics and assessment was performed using Image-J. After complete wound closure wounds were excised and taken for immunhistological evaluation.

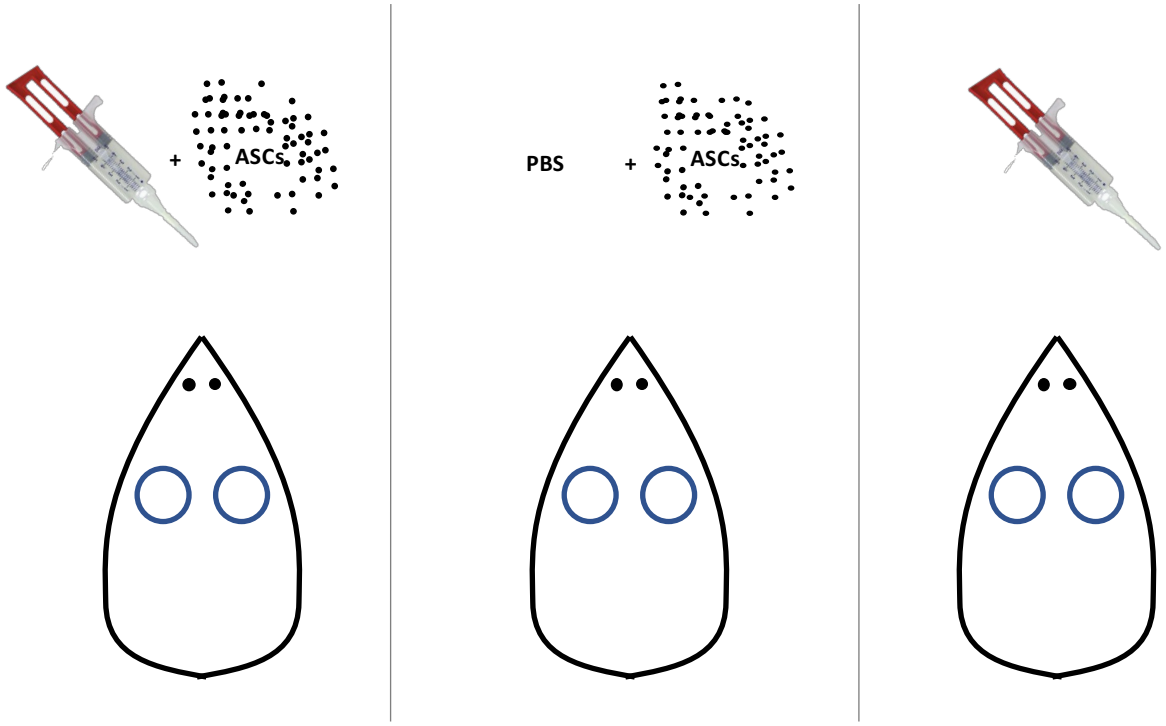


Figure 29 Set-Up of the experiment- two full-thickness excisional wounds were created at the dorsum of each mouse and treated either with Fibrin Glue and ASCs, PBS and ASCs or with Fibrin Glue alone.

5.3.5 In vivo imaging System

To evaluate survival rate and time, an In vivo imaging system (IVIS- Caliper Life Sciences, Hopkinton, MA) was used. Unlike in other models, it was possible to specifically stain single ASCs using luminescence dye.

On day 0,3,5,7 and 9, db/db mice, that received ASC-treatment from luciferase positive mice, were anaesthetised by using a chamber filled with 2.5% isoflurane. Then we injected 200 uL of D-Luciferin (Perkin Elmer, Walham, MA) intraperitoneally. While constant anaesthesia by isoflurane 1.5% for 60 seconds, luminescence signal of wounds was evaluated and quantified. Therefore, photons per centimetre squared per steradian were calculated (342).

5.3.6 Wound Healing Kinetics

As shown previously we used a CD31 and DAPI staining to assess wound healing.

5.3.7 Statistical Analysis

For all testing, mean, Standard Deviation as well as the SEM were calculated. A p-value of <0.05 was considered as statistically significant. A student´s T-test was used to compare groups. The Bonferroni method was used to correct the result.

6 Conclusion

While ASCs represent a novel and effective method for enhancing regenerative medicine in many different fields, many uncertainties are hindering their clinical translation. For preclinical studies, ASCs obtained from different donor-regions are used, but no currently available studies investigate effects of donor region on ASC characteristics. Therefore, we analysed subcutaneous and visceral murine ASCs and found significant differences on gene expression level, but no differences on protein level and in testing their ability to close wounds.

Constant evaluation of novel liposuction methods and their effects on ASCs is essential to ensure maximal effectiveness. Therefore, we compared the regenerative potential of ASCs harvested by two different Ultrasound assisted Liposuction methods and found significant differences regarding their differentiation profile. When comparing Ultrasound assisted Liposuction with Suction assisted Liposuction and ASCs harvested by Abdominoplasty, only slight differences in genetic expression of kex cytokines was found.

To optimize delivery method, Fibrin glue, a novel, simple method was used to enhance function and efficacy of ASCs by delivering them to chronic wounds. This might represent a novel therapy with no translational hurdles and open the possibility of stromal cell–based therapy for many approaches.

7 References

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8 Publications

8.1 Original Articles

Hopfner, U.*, Aitzetmueller, M. M.*, Neßbach, P., Hu, M. S., Machens, H. G., Maan, Z. N., & Duscher, D. (2018). Fibrin glue enhances adipose-derived stromal cell cytokine secretion and survival conferring accelerated diabetic wound healing. Stem cells international, 2018.

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8.2 Book Chapter

Aitzetmüller, M. M., Machens, H. G., & Duscher, D. (2019). Challenges and Opportunities in Drug Delivery and Wound Healing. In Regenerative Medicine and Plastic Surgery (pp. 27-38). Springer, Cham.

Mihalceanu, S., Aitzetmüller, M. M., Machens, H. G., & Duscher, D. (2019). Stem Cell Therapies for Tissue Regeneration and Wound Healing: Strategies to Enhance Therapeutic Effectiveness. In Regenerative Medicine and Plastic Surgery (pp. 187-199). Springer, Cham.