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der Heinrich-Heine Universität Düsseldorf
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Comparative Evaluation of Organic and Synthetic Matrix Structures Combined with Adipose Tissue-Derived Multipotent Cells in a Murine Skin Wound Model

Dissertation

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Meiner Familie und Freunden

„Man darf nie an die ganze Straße auf einmal denken, verstehst du? Man muss nur an den nächsten Schritt denken, an den nächsten Atemzug, an den nächsten Besenstrich. Und immer wieder nur an den nächsten.“ Wieder hielt er inne und überlegte, ehe er hinzufügte: „Dann macht es Freude; das ist wichtig, dann macht man seine Sache gut. Und so soll es sein.“ (Auszug aus Momo, Michael Ende, 1973)

I. Summary

In reconstructive surgery, tissue engineering opens up magnificent alternative treatments. As one prospective source adipose derived multipotent cells keep the ability to enhance wound healing[1,2] in order to decrease the patient's pain and most likely the time of hospitalization[3]. Therefore the adipose-derived stromal vascular fraction (ASVF), deviating from adipose-derived stem cells (ASC) which are laboratory processed for a certain time, provide a potential future source for soft tissue wound regeneration[4,5].

Aim of this study was to determine the potential of these fresh cell aspirates collected from liposuction procedures. The hypothesis was that this initially less passaged, less differentiated, untreated and unselected heterogenic population (ASVF) represents an attractive alternative source for potential applications in regenerative medicine.

To verify and compare the effect of ASVF and ASC on wound healing two prospective in vivo study designs were established and the results further analyzed. Prior to this ASVF and ASC were phenotypically characterized by fluorescence activated cell sorting analysis. In the Collagen Matrix Study the effect of directly applied ASVF and ASC suspension respectively in combination with a collagen matrix on wound healing was investigated. The Seeded ADM Study examined the efficiency of an ASVF loaded human acellular dermal matrix. To monitor wound healing progression over time the area of reepithelialization and microvascular density were analyzed by using planimetric calculations and antibodies directed against the endothelial vWF glycoprotein.

In the first study the effect of ASVF as well as ASC on wound healing was not significant. Possibly applied cell populations need a niche, a suitable carrier vehicle, to survive and support wound healing. In the second study design a contribution of ASVF to wound healing, as proven before for ASC, could not be finally clarified for ASVF because of the undetermined interaction of ASVF and the applied carrier matrix, maybe in respect to cell adherence and viability. Generally speaking, more parameters than the area of reepithelialization and vascularization over time need to be considered to define the conditions of effective wound healing using fresh cell aspirates. Finally there is no consensus to rule out the benefits of tissue reconstruction by using ASVF.

To realize a final explanatory framework for the conditions of ASVF in facilitating wound healing I suggest more fundamental research in respect to the properties and potentials of ASVF. This study was an attempt to minor this gap. It is important that further research is run on this topic to approach the ambitious goal of a manufactured skin substitute that matches the quality of autologous skin.

I. Zusammenfassung

In der plastischen Wiederherstellungschirurgie eröffnet die Gewebezüchtung (tissue engineering) eine Vielfalt an alternativen Behandlungsmöglichkeiten. Aus Fettgewebe isolierte Stammzellen (adiopse-derived stem cells) haben das Potenzial Wundheilung zu verbessern[1,2] und Schmerzen sowie den Hospitalisierungszeitraum des Patienten zu verringern[3]. Eine vielversprechende Quelle zur Therapie oberflächlicher Wundheilungsstörungen stellen insbesondere stromavaskuläre Zellfraktionen, die aus Fettgewebe gewonnen werden (adipose-derived stromal vascular fraction (ASVF)), dar. Diese sind im Gegensatz zu Stammzellen, die aus dem Fettgewebe isoliert und über einen längeren Zeitraum im Labor kultiviert werden (adipose-derived stem cells (ASC)), in kürzester Zeit für die Wundtherapie verfügbar[4,5].

Ziel dieser Arbeit war es, das Potenzial dieser durch elektive Fettabsaugung gewonnenen, unbehandelten Zellfraktion herauszuarbeiten. Die Hypothese war, dass diese weniger passagierte, weniger ausdifferenzierte und unbehandelte heterogene Zellpopulation (ASVF) eine attraktive Alternative in der regenerativen Medizin darstellt.

Um den Einfluss von ASVF und ASC auf Wundheilung zu verifizieren und zu vergleichen, wurden zwei prospektive in vivo-Studien durchgeführt und deren Ergebnisse analysiert. Im Vorhinein wurden ASC und ASVF mittels fluoreszenzbasierter Durchflusszytometrie (FACS-Analyse) phänotypisch charakterisiert. In der Kollagenmatrix-Studie (Collagen Matrix Study) wurde der Effekt auf die Wundheilung von direkt applizierten ASVF respektive ASC unter Verwendung einer Kollagenmatrix untersucht. Die Hautmatrix-Studie (Seeded ADM Study) prüfte die Leistungsfähigkeit einer ASVF beladenen humanen azelluären Hautmatrix. Um den Fortschritt über die Zeit zu beobachten, wurden die reepithelialisierte Fläche und die mikrovaskuläre Dichte mittels Wundflächenvermessung und Antikörperfärbung analysiert.

In der ersten Studie zeigte sich kein signifikanter Effekt von ASVF und ASC auf die Wundheilung. Um ihr vollständiges Potenzial in der Wundheilung zeigen zu können, brauchen stammzellnahe Zellen zum Überleben möglicherweise ein adäquates Transportmedium, eine Art Nische. Ebenso konnte der zuvor demonstrierte Beitrag von ASC zur Wundheilung in der zweiten Studie unter Verwendung von ASVF nicht reproduziert werden. Die Interaktion zwischen ASVF und angewendeter Trägermatrix kann nicht eindeutig definiert werden, weil die Zelladhärenz und Überlebenszeit von ASVF auf der Trägermatrix unklar bleiben. Insgesamt zeigen die Studien, dass es mehr Parameter als die Reepthelialisierung und Vaskularisierung braucht, um die Bedingungen effektiver Wundheilung unter Anwendung von stromavaskulären Zellfraktionen zu definieren. Insgesamt bleiben die Vorteile von in der Wundheilung eingesetzten ASVF ungeklärt.

Um ein ganzheitliches Erklärungsmuster für die Bedingungen von ASVF und deren Förderung von Wundheilung zu erhalten, empfehle ich weitere grundlegende Forschung zu Stammzellen, insbesondere der stromavaskulären Zellfraktion, die aus Fettgewebe isoliert wurden, deren Eigenschaften und Potenzialen. Diese Studien waren ein erster Versuch, diese Lücke zu verkleinern. Um das ambitionierte Ziel, ein Hauttransplantat herzustellen, das der Qualität eines autologen Transplantates entspricht, zu erreichen, ist es von elementarer Bedeutung, dass weiterhin auf diesem Gebiet geforscht wird.

II. Abbreviations

ACUF	Animal Care and Use Form
ADM	human acellular dermal matrix (AlloDerm™)
ASC	adipose-derived stem cells
ASVF	adipose-derived stromal vascular fraction
BM-MSC	bone marrow-mesenchymal stem cells
CD	cluster of differentiation
CMF	Collagen Matrix Film™
CSS	cultured skin substitute
DAPI	4´,6-diamidino-2-phenylindole, dihydrochloride
ECM	extracellular matrix
EDTA	ethylenediamine tetracetic acid
e.g.	exempli gratia (for example)
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate-conjugated
GM	growth medium
HSC	human stem cells
KGF	keratinocyte growth factor
MSC	mesenchymal stem cells
PBS	Phosphate Buffered Saline
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
rpm	rounds per minute
SD	standard deviation
Sca-1	stem cell antigen 1
SSEA-4	stage specific embryonic antigen 4
U	units
vWF	von Willebrand Factor
VEGF	vascular endothelial growth factor

III. Table of Content

1. Introduction

1.1 Tissue Engineering

Given recent progress in tissue engineering methods regarding the substitution of diseased or damaged tissue, alternative ways to repair wounds which have been difficult to manage before are emerging in reconstructive surgery. So far tissue as well as organ loss due to oncologic or traumatic processes are mainly replaced by organ transplants. In contrast, in other fields artificial spare parts e.g. heart valves or hip-prothesis are commonly used. Recent advances in stem cell research and tissue engineering represent a prospective source for therapy of acute and chronic skin wounds.

The term “tissue engineering” evolved in 1987 during a meeting sponsored by the National Science Foundation and implies the use of living cells, biomolecules and/or biomaterials[6]. An important task of tissue engineering is the development of skin grafts for the treatment of large lesions after traumatic insults or burns. The ultimate goal of tissue engineering is to provide a skin that matches the quality of the autologous skin graft[7]. A major consideration is to identify, analyze and evaluate suitable sources of cells and biomaterials as well as protocols which allow to form an artificial skin to promote wound healing properly.

1.2 Skin

The skin is the largest organ of the human body. Depending on the individual body size it may weigh up to 3kg, including the hypodermis even up to 20kg. The skin covered surface area is approximately $2m^2$.

The skin functions are essential to shield the body from exterior mechanical (e.g. wounding), chemical and thermal damages. Skin by its vascular architecture and sweat glands is involved in processes like thermal regulation. In its complexity it also functions as a sensor for surrounding stimuli. Impression, pain and temperature are detected by its corresponding receptors. In addition, skin provides a sufficient immune barrier against microbial invasion from the external world.

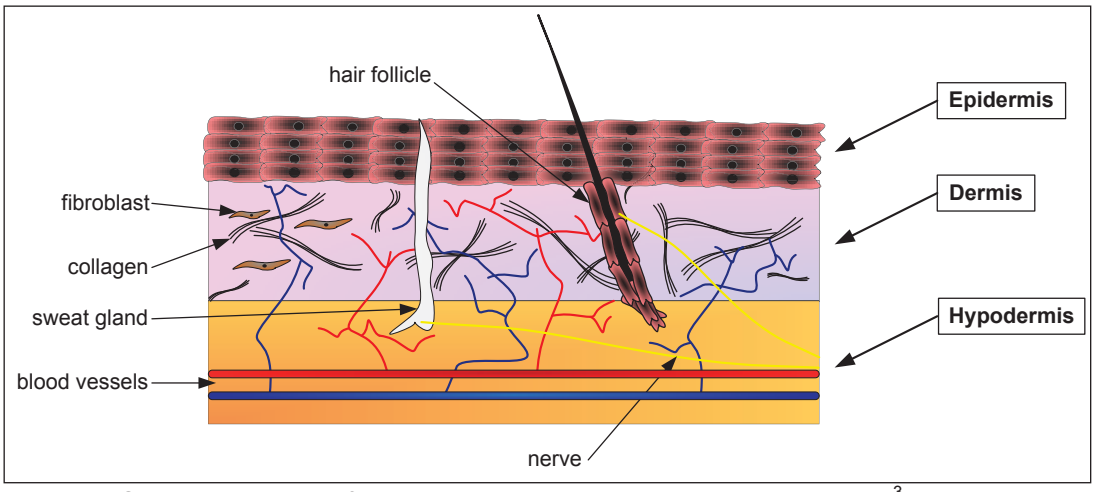

Figure 1. *Schematic model of the skins structure* (own illustration based on [3])

The skin consists of three layers. The epithelial layer (epidermis) includes a regenerative squamous epithelium including keratinocytes, melanocytes for pigmentation and mechanoreceptors. This layer and its appendages (hair, nails and sweat glands) is maintained by constant recycling of the basal layer. Newly generated cells migrate to the surface and replace damaged cells of the epithelial surface. One factor contributing to surface cell damage is e.g. ultraviolet radiation. The second layer (dermis) is directly adjacent and toothed to the epidermis. It contains a rich vascularization and the major cell type in this layer is the fibroblast. By producing collagen, elastin and glycosaminoglycans fibroblasts keep the mechanical strength of the cutis. Furthermore enzymes like proteases and collagenases produced by these cells play an important role in the wound healing processes. The third layer, the hypodermis (subcutis), which is mainly composed of adipose and loose connective tissue forms the connection between cutis and body structures like fascia and periosteum. It functions as a pressure pad and shifting layer. In addition, it contributes to the thermoregulatory properties of the skin by its rich vascularization[3,8,9].

1.3 Skin Substitutes

Chronic ulcers, trauma or large burns require an instant coverage to ease repair and reinstate skin function. The autologous skin graft (Table 1) is still the surgical method of choice in an attempt to repair large lesions[10,11].

Graft take is defined as the course of the union of the graft, the recipient area and its vascularization. Graft take is among other contributing factors dependent

on antigenic compatibility, ability of the recipient area to perform neoangiogenisis and adherence of the graft to the wound site. As well the recipient area should be not infected with a proper wound debridement, not excessively exsudative and capable of producing granulation tissue. A moist environment with reduced fluid loss, maintained homeostasis and a tight link by a proper bandage is essential to prevent graft detachment[10]. The capillary merge and anastomosis of autograft and recipient as well as the non immunogenicy of total skin grafts is still a medical challenging topic[12].

However, removing skin for autologous grafting is a painful process and the quantity of skin that can be harvested is limited. Regarding to origin and dimension of the lesion autologous skin grafting may be limited e.g. in patients with extensive burn lesions[11,13].

In view of these obstacles tissue engineering of artificial skin that matches the quality of the autologous skin graft[7] and fulfills the manifold functions of human skin[14] is of particular medical need.

Grafttype	Epidermis	Dermis	ECM Equivalent	Durability
Total Skin Graft				
Split Skin Graft	*autolog*	*autolog*	*autolog*	*permanent*
Karoskin™	*allogen*	*allogen*	*allogen*	*temporary*
EZ-Derm™	–	*porcine derived xenograft of collagen crosslinked with aldehyde*		*temporary*
Acellular Skin Substitutes				
Integra™	*Siliconpolymer layer*	*biodegradable bovine collagen with Chondroitin-6-sulfate glycosaminoglycan*		*temporary*
AlloDerm™	–	*processed allogenic cadavar donor tissue*		*permanent*
Cellular Allogenic Skin Substitutes				
Apligraf™	*neonatal keratinocytes*	*neonatal fibroblasts*	*bovine collagen matrix gel*	*temporary*
Orcel™	*neonatal keratinocytes*	*neonatal fibroblasts*	*bovine collagen matrix sponge*	*temporary*
Cellular Autologous/Xenogenic Skin Substitutes				
Epicel™	*autologous keratinocytes in coculture with proliferation arrested murine fibroblasts*	–	–	*permanent*
Cellular Autologous Skin Substitutes				
	autologous keratinocytes	*autologous fibroblasts*	*Collagen - Glycosamino-glycane*	*permanent*

Table 1. *Systematic overview of relevant available skin substitutes* (own illustration based on[11,13,15–20])

Promising allogenic and xenogenic skin substitutes for restoration of wounds like Karoskin™, an allograft (Table 1), also distributed by the European tissue bank or EZ-Derm™, a porcine-xenograft (Table 1), provide possible ready to use off the shelf solutions in cases of autologous donor graft shortage. These skin grafts can be used as temporary covers, as a bridge to transplant to reduce thermal and fluid loss as well as to reduce infection rates and progression of granulation tissue[13,21].

Although temporary pain relief [16] and the previously described features are rewarding, there are disadvantages regarding these total skin grafts. Both grafts are immune rejected by the recipient, do not get incorporated into the wound bed and need to be removed after a certain time[16]. Finally, allogenic skin grafts bear the risk of transmitting viruses such as Hepatitis B Virus and Human Immunodeficiency Virus[13]. Noticeably, in partial thickness burns Healy et al. showed no difference between xenogenic grafts and petrolatum non-adherent gauze dressing for bacterial contamination rates, healing time, pain relief and frequency of wound dressing change[22].

Acellular skin substitutes like Integra™ mimicking the skin structure are already in use and supposed to be the gold standard for dermal substitutes[23]. Integra™ is assembled of two layers: a biodegradable dermal bovine collagen-chondroitin-6-sulfate matrix and an artificial silicone polymer epidermal-like layer (Table 1). The artificial epidermal layer creates a protective barrier to minimize vapor loss and microbial contamination. Resident cells at the wound bed infiltrate into the collagen matrix and spontaneously convert into a dermis-like tissue. Once the donor sites are available for autografting (approximately 21 days after grafting) the epidermal-like layer is removed and the final autograft is applied to the wound bed[11,24,25].

AlloDerm™ is a similar skin substitute that has successfully been used for reconstruction[26,27]. This human acellular dermal matrix (ADM) is derived from donated human skin (Table 1). To prevent graft rejection the human donor tissue undergoes a multi-step process. All cellular components and transmissible pathogens are removed and finally the tissue matrix is preserved with a freeze-drying process. After this process a framework of human extracellular matrix (ECM) is left [28]. AlloDerm™ gets incorporated with

revascularization and cellular repopulation without evidence of rejection or replacement by scar tissue[29]. As well AlloDerm™ prevents wound contraction and provides mechanical stability. The lack of an epidermal layer requires the use of thin autograft[13].

Commercially available skin substitutes like AlloDerm™ in combination with an autograft have successfully resurfaced full thickness burn lesions[29–31].

1.4 Skin Substitutes and Living Cells

Cellular allogenic skin substitutes like Apligraf™ and OrCel™ have effectively been used in clinical wound treatment. Both substitutes have a similar composition and contain allogenic fibroblasts as well as keratinocytes derived from neonatal foreskin (Table 1).

Apligraf™ decreased the time for wound closure using a collagen gel as matrix and increased the percentage of healed wounds in clinical application. Moreover, no graft rejections were noticed[32–34]. The considered mechanism of action by which Apligraf™ may stimulate wound healing are persistence of graft cells at the wound site, where they may produce growth factors which stimulate wound healing by secondary recruitment of cells from the wound edges[20]. However, the temporary nature of the allogenic cells used in Apligraft™ that survive for 1 to 2 months requires co-grafting with an autologous epithelial source e.g. split skin graft. The short shelf life of 5 days and the allergic potential of the bovine component as well as the recommendation of a special training for the operator e.g. nurses and doctors face the complexity and the limitations of this substitute[16,20].

OrCel™ provides a collagen sponge as scaffold (Table 1) and mediates significant faster healing rates and reduced scarring in burn patients[19]. Beneficial treatment was attributed to the sponge's presence and the appearance of cytokines and growth factors produced by the living cells. Among others, multiple cytokines and growth factors such as FGF-1, KGF-1, PDGF-1, VEGF and TGF-α secreted by the living cells form an adequate wound bed for further skin graft placement[16,19].

There is still a limitation regarding these materials because the cell engraftment cannot be guaranteed. All of the substitutes are suitable for provisional wound

coverage and an advantage is that they are available for application whenever they are needed.

It appears that optimal skin substitutes for constant wound closure need to be settled by autologous cells because once a substitute is engrafted the natural stability is restored and a permanent wound closure is achieved. Therefore, transplantation of autologous cells might represent an important strategy to improve the outcome of skin substitute application. Due to the need of cell isolation and expansion only delayed application is feasible. The time until application of autologous seeded skin grafts varies depending on the size of the lesion as well as the number and growth rate of cells used for seeding.

This fact is illustrated by an approach using a graft composed of autologous keratinocytes harvested from hair follicles and plated onto silicone dishes for expansion. Although it was shown to successfully improve the outcome of a small leg ulcer it takes about 6 weeks to prepare a cell population of sufficient size[35].

For larger wounds Epicel™, a cultured epidermal autograft, has shown its benefit in patients with large burn lesions[36]. The nomenclature autograft is confusing because the autologous keratinocytes are cocultured in vitro with proliferation arrested murine fibroblasts (Table 1). Therefore the Federal Drug Agency (FDA) considers it as a xenotransplantation product[17]. The material is fragile and even small amounts of friction during engrafting result in blisters. High costs in preparation as well as the required intensive care are mentioned as disadvantages by the author[36]. Other disadvantages included aesthetic issues like scarring and hyperkeratosis. The most important aspect to be mentioned is that Epicel™ substitutes only the epidermal layer and lacks an integrated dermal component[11,17].

An advanced procedure of cultivating autologous fibroblasts and keratinocytes collected from a small skin biopsy creates a cultured skin substitute (CSS) (Table 1)[37,38], providing a skin replacement which showed excellent results in clinical application[18]. Still there is a lack of vascularization which consequently ends up in a deficit of cell nutrition at the graft site and increases the risk of microbial contamination. Studies addressing this issue found that CSS

incubated with nutrients improve wound healing[39]. Irrigation with a specific antimicrobial solution may also improve CSS engraftment[40]. Attempts to increase vascularization have been made but their benefit still needs to be demonstrated[41]. A known limitation regarding this effort is that cultivating autologous endothelial cells, isolated from the same biopsy like fibroblasts and keratinocytes, delay the CSS preparation and implantation[42]. At the moment no manufactured skin substitute has shown results akin to an autograft.

1.5 Cell Source

In search of alternative cell sources, bone marrow-mesenchymal stem cells (BM-MSC) might be an appropriate source for tissue regeneration due to their capability of self renewal and their potential to differentiate into mesenchymal phenotypes such as osteoblasts, chondrocytes, fibroblasts and adipocytes[43,44]. In view of the low frequence of mesenchymal stem cells (MSC) within the bone marrow other sources for MSC with similar properties might be more suitable. Besides this, the invasive procedure to extract MSC is another critical factor[45].
MSC with similar properties have been isolated from sources like adipose tissue and other somatic tissues[46,47]. Adipose-derived stem cells (ASC) keep the possibility to differentiate into mesenchymal lineages similar to BM-MSC[48]. Therefore, fat may be a rich source of MSC[49]. Subcutaneous adipose tissue depots are easy to access, abundant and replenishable. Also the abundance of MSC in adipose tissue exceeds that of MSC in bone marrow[50]. Other advantages are that ASC are easy to culture and expand in the laboratory.

1.6 ASC and Soft Tissue Wounds

ASC can be obtained by a relatively low-risk surgical procedure. By processing of (formerly discarded) liposuction aspirates from adipose tissue in elective procedures a significant number of ASC can be applied therapeutically to treat soft tissue injuries[51,52].
Today, there is high evidence that residents of a microvascular niche stand by for soft tissue repair when needed. ASC line the outer surface of vessels in a perivascular location. Stabilization of newly formed endothelial cell networks as well as paracrine crosstalks have been described[53]. However, in the context of

large burn lesions, chronic wounds and extensive tissue damages the compensation of resident cells is putatively insufficient to correct the deficiency. In soft tissue injuries ASC have been reported to award benefits regarding to multi-lineage restoration and induce angiogenesis[1,54].

Seeding cells (e.g. keratinocytes and fibroblasts from neonatal foreskin or mesenchymal aminocytes) on ADM reduces contraction, improves epidermis formation and enhances vascularization of the substrate. This means that the material becomes more dynamically integrated into the wound bed and the mechanical as well as functional performance is enhanced[55,56]. Previous studies have shown enhanced wound healing by ASC delivered via ADM. It was shown that ASC survived and differentiated into cells with endothelial, fibroblastic and epidermal characteristics[2].

1.7 ASVF, a prospective source for tissue engineering? - Study's Approach

In the context of soft tissue reconstruction with living cells an important experimental challenge is, due to the necessary extensive cell expansion and substitute processing, to receive an adequate number of cells for grafting procedures and to minimize the delay. Ferguson and Metcalfe extensively review this issue. The authors assume and highlight that new protocols for faster processing would alleviate the patient's pain (e.g. acute burned patients) and most likely decrease the time of hospitalization[3].

Therefore in this thesis the efficiency of stem cell seeding without prior expansion was examined. The hypothesis was that application of tissue engineered skin substitutes using autologous cells collected from liposuction procedures followed by their direct application to the wound would enhance healing of skin lesions. The adipose-derived stromal vascular fraction (ASVF) as one prospective cell source for soft tissue reconstruction deviates from ASC as they are minimally processed and not plated or passaged in the laboratory[5]. ASVF vary in phenotype from ASC because its preparation contains a higher amount of CD34+ cells representing an undifferentiated stem cell population. ASC have been defined as a subpopulation of ASVF which adheres to plastic culture dishes and can be passaged later on. Due to passaging ASC exhibit

different features and properties in comparison to the pure and untreated ASVF[4]. Mitchell et al. e.g. compared the immunophenotype of ASVF and serially passaged ASC. Adherence to plastic and subsequent expansion selects and enriches for a relatively homogenic cell population expressing stromal markers like CD44, CD73 and CD90. In contrast, ASVF are heterogenous cells including CD34+, a subset of stromal markers and a low percentage of CD45+ hematopoietic precursors[5,51].

It needs to be determined if this initially less passaged, less differentiated, untreated and unselected heterogenic ASVF represents an attractive source for potential applications in regenerative medicine. Aim of this study was to investigate the potential of fresh cell aspirates for soft tissue wound regeneration. Therefore, ASVF was collected, phenotypically characterized and directly applied without prior processing to the wound sites in order to study its potential to regenerate skin lesions. ASVF was used in combination with a bio compatible collagen substrate and ADM, respectively.

2. Materials and Methods

2.1 Materials

2.1.1 Animals

Athymic nude mice of the nu/nu strain	Charles River Laboratory, Wilmington, USA

2.1.2 Cells

ASVF	established from subcutaneous tissue of patients undergoing elective liposuction.
ASC	established from subcutaneous tissue of patients undergoing elective liposuction.

2.1.3 Tissue Culture

Liberase Blenzyme 3	F.Hoffman-La Roche Ltd., Basel, Switzerland
Phosphate Buffered Saline (1x)	Invitrogen, Carlsbad, CA, USA
40µm filtration membrane (Steriflip)	Millipore Corporation, Billerica, MA, USA
Alpha Minimum Essential Medium	Invitrogen, Carlsbad, CA, USA
Fetal bovine serum	Invitrogen, Carlsbad, CA, USA
L-glutamine	Invitrogen, Carlsbad, CA, USA
Penicillin	Sigma, St. Louis, MO, USA
Streptomycin	Sigma, St. Louis, MO, USA
Culture flasks T75	Greiner bio-one, St. Louis, MI, USA
Centrifuge tubes 50ml	Fisher Scientific, Pittsburgh, PA, USA
Trypsin-EDTA	Invitrogen, Carlsbad, CA, USA

2.1.4 Antibodies

unconjugated-anti-human CD31	BD Biosciences, San Jose, CA, USA
unconjugated-anti-human CD34	BD Biosciences, San Jose, CA, USA
unconjugated-anti-human CD44	BD Biosciences, San Jose, CA, USA
unconjugated-anti-human CD71	BD Biosciences, San Jose, CA, USA
unconjugated-anti-human CD73	Invitrogen, Carlsbad, CA, USA
unconjugated-anti-human CD90	BD Biosciences, San Jose, CA, USA
unconjugated-anti-human CD105	BD Biosciences, San Jose, CA, USA
unconjugated-anti-human CD117	R&D Systems, Minneapolis, MN, USA
unconjugated-anti-human CD146	R&D Systems, Minneapolis, MN, USA
unconjugated-anti-human Sca-1	Beckman Coulter, Inc., Fullerton, CA, USA
unconjugated-anti-human SSEA-4	R&D Systems, Minneapolis, MN, USA
2nd Anti-mouse IgG FITC	Jackson Laboratories, West Grove, PA, USA

2.1.5 FACS and Histology Reagents

Paraformaldehyde	Sigma-Aldrich Inc., St. Louis, MO, USA
Goat serum	Invitrogen Corporation, Carlsbad, CA, USA
Formalin	Fisher Scientific, Pittsburgh, PA, USA
O.C.T Compound	Sakura Finetek Inc., Torrance, CA, USA
HistoPrep Xylenes	Fisher Scientific, Pittsburgh, PA, USA
100% EtOH	Sigma-Aldrich Inc., St.Louis, MO, USA
Antibody Diluents Solution	Invitrogen Corporation, Carlsbad, CA, USA

vWF antibody, polyclonal rabbit anti-human	Dako North America, Carpinteria, CA, USA
2nd AB, Alexa Fluor 488 donkey anti-rabbit	Invitrogen Corporation, Carlsbad, CA, USA
4´,6-diamidino-2-phenylindole	Invitrogen Corporation, Carlsbad, CA, USA
Fluorescent Mounting Medium	Dako North America, Carpinteria, CA, USA
Cryomold, disposable specimen molds	Sakura Finetek Inc., Torrance, CA, USA

2.1.6 Operative Equipment

human acellular dermal matrix (ADM)	AlloDerm, LifeCell, Branchburg, NJ, USA
Biopsy Punch	Militex, Inc., York, PA, USA
96-well plate	Fisher Scientific, Pittsburgh, PA, USA
6-well plate	Fisher Scientific, Pittsburgh, PA, USA
inhalational Isoflurane (Isoflo)	Abott Laboratories, Chicago, IL, USA
Chlorhexidin PLUS Gluconate solution	DVM Pharmaceuticals, Inc. Florida, USA
Sodium Chloride Irrigation, USP	Baxter Healthcare, Deerfield, IL, USA
Collagen Matrix Film	Collagen Matrix Inc., Franklin Lakes, NJ, USA
Tegaderm	3M Health Care, St. Paul, MN, USA
Triple Antibiotic Ointment (Bacitra Zinc – Neomycin sulfate –Polymyxin B Sulfate)	Alphapharma USPD Inc., Baltimore, MD, USA
6-0 prolene suture	Ethicon, New Brunswick, NJ, USA
3-0 PDS* II (polydioxanone) suture	Ethicon, New Brunswick, NJ, USA
Normlgel (0.9% saline gel)	Moelnlycke Healthcare, Gothenburg, SE
Buprenex (Buprenorphine)	Reckitt Benckiser Healthcare Ltd. Hull, GB
Cefazolin	Sandoz, Princeton, NJ, USA

2.1.7 Equipment

Canon G7 high resolution digital camera	Canon Company Ltd., Hong Kong, CN
COULTER EPICS XL-MCL Cytometer	Beckman Coulter, Inc. Fullerton, CA, USA
Zeiss Axiovert microscope	Carl Zeiss AG, Oberkochen, Germany
Canon G7 digital camera adapter	Canon, Lake Success, NY

2.1.8 Software

Image J software package	ImageJ launcher, Broken Symmetry Software
Win-MDI 2.8	Purdue University, IN 47907, USA
TIBCO Spotfire S+	TIBCO software Inc., Palo Alto, CA, USA

2.2 In Vitro Methods

2.2.1 Cell Fraction Isolation

In accordance to the guidelines of the MD Anderson Institutional review board human adipose tissue specimens were obtained from patients undergoing elective body-contouring and reconstructive procedures.

Adipose tissue was generally processed according to the following protocol: Lipoaspirate was incubated in a digestion buffer at 37°C on a shaker at 70rpm for 35min. As digestion buffer a Phosphate Buffered Saline (PBS) solution of 0.07% Liberase Blenzyme 3 was used. After gentle trituration digested tissue was passed through a 40µm sterilized filtration membrane under vacuum. The filtered preparation was centrifuged at 1500rpm for 10min[5,49,57,58].

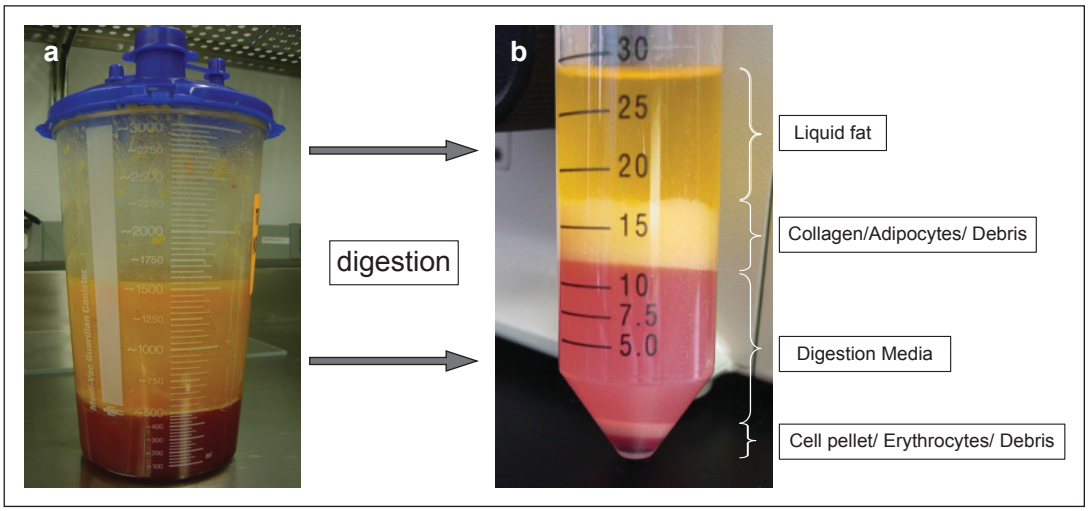

Figure 2. *Cell fraction isolation*
a) Human fat obtained during surgical liposuction and processed for isolation of ASVF (adipose-derived stromal vascular fraction) and ASC (adipose-derived stem cells).
b) Human fat tissue after digestion, filtration and centrifugation steps. The pellet contains the target cells which were subsequently used for experiments or cultured in T75 flask.

The supernatant and digested tissue was discarded and pelleted cells were resuspended in 10ml of growth media. Alpha Minimum Essential Medium was supplemented with 20% fetal bovine serum (FBS), 2mM L-glutamine, 100 u/ml penicillin and 100 µg/ml streptomycin and used as growth medium (GM).

2.2.2 Tissue Culture

2.2.2.1 ASC Incubation

The resulting cell suspension was placed in a T75 tissue culture flask and incubated in a 5% CO_2 containing humidified atmosphere at 37°C. After 24h non-adherent cells and debris were removed and plastic-adherent cells were expanded with media changes every 3 days. Passaging was carried out at a cell density of approximately 80%. Cells at passage 1 to 5 were used for all experiments.

2.2.2.2 ASVF Incubation

The resulting cell suspension was placed in a 50ml conical tube and held in suspension with constant shaking in a 5% CO_2-containing chamber at 37°C.

2.2.3 Flow Cytometry

To characterize ASVF, specific surface proteins were labeled by fluorescein isothiocyanate-conjugated (FITC) antibodies and subsequently analyzed using flow cytometry. Flow cytometric analyzes and preparation was generally performed based on the following protocol:

ASVF (approx. $1x10^6$ cells/ml aliquot) were washed twice in labeling buffer (PBS with 2% goat serum). Subsequently, samples were incubated on ice for 15min with one of the following unconjugated primary anti-human antibodies: CD31, CD34, CD44, CD71, CD73, CD90, CD105, CD117, CD146, Sca-1 and SSEA-4. Dilutions of 1:500 to 1:1250 were used throughout the experiments. Samples were then centrifuged and washed with labeling buffer at 1300rpm for 3min. After incubation with a fluorescent secondary antibody on ice for 15min, samples were resuspended in labeling buffer and 2% paraformaldehyde (PFA)-PBS for fixation. These fixed samples were stored at 4°C over night. In all cases fluorescent activated cell sorting (FACS) analysis was performed on the next day. Immediately prior to analysis 1ml of labeling buffer (PBS with 2% goat serum) was added per sample. To assess the specificity of labeling and to confirm suitable fluorescence over background for antigen positive cells controls using only the secondary antibody were performed in addition to the reactions

above. Flow cytometry was performed using a fluorescence-activated cell sorter and data analysis was performed with WinMDI (Windows Multiple Document Interface) for Flow Cytometry.

2.2.4 ADM Graft Preparation

ADM was obtained from a commercial supplier and processed according to the manufacturer's instructions. ADM is obtained from cadaveric donors, rendered acellular and finally preserved by freeze-drying[28]. The materials thickness ranged from 0.53-0.76mm.

Sterile technique was maintained throughout the manipulation of ADM as described below. ADM was washed for 60min in PBS. Graft-specimens were cut into pieces using a 6mm diameter biopsy punch and then placed on the well bottom of 96-well plates. Grafts were placed with the papillary dermal surface facing up and then covered with either 200µl of GM or with a suspension of $5x10^5$ freshly isolated ASVF resuspended in 200µl GM (seeded graft group). Grafts were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24h (Figure 3).

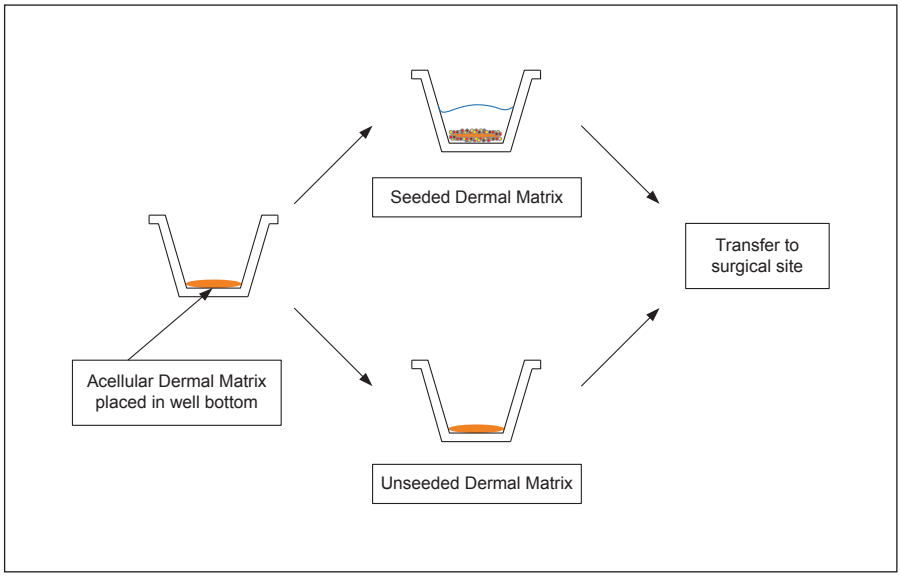

Figure 3. *Seeded and unseeded graft preparation* (own illustration)

Subsequently, the medium was removed from the wells by pipette aspiration and grafts were placed with the reticular surface facing up in the well bottom. Grafts were covered with either GM alone or GM containing $5x10^5$ freshly isolated cells as described above. Grafts were incubated for an additional 24h

and then transferred to the operating suite for surgical engraftment. Immediately before operation, grafts were transferred to a sterile 6-well plate and washed gently in 2x500µl PBS for 5min to remove non-adherent cells or any residual GM prior to engraftment[2].

2.3 In Vivo Methods

2.3.1 Operative Design: Murine Skin-Wound Model

All in vivo procedures were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee (ACUF protocol #05-08-04931). Immunodeficient athymic nude mice were used because of their ability to accept human cells without generating an immune reaction.

To induce general anesthesia animals were placed in an airtight chamber with 3% inhalational Isoflurane for 90sec. Subsequently, mice were placed on the operating table and connected to a circuit providing a constant flow of 1.5% Isoflurane for maintenance of anesthesia during the operation procedure. The entire dorsal skin was scrubbed with 2% gluconate solution, washed with 0.9% Sodium Chloride and subsequently draped to achieve a sterile field.

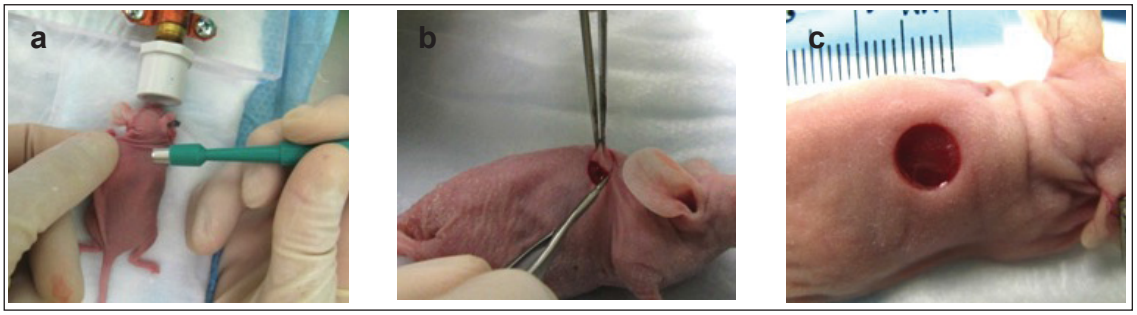

Figure 4. *Surgical procedure of the murine skin-wound model*
a) creation of a 6mm diameter circular impression by biopsy punch placement. b) removal of native skin. c) full thickness skin defect.

To create a full thickness skin defect a 6mm diameter circular impression was made by placing a biopsy punch with a moderate force on the paramedian dorsum of the mouse (Figure 4a). Next, the middle of the outlined region was grasped and elevated with Adson forceps to excise a 6mm diameter region using a fine Metzenbaum scissor (Figure 4b). The excised tissue was full-

thickness skin deep, leaving the subcutaneous dorsal muscle exposed after excision (Figure 4c). After procedure completion animals were released from general anesthesia and supplemented with 100% O_2. The post-operative recovery was monitored for 15min before the animals were placed in individual cages to resume activity ad-libitum (own modification based on[2]).

2.3.2 Wound Treatment

2.3.2.1 Collagen Matrix Study

The ASVF and ASC were obtained as described above. The ASC were harvested by treatment with 0.05% trypsin and 0.53mM ehthylenediamine tetracetic acid (EDTA). Cell suspensions were transported on ice to the operating suite, pelleted by centrifugation at 1500rpm and washed with PBS. After a second centrifugation cell pellets were resuspended in an adequate volume to gain a cell suspension of $0.5x10^6$ cells per 20µl PBS and held on ice until usage.

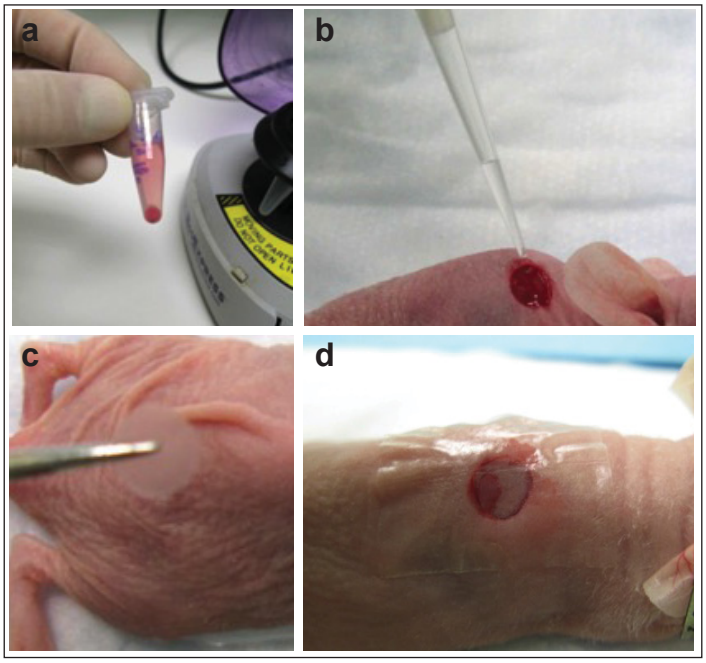

Figure 5. *Cell application and wound taping in the Collagen Matrix Study*
a) either ASVF (adipose-derived stromal vascular fraction) or ASC (adipose-derived stem cells) were pelleted and resuspended at surgical side, b) right before application to the wound-bed. c) CMF (Collagen Matrix Film™) was placed and d) operative site was finally sealed with Tegaderm™.

Animals in all groups received one full thickness skin lesion on the right paramedian dorsum. Experiments were carried out on 3 different days. A total number of 32 animals were randomized to 1 of the 4 treatment groups:

a) ASVF/CMF (ASVF suspension, n=8)
b) ASC/CMF (ASC suspension, n=7)
c) CMF (PBS, n=11)
d) control (no treatment, n=6)

After application of either treatment a)-c) (Figure 5b) 6mm diameter pieces of Collagen Matrix Film™ (CMF) were placed into the wound bed (Figure 5c). After completion of the operation wounds were covered with Tegaderm to keep the CMF at the wound site (Figure 5d)[1].

2.3.2.2 Seeded ADM Study

Two wounds were created on the paramedian dorsum of 15 rodents. One wound was placed contra lateral to the other. Respectively 30 wounds were created and each animal received two treatments. The three treatment groups were randomized and distributed to 2 procedure days. A total number of 15 rodents were randomized to their receiving treatment.

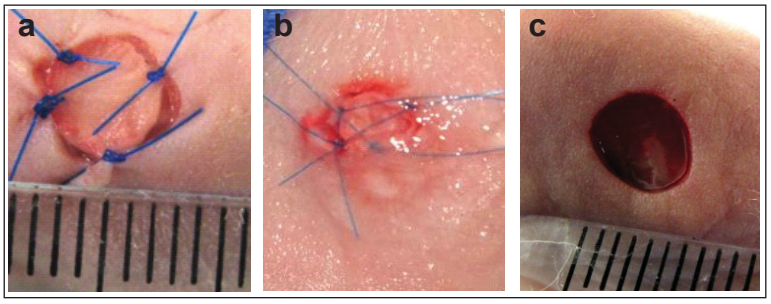

Figure 6. D*esignated treatment groups in the Seeded ADM Study*
Wounds were treated with a) ASVF/ADM (adipose-derived stromal vascular fraction + human acellular dermal matrix) or b) ADM alone and secured into place with tacking sutures. c) as Control (no treatment) spontaneous wound healing was monitored.

Overall the group size was:

a) ASVF/ADM (seeded graft, n=9)
b) ADM (graft alone, n=8)
c) Control (no treatment, n=11)

A triple antibiotic ointment was applied in a thin layer to animals receiving no treatment. For graft placement in the ASVF/ADM seeded graft and ADM group, grafts were removed from PBS and placed into the dorsal wound. Grafts were secured in place with 4-5 interrupted stacking stitches with either 6-0 prolene or 3-0 PDS*II tacking sutures. Operative sites were covered with a thin layer of 0.9% saline gel[2].

2.3.3 Peri Operative Animal Care

Animals were housed and kept in an air-filtered barrier facility receiving autoclaved nutrition and maintenance of a sterile working and housing environment. Initially animals were housed in groups of 4 per cage. Post-operatively mice were housed at one per cage, fed chow ad libitum and physically examined daily. Post operative application of Buprenorphine for analgesia was administered by a subcutaneous injection immediately after completion of surgery on day 0 (0.5-2.5mg/kg body weight). Additionally all mice received a subcutaneous injection of 15mg/kg Cefazolin on day 0 to achieve peri-operative antibiosis.

- Collagen Matrix Study: Tegaderm™ wound tape was removed on post-operative day 2
- Seeded ADM Study: General anesthesia was induced as before on post-operative day 4 for suture removal.

2.3.4 Planimetric Analysis

Animals were removed from their cages and wound sites were photographed using a Canon high resolution digital camera. At least three independent pictures per wound were taken. Photographs were uploaded on a computer platform and analyzed using the Image J software package.

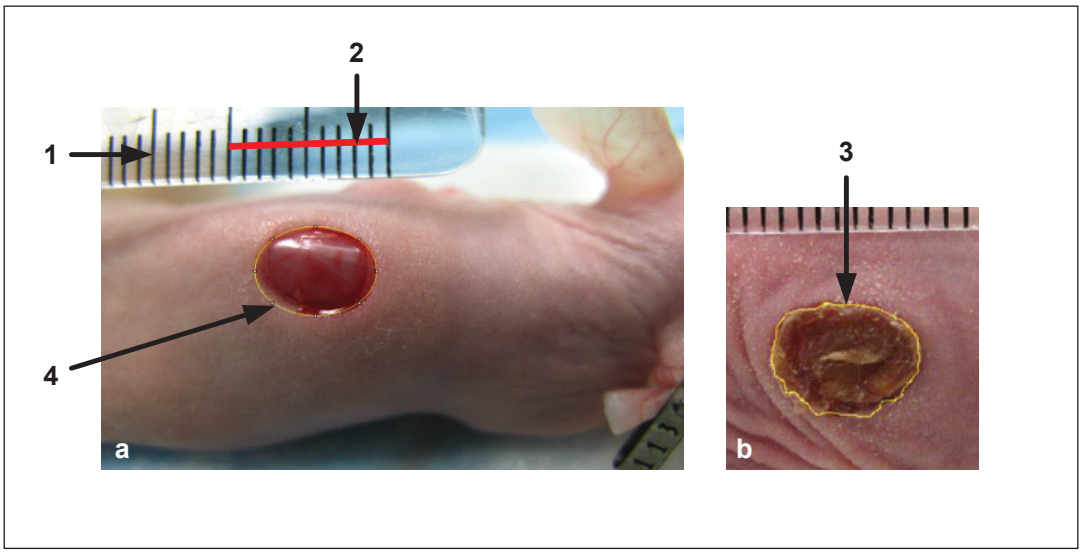

Figure 7. *Wound site pictures for planimetric analysis*
Once uploaded on a computer platform Image J software package was used for calculations. a) ruler (1) was placed adjacent to the mouse to provide distance calibration and standardization (2). Wound areas were captured using a virtual rope (3,4). b) magnified wound site image showing the captured non-epithelialized area outlined by a Yellow circumference (3)

For calibration and standardization a metric ruler was placed adjacent to the mouse in each case (Figure 7a). Mean value of 3 independent pictures was used for further analysis.

- Collagen Matrix Study: Animals were photographed on post operative day 0, 2, 7 and 12. Animals of the third sample day were additionaly photographed on day 4.
- Seeded ADM Study: Species were photographed on post operative day 0, 2, 4, 7 and 12.

The rough non-epithelialized area at the wound site was considered to be unhealed wound area and outlined on the digital image (Figure 7b). This outlined non-epithelialized region was subsequently subjected to surface area calculation. Reepithelialized area in mm^2 was defined as [wound area of day 0] – [wound area of e.g. day 2].

2.3.6 Standard Histology

Tissue biopsies were obtained from the wound area by sharp dissection and placed either into 10% formalin for fixation and subsequent paraffin embedding

or into molds for immersion in optimum cutting temperature freezing medium on dry ice for frozen block preparation. All histology sections were prepared from tissue specimens harvested at post operative day 14. Serial sectioning was performed resulting in 5µm sections for frozen tissue and 4µm for paraffin embedded tissue. To examine tissue architecture representative sections underwent H&E staining. Additionally serial sections were prepared as unstained slides for immunofluorescent studies. H&E-stained Z-sections were studied and analyzed using a microscope equipped with a high resolution digital camera for image acquisition.

2.3.7 Microvascular Density Analysis

To determine microvascular density paraffin embedded sections were deparaffinized and stained with primary antibody directed against von Willebrand Factor glycoprotein (vWF). Slides were deparaffinized in serial xylene and ethanol washes and subsequently exposed to target retrieval solution at 95°C for 20min and kept in this solution for additional 20min. After cooling and serial washing steps in distilled water and PBS slides were blocked in 10% goat serum for 10min at room temperature. Specimens were then incubated with a 1:100 dilution of primary antibody at 4°C for 24h. On the following day slides were washed in PBS and incubated in a 1:1000 dilution of secondary antibody in PBS for 40min at 37°C. Slides were then washed and incubated with DAPI (to stain nuclei) for 15min at room temperature. Slides were finally mounted with cover slips for viewing.

The microvessel quantification assay was based on an adaption of established protocols[59,60]. One section from the wound center was examined per animal. 10 randomized view fields were examined using 40fold magnification. Each VWF positive cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered as a single microvessel. The presence of a vascular lumen was not necessary for structure to be defined as a microvessel. Vessels of diameter ≤50µm were counted excluding macrovessels which were characterized by a diameter >50 µm.

3. Results

3.1 Phenotypic Characterization of ASVF

In vitro flow cytometric analysis was performed on each batch of freshly isolated ASVF to be used for animal studies. Figure 8 shows representative flow histograms. The ASVF population was screened for progenitor/ stem cell markers (CD34, Sca-1 and SSEA-1), stromal cell associated marker (CD44, CD73, CD90, CD105), endothelial progenitor cell marker (CD146), endothelial marker (CD31) as well as panhaematopetic lineage marker (CD45).

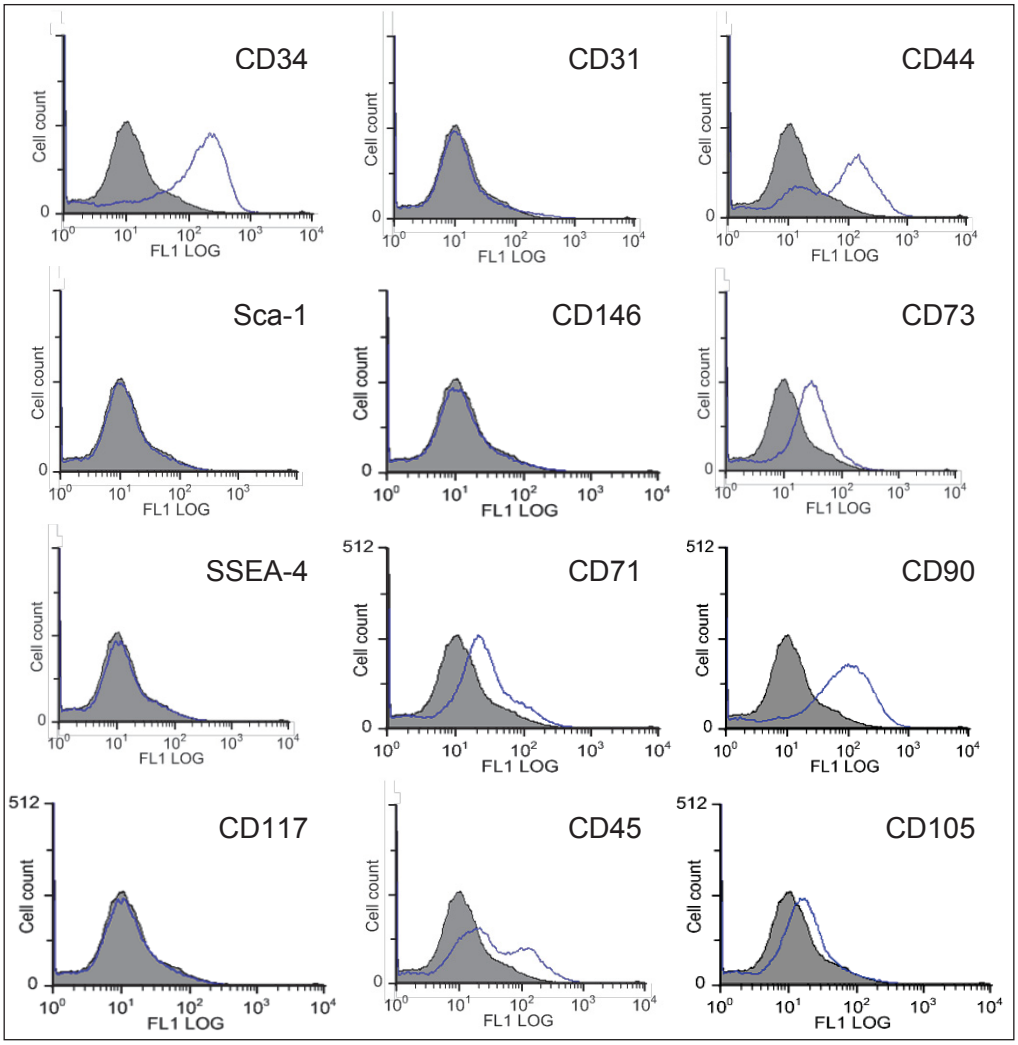

Figure 8. *Phenotypic characterization of ASVF*
Representative flow cytometry histograms demonstrate the results of phenotypic analysis of human adipose-derived stromal vascular fraction (ASVF). The blue line indicates cells stained for the cluster of differentiation (CD) marker. The isotype matched monoclonal antibody control is indicated by the defined grey integral.

As summarized in Table 2 the presence of surface markers was always positive for the markers CD34, CD90, CD44 and CD73. All preparations were negative for CD31, CD117, Sca-1, SSEA-4. Incoherent and slight expression was found for CD45, CD146, CD71 and CD105.

Preparation	CD34	CD90	CD44	CD45	CD31	CD117	Sca-1	SSEA-4	CD146	CD73	CD71	CD105
I	++	++	++	+/-	-	-	-	-	-	+	-	-
II	++	++	++	-	-	-	-	-	-	+	+	+
III	++	++	++	+/-	-	-	-	-	+	+	+	+
IV	++	++	++	-	-	-	-	-	-	+	+	-

Table 2. *Comparison of 4 independent ASVF preparations used in the rodent studies*
CD marker comparison. 10fold increase or more in fluorescence intensity is marked as (++). More than 2fold up to 10fold indicated as (+) and less than a 2fold difference labeled as (-). Vague increased expression is marked as (+/-).

The ASC population used in this study has previously been characterized via flow cytometry by Altman et al. and Bai et al. [2,61]. Table 3 shows the expressed surface marker profile of ASVF in comparison to ASC used in these studies. Both populations are positive for the stromal associated marker CD90, CD44 and CD105. The panheamatopoetic marker CD45 was negative in the ASC and incoherent in the ASVF. CD34 a progenitor/stemm cell marker was only positive in the ASVF population.

Surfacemarker	ASVF	ASC [2,61]
CD34	+	-
CD90	+	+
CD44	+	+
CD105	+/-	+
CD45	+/-	-

Table 3. *Similarities and differences in surface marker profile*
Freshly isolated adipose-derived stem cells (ASVF) and cultured adipose-derived stem cells (ASC) were both positive for stromal associated marker CD90, CD44, CD105. The panhaematopoetic marker CD45 was negative in ASC and incoherent in ASVF. The progenitor/ stem cell marker CD34 was only positve in the ASVF population.

3.2. Collagen Matrix Study

To determine the effect of a synthetic matrix combined with ASVF or ASC in wound healing a murine skin-wound model was established. As shown in Table 4 different treatment groups were designed. All animals were subjected to the standardized skin wound procedure leading to a 6mm diameter full thickness wound defect.

Treatment groups	Cell-type	Material	Wound tape
ASVF/CMF (n=8)	adipose-derived stromal vascular fraction	Collagen Matrix Film™	Tegaderm™
ASC/CMF (n=7)	adipose-derived stem cells	Collagen Matrix Film™	Tegaderm™
CMF (n=11)	-	Collagen Matrix Film™	Tegaderm™
control (n=6)	-	-	-

Table 4. *Group designation in the Collagen Matrix Study*
CMF = Collagen Matrix Film

In the ASVF/CMF as well in the ASC/CMF group a cell suspension of $5x10^6$ ASVF and ASC respectively was directly applied to the wound bed. Subsequently, a 6mm diameter piece of Collagen Matrix Film (CMF) was placed onto the wound which was then covered with Tegaderm™ to fix the CMF to the wound site. To determine differences in sample quality experiments were carried out in three experimental series (series I-III).

3.2.1 Planimetric Analysis of Wound Area

Series I

Wounds received three different treatments: ASVF/CMF (n=2), ASC/CMF (n=2) or control (n=3). Wound area measurements were assessed to detect differences in wound closure progression. The gross non-epithelialized area at the wound site was considered to be unhealed wound area outlined on digital images and subjected to surface area calculation. The control group reflects spontaneous wound healing of a full thickness skin defect with an initial surface area of $28mm^2$. Representative pictures were taken on post operative day 6 and 12. Comparative wound healing progression is shown in Figure 9A(a-f).

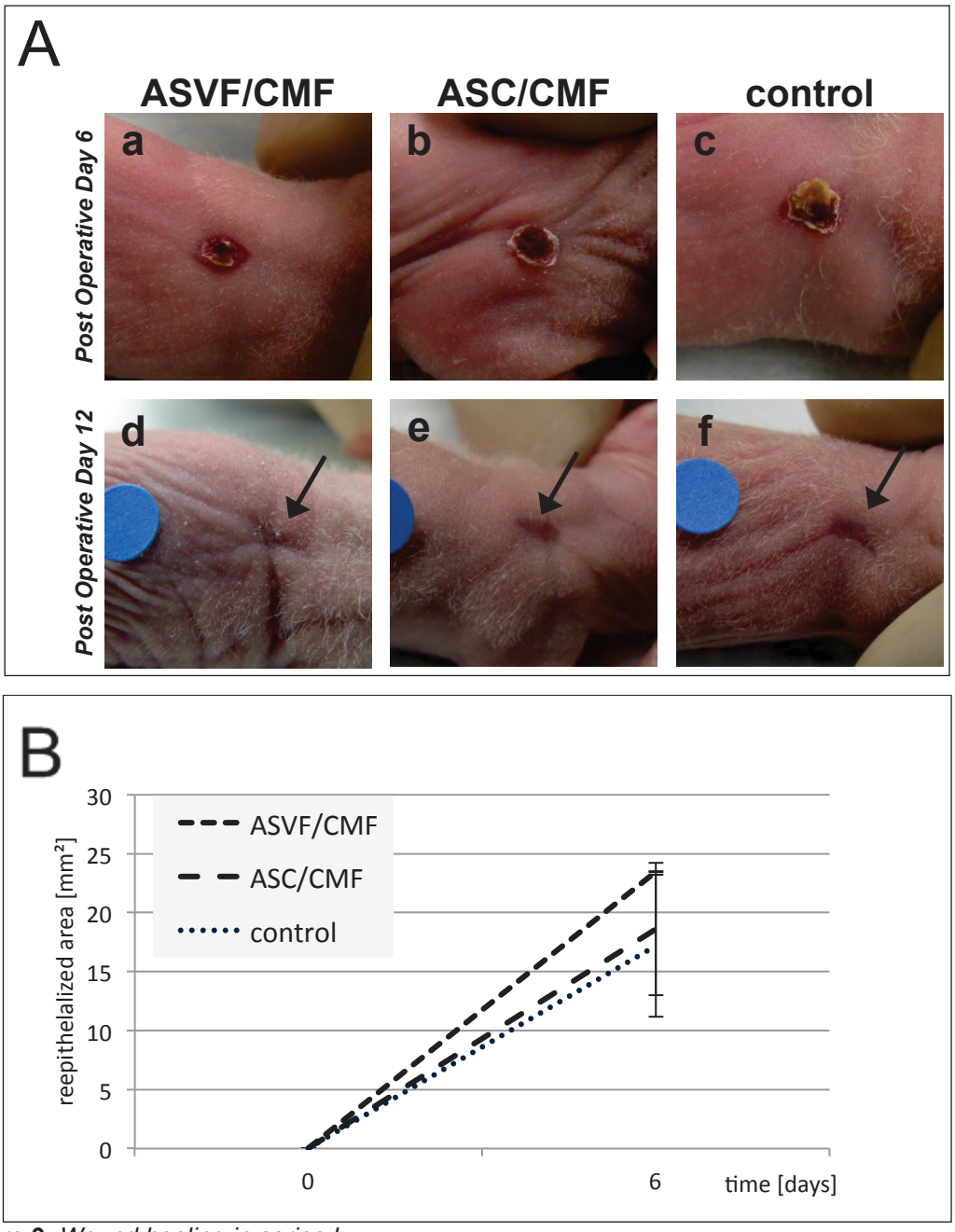

Figure 9. *Wound healing in series I*
A: Representative images of wound healing progress over time of 3 experimental groups. Post operative images of day 6 and 12 of ASVF/CMF (adipose-derived stromal vascular fraction + Collagen Matrix Film™, n = 2), ASC/CMF (adipose-derived stem cells + CMF, n =2) and control group (n = 3) are shown. Blue adhesive labels have been used to provide proper distance calibration and standardization to achieve an appropriate metric analysis. Arrow indicates wound area.
B: The Graph shows the time course of wound reepithelialization at post operative day 0 and 6 for the ASVF/CMF (n=2), ASC/CMF (n=2) and control group (n=3). Mean values of reepithelialized area ±SD are plotted.

For calibration a metric ruler or a defined circular adhesive label (Figure 9A(d-f)) was placed adjacent to the wound site. A pronounced circumferential region of hyperemic, erythematous tissue at the wound's margins of epithelialization was

consistently observed in the ASVF/CMF group (Figure 9Aa). This was not found in the ASC/CMF (Figure 9Ab) and control group (Figure 9Ac). At post operative day 12 almost all wounds were closed and a distinguishable scaring area remained (Figure 9A(a-f)). The quantitative data for reepithelialization is shown in Figure 9B. Mean values of all groups are plotted. The initial punch instruments area was set as baseline area for all groups on post operative day 0. After six days the mean reepithelialized area was 23.5±0.05mm^2 in the ASVF/CMF group, 18.6±5.6mm^2 in the ASC/CMF group and 17.2±6.0mm^2 in the control group.

Series II

To estimate wound healing kinetics over time images of series II animals were captured on post operative day 0, 2, 4, 7 and 12. For this purpose animals of series II were wounded according to the standard practice and subsequently treated with ASVF/CMF, CMF or left unaltered as control. Representative pictures show no notable morphological differences between the ASVF/CMF (n=6), CMF (n=6) and control (n=3) group at post operative day 2 (Figure 10A(a-c)). Mean area of reepithelialization at day 0 was 0±9,5mm^2 for the ASVF/CMF group, 0±4,3 mm^2 for the CMF group and 0±2,5 mm^2 for the control group (data not shown). Since there were interindividual differences and deviation in wound size immediately after wounding data were normalized for the wound area at day 0. The reepithelialized area over time showed no significant difference between the three treatment groups at any point in time. The graph in Figure 10B shows the relative area of reepithelialization over time. However, the normalized data did not show any significant differences.

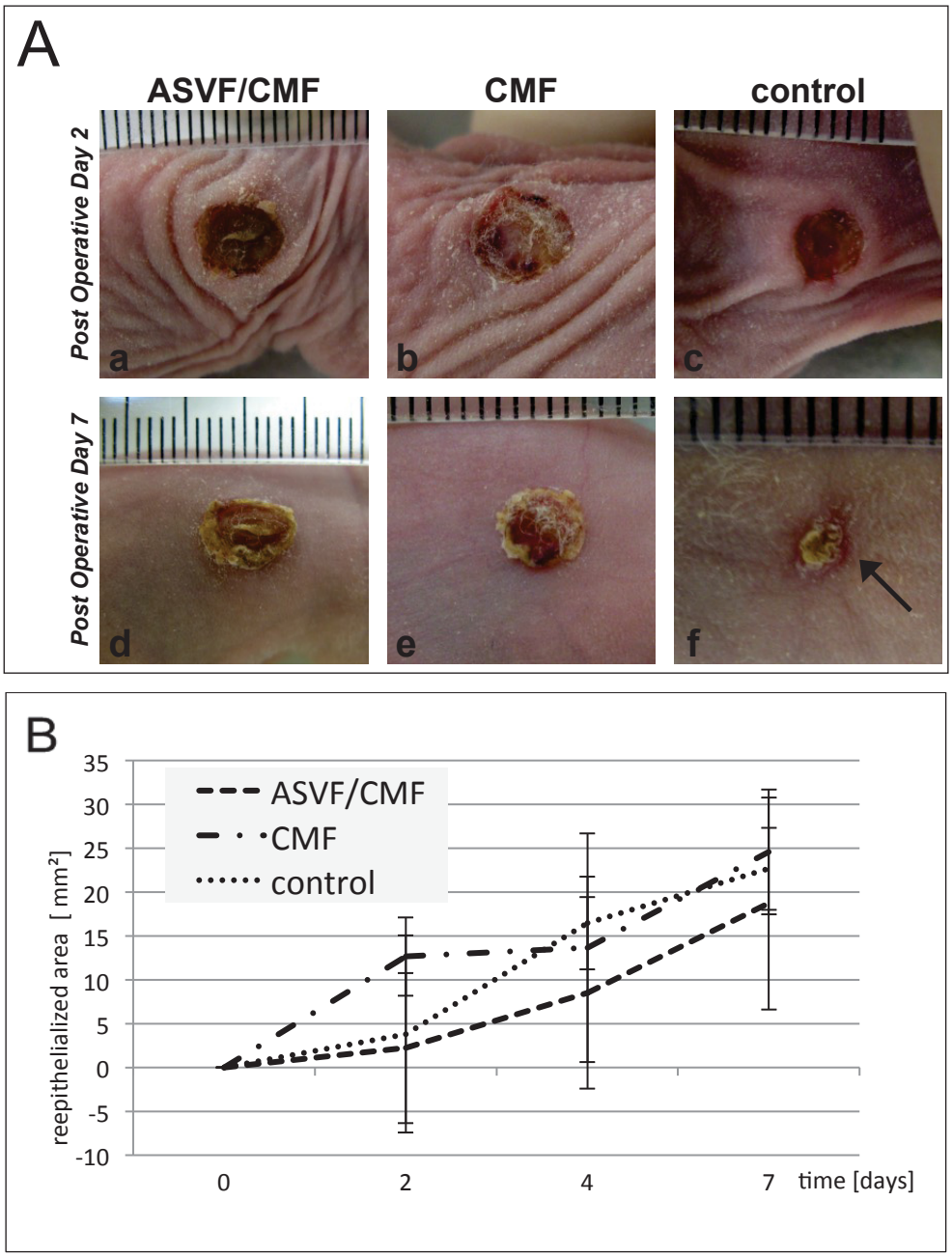

Figure 10. *Reepithelialization in series II*
A: Representative macroscopic appearance of wound sites over time from the three experimental groups of series II at post-operative day 2 and 7. Either ASVF/CMF (adipose-derived stromal vascular fraction + Collagen Matrix Film™, n=6) or CMF (n=6) was initially applied to wound beds. Control (c, f) represents the unaltered group (n=3). A metric ruler has been used to achieve calibration. Arrow indicating wound area.
B: The graph shows the time course of reepithelialization at post operative day 0, 2, 4 and 7 for the ASVF/CMF (n=6), CMF (n=6) and control (n=3) group. Mean values of reepithelialized area ±SD are plotted.

Series III

In series III it was analyzed to what extent ASC might influence wound healing. As shown in Figure 11A macroscopic appearance did not show major

differences between the ASC/CMF and CMF group at post operative day 2. On post operative day 7 a pronounced circumferential region (Figure 11Ac arrows) of hyperemic, erythematous tissue at the wound's edge was detected in the ASC/CMF group (n=5) (Figure 11Ac) but not in the CMF group (n=5) (Figure 11Ad).

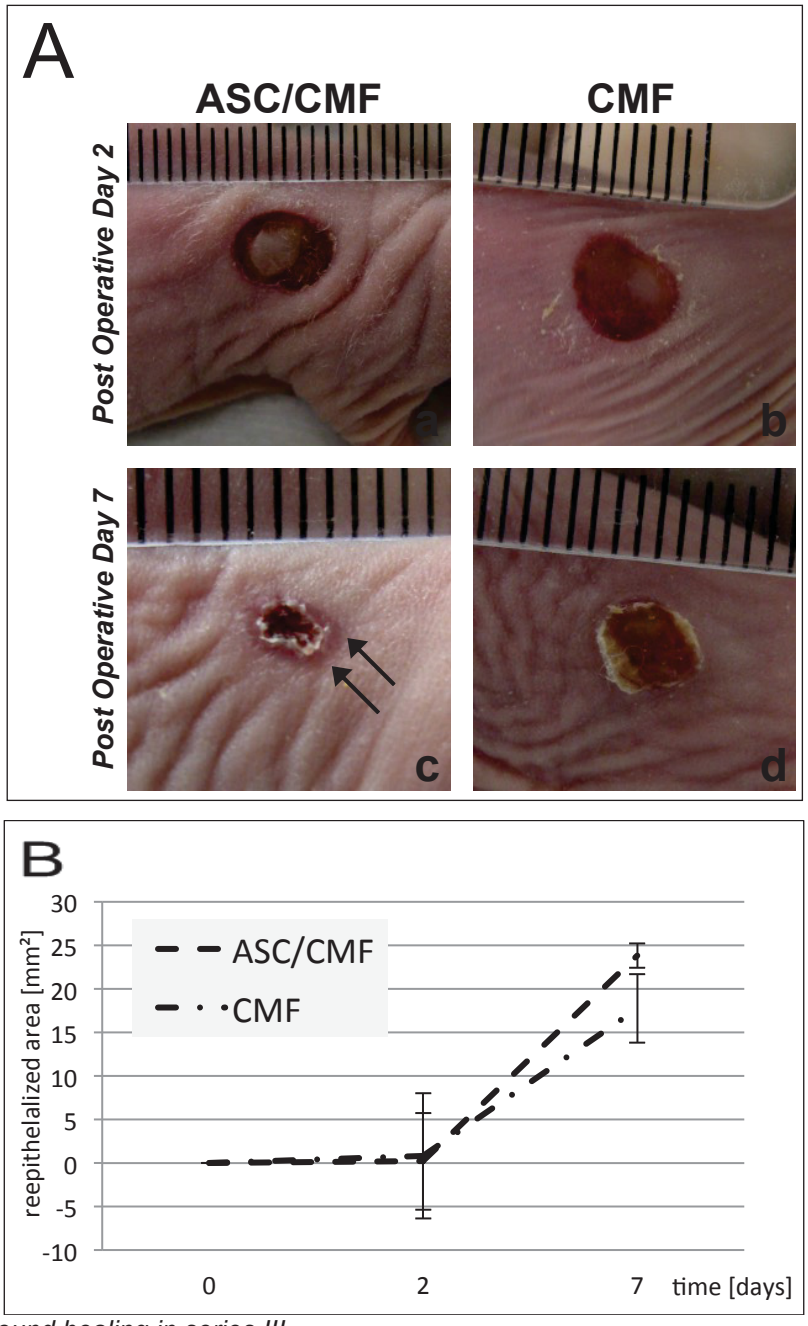

Figure 11. *Wound healing in series III*
A: Typical images of wound healing progress over time at post operative day 2 and 7. Wounds either received ASC/CMF (adipose-derived stem cells + Collagen Matrix Film™, n=5) or CMF (n=5). A metric ruler has been used to achieve an appropriate metric analysis.
B: To establish the quantitative development of reepithelialization measurements of ASC/CMF (n=5) and CMF (n=5) treatment groups were plotted (Mean values of reepithelialized area ±SD).

This morphological characteristic was prior observed in the ASVF/CMF group in series I (see p.28 Figure 9). Additionally the ASC/CMF group differs from the CMF group in less granulation tissue in the wound center (Figure 11Ac,d). The wound area showed no differences at post operative day 2 (ASC/CMF 0.2±5,6mm^2; CMF 0.8±7,2mm^2). The computed values at day 7 tended to be smaller in the ASC/CMF group (Figure 11Ac and Figure 11B). However differences in mean values of reepithelialisation (ASC/CMF 23,8±1,4mm^2; CMF 17,8±3,9mm^2).did not reach the significance level.

3.2.3 Assessment of Vascular Staining

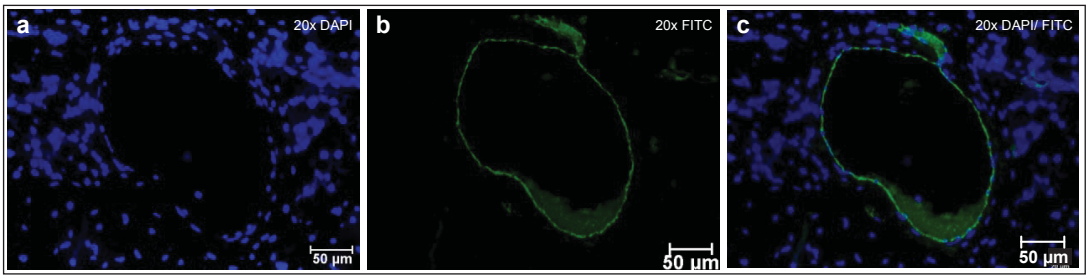

Figure 12. *Representative picture of immunofluorescent vascular wound bed staining*
a) Nuclei were stained with DAPI. b) the vessels endothelial were stained with a vWF antibody resembling the vascular circumference. c) shows the overlay of a) and b).

To evaluate the impact of ASVF and ASC on the microvascular density in wound bed biopsies at post operative day 14 an antibody directed against the vWF glycoprotein was used. The VWF glycoprotein is expressed on the surface of endothelial cells and platelets. A representative vWF staining of a large vessel (approx. 200µmØ) of a typical wound biopsy is shown in Figure 12. Staining with vWF-antibody highlighted the vascular endothelial layer (Figure 12b). To define vascular structures an overlay of DAPI and VWF was performed, clearly marking the vascular lumen (Figure 12c).

To analyze vascularisation of the wound tissue sections in the Collagen Matrix Study (n= 30) and Seeded ADM Study (n= 24) species were analyzed for VWF positive signals.

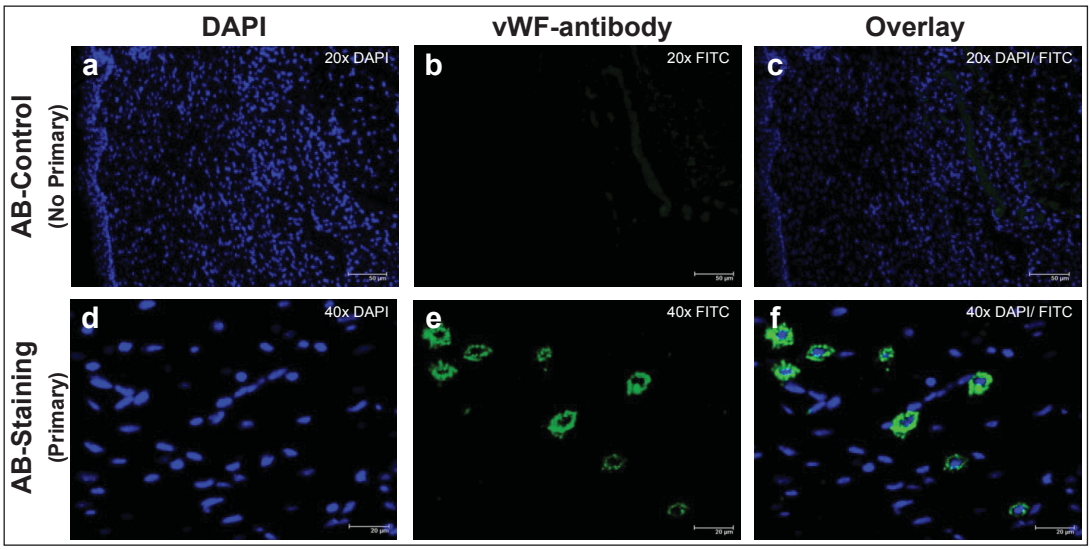

Figure 13. *Representative picture of antibody (AB) specificity and microvascular staining* The No Primary Control a) DAPI, b) VWF-antibody and c) overlay did not show any significant fluorescent signal for the vWF protein staining. Stained microvessels d) DAPI, e) vWF-antibody and f) overlay were quantified in each sample.

Figure 13 shows a representative picture of this analysis. In contrast to the negative control without primary vWF-antibody (Figure 13b) the complete assay clearly demarcated vWF positive cells (Figure 13e,f). The quantification of microvascular density for the Collagen Matrix Study is shown in Figure 14 and Figure 18 respectively for the Seeded ADM Study. To assess VWF positive cells/ microvascular density 10 randomized view fields of wound center section per animal were examined.

3.2.4 Microvessel Quantification

Comparison of the mean microvascular density at post operative day 14 of the CMF group (n=14) to the control group (n=3) showed no significant differences (12.3±2 vs. 10.7±2.5). Mean values for the ASVF/CMF (n=7) and the ASC/CMF (n=6) group indicate no difference to the control group (10.9±2.3 and 11.8±2.8 in comparison to 12.3±2).

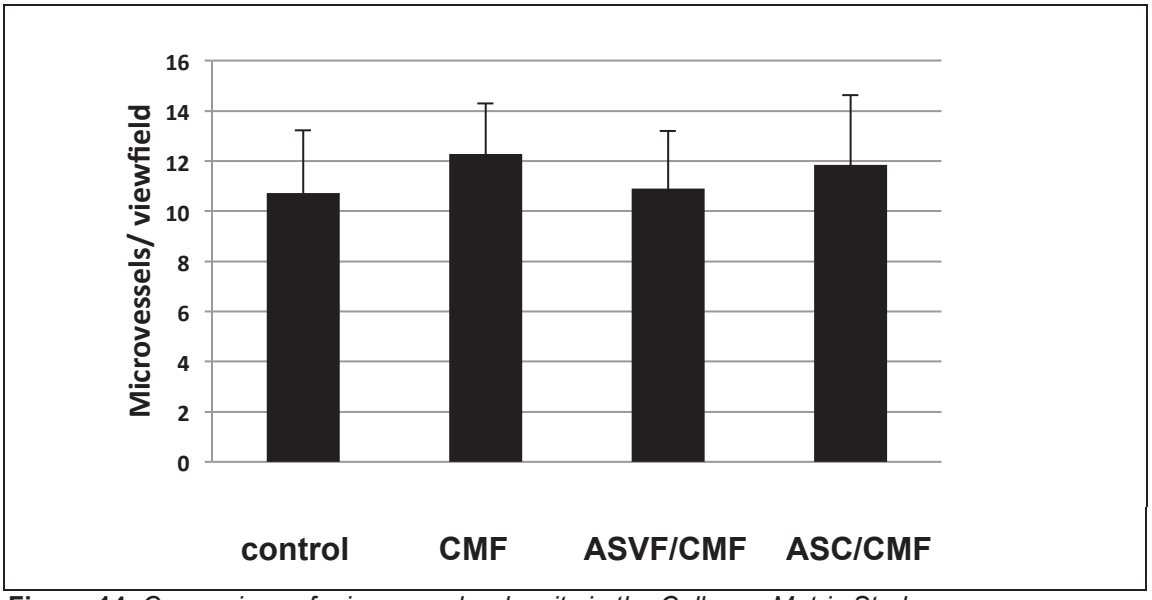

Figure 14. *Comparison of microvascular density in the Collagen Matrix Study*
The treatment groups control (n=3), CMF (Collagen Matrix Film™, n=14), ASVF/CMF (adipose-derived stromal vascular fraction + CMF, n=7) and ASC/CMF (adipose-derived stem cells + CMF, n=6) are listed on the abscissa. The ordinate scales the correlating mean number of microvessels ±SD per 40x magnified viewfield.

3.3 Seeded ADM Study

Efficiency of an ASVF loaded ADM was investigated in an athymic murine full-thickness cutaneous wound model. In this experimental setting two contra lateral full thickness wounds on the paramedian dorsum of athymic nude mice were created and then three different treatments were applied in a previously randomized manner. The three potential treatments are listed in Table 5.

Treatment groups	Cell-type	Material
ASVF/ADM (n=9)	adipose-derived stromal vascular fraction	human acellular dermal matrix
ADM (n=8)	-	human acellular dermal matrix
control (n=11)	-	-

Table 5. *Seeded ADM Study design*
The table shows the 3 groups included in this study: ASVF/ADM (n=9), ADM (n=8) and control (n=11). Groups differed in the presence of cells and application or absence of ADM.

In the ASVF/ADM group (n=9) dermal matrices were incubated with a preparation of $1x10^6$ freshly isolated ASVF. As relevant controls ADM (n=8) and untreated control (n=11) groups were set-up. In the two following weeks

wounds were repeatedly examined and wound bed biopsies were taken after 14 days for further standard histology and immune-histochemistry analysis.

3.3.1 Planimetric Analysis of Wound Area

Throughout the post operative period differences in wound site appearance became striking. In the control group the wounds were substantially closed at post operative day 7 (Figure 15d). However the star-like patterned rankles (Figure 15d) reflected that a high level of tissue contraction contributed to this result rather than reepithelialisation. Graft groups (ASVF/ADM and ADM) showed a delayed wound closure rate and the scab in either group is much longer present (Figure 15c,d). The variation in graft incorporation shows a less embossed graft in the ASVF/ADM group (Figure 15c) at post operative day 7 when compared to the ADM group (Figure 15d).

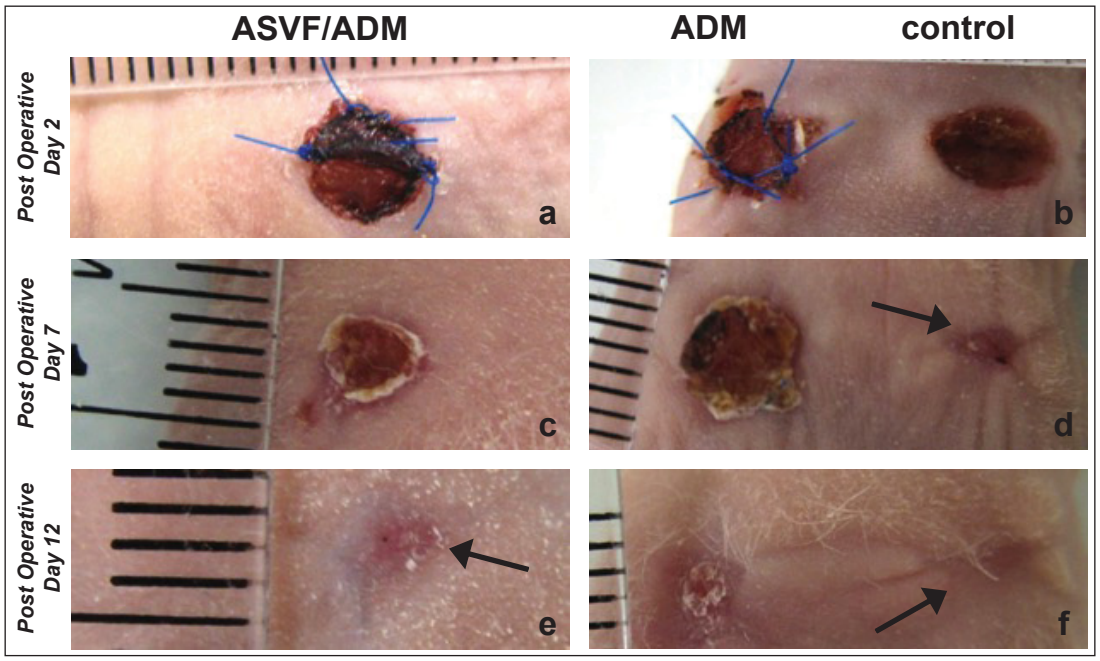

Figure 15. *Treatment effect over time in the Seeded ADM Study*
Wounds were treated with a) ASVF/ADM (adipose-derived stromal vascular fraction + human acellular dermal matrix, n=9), b) ADM (n=8) or c) left unaltered as control (n=11). Representative pictures show the engraftment at a/b) post operative day 2, c/d) 7 and e/f) 12 of the three different treatment groups. To provide proper calibration a metric ruler was placed adjacent to wound sites. Arrow indicates wound area.

Furthermore the link between ADM and adjacent wound margin appears more homogeneous in the ASVF/ADM in comparison to the ADM group. Besides the less embossed graft less rankles are present. The hyperemic erythematous

reepithelialized area visibly separates the granulation tissue from the unwounded skin in the ASVF/ADM group (Figure 15c). This observation cannot be made in the ADM group (Figure 15d). Facing asthetic complexion as a feasible endpoint the scars contour and it's rankles are less impressive in the ASVF/ADM group (Figure 15e) in comparison to the other groups (Figure 15f) at post operative day 12. As before the enormous contribution of contraction in the control group persists (Figure 15f).

To quantify the effect of ASVF/ADM seeded grafts on reepithelialization Figure 17 shows the reepithelialized area over time via planimetric calculations. In contrast to the previous observed morphological differences the planimetric calculations of the three treatment groups did not show a significant difference. There is no verifiable metric effect of ASVF/ADM and ADM respectively.

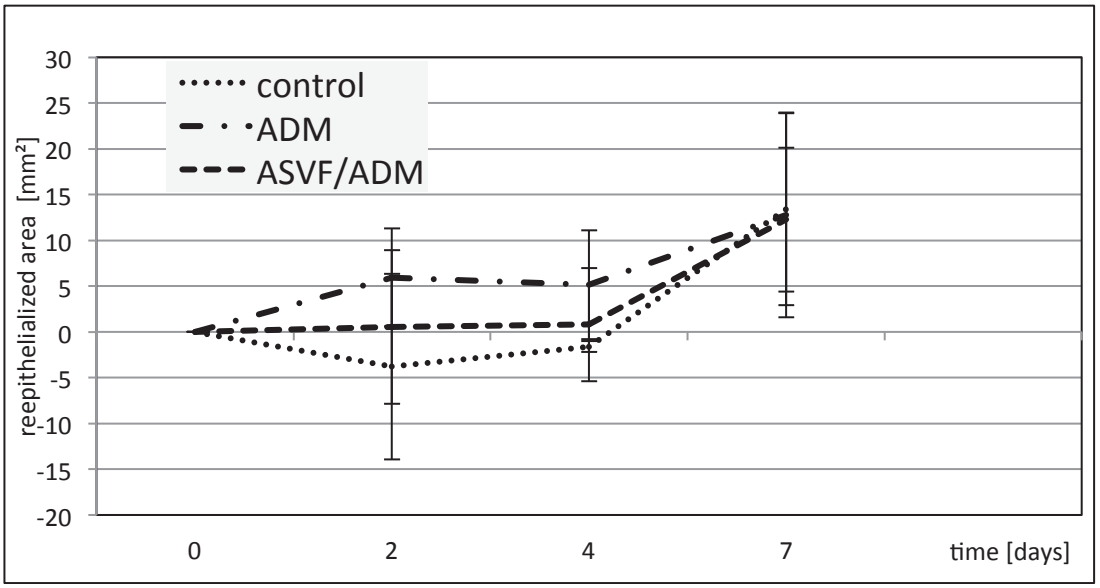

Figure 17. *Reepithelialization of the three designated groups in the Seeded ADM Study*
Graph shows time course of wound reepithelialization kinetics at post operative day 0, 2, 4 and 7 for the ASVF/ADM (adipose-derived stromal vascular fraction + human acellular dermal matrix, n=9), ADM (n=8) and control (n=11) group. Mean values of reepithelialized area ±SD are plotted.

3.3.3 Microvessel Quantification

Quantified microvascular density for the seeded and unseeded graft groups is shown in Figure 18. The mean number of microvessels at post operative day 14 for the ADM group (12.6±4.3, n=7) and ASVF/ADM group (14.3±4.3, n=9) is not significantly deviating in comparison to the control group (14.1±2.3, n=9).

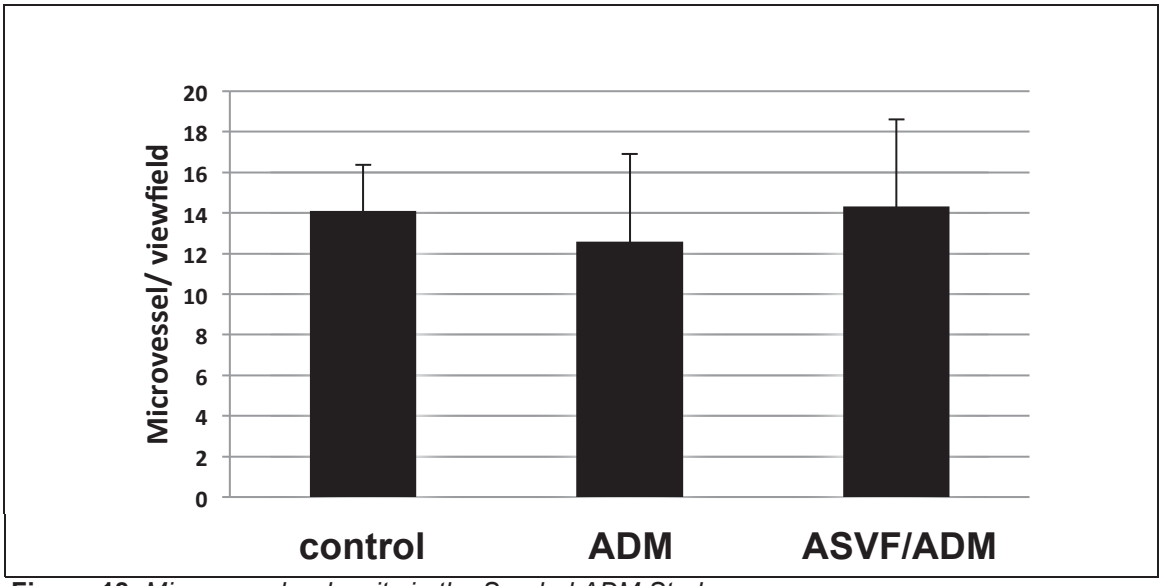

Figure 18. *Microvascular density in the Seeded ADM Study*
The treatment groups control (n=9), ADM (human acellular dermal matrix, n=7) and ASVF (adipose-derived stromal vascular fraction, n=9) are listed on the abscissa. The ordinate scales the correlating mean number of microvessels ±SD per 40x magnified viewfield.

4. Discussion

The studies objective was to evaluate the potential of an adipose-derived stromal vascular fraction (ASVF) as a prospective skin graft for the therapy of large skin defects. If applicable, the patient may benefit from a decreased time of hospitalization and a reduction of pain[3,6].

Of particular interest for soft tissue wound regeneration is the potential of fresh cell aspirates which have not been expanded in the laboratory prior to usage. It needs to be determined if ASVF represents an attractive alternative source to adipose-derived stem cells (ASC) which have to be cultivated prior to their application in regenerative medicine.

Two major study designs were compared: First, in the Collagen Matrix Study the effect of directly applied ASVF and ASC suspension in combination with a Collagen Matrix Film™ (CMF) on wound healing was evaluated. Second, the efficiency of an ASVF loaded human acellular dermal matrix (ADM) was analyzed in the Seeded ADM Study.

The regenerative potential of ASC was described by Kim et al.[1], and involves the improvement of reepithelialization of full thickness skin defects. To determine ASVF phenotypic characteristics and its differences to ASC the ASVF was analyzed in vitro prior to in vivo studies.

It was highlighted that ASC contribute to microvessel formation e.g. by bidirectional paracrine interaction and secretion of proangiogenic factors[53]. Kim et al. showed, that ASC mediate vascularization[54]. Moreover, its ability to transdifferentiate into vascular endothelial cells has been investigated by Moon et al.[62]. Planat-Bernat et al. discovered that ASVF also have the potential to acquire an endothelial cell phenotype and may participate in blood vessel formation[63]. Brem mentioned that besides numerous other factors, revascularization is crucial for sufficient wound healing. Consequently in this study microvascular density has been analyzed, because angiogenesis is an important process contributing to wound healing[64].

These studies yielded the following key results:

- The phenotypic characteristics of the freshly isolated cells used in these studies resembles consistent with the literature, a heterogenic phenotype, the so called ASVF. Stem Cell associated markers as well as stromal associated markers are expressed. The panhaematopoetic linage marker was expressed only weakly. Endothelial lineage marker expression was not detected. The surfacemarker profile of ASVF was different to ASC.
- There is no significant detectable contribution of neither ASVF nor ASC to reepithelialisation of full thickness skin defects and microvascular density in the Collagen Matrix Study.
- An ASVF seeded ADM did not improve reepithelialisation and microvascular density when compared to no treatment controls in the athymic nude mice model.
- The ASVF seeded ADM approach appeared to induce the formation of a more smooth and less wrinkled scar tissue an might therefore be taken into consideration for further studies facing aesthetic aspects of ASVF application.

Phenotypic Characterization

Before the in vivo studies were started a phenotypic surface marker analysis showed a constant presence of the stem cell associated marker CD34 as well as the stromal associated markers CD90, CD44 (a hyaloronic receptor) and CD73 throughout all series.

The presence of human stem cells (HSC) associated marker CD34, a sialomucin and L-selectin ligand[65], in the initial ASVF has been shown by Mitchell et al. as well. Furthermore Mitchell et al. observed, that the CD34 presence declines significantly during successive passaging in vitro[5]. This observation is consistent with prior findings by Altman et al. and Bai et al.[59,61] and possibly segregates ASVF from ASC by phenotypic characteristics.

The continuous presence of stromal cell associated markers like CD90, CD44 and CD73 rises during laboratory processing as shown by Mitchell et al. This depicts the enrichment by laboratory processing and cell expansion of a

homogenous stromal phenotype of a former heterogenic cell fraction[5]. In consequence the enrichment of a stromal cell phenotype can be regarded as one relevant factor for suitable wound healing capacities.

The panhematopoietic marker CD45 was only slightly positive in one sample. McIntosh et al. [4] described the presence of hematopoietic marker CD45 in the ASVF but in this study only a low amount of CD45 was found. Furthermore CD31 an endothelial lineage marker was not detectable throughout all preparations; this finding is inconsistent to Mitchell et al.[5]. Among other stem cell associated markers CD34 and CD31 have been used to classify endothelial progenitor cells in adipose-derived cell populations.

Even if the phenotypic characteristics differ from data by Mitchell et al., and McIntosh et al. Miranville et al. stress that a cell pool containing CD34+/CD31-/CD45- is capable to differentiate into endothelial cells and, moreover, improves vasculogenesis[66].

I agree with Kondo et al. who stated that it might need more parameters to investigate than CD markers, antigens or enzymes to exclusively define adipose tissue-derived cell fractions[67]. Therefore, it remains unclear if the conclusion of the causality to mediate wound healing can be drawn. For future experiments more features and abilities of cell fractions and their differences might help to clarify this issue.

Collagen Matrix Study

The first in vivo study addressed the question if application of ASVF and ASC in combination with a CMF enhances wound healing. The quantative analysis of reepithelialized area did not point out any significant variation in reepithelialization over time with respect to the different treatment groups.

A probable explanation could be that the direct application of ASVF and ASC in this model weakens their regenerative potential in this experimental setting. Instead, other application methods using similar cell populations have proven to be effective. Falanga et al. used bovine collagen as a sufficient matrix carrier for fibroblasts and keratinocytes to treat venous ulcers[34]. Furthermore Nambu et al. enhanced wound healing by using ASC linked to collagen gel in an impaired wound model[68]. Kim et al. showed that collagen gel as ASC carrier and

Tegaderm™ as wound cover in vivo accelerate wound healing without signs of abnormal wound healing such as inflammatory cell infiltration and epithelial hyperplasia[1].

In addition Scadden illuminated the importance of ECM as stem cell niche. By the absence of a niche cells might not develop proper functionality to contribute to tissue regeneration. Cell depletion might take place without any niche[69]. Without an appropriate matrix support cells applied to the wounded area might spread to a major extent throughout the body. Indeed Meyerrose et al. showed that ASC administration via various routes: e.g. subcutaneous injection leads to ASC migration throughout multiple organs[70].On the other hand Altman et al. showed that ASC engraftment combined with a proper delivery construct (ADM) lets cells stay at the site of engraftment without observable systemic distribution [2].

It seems, that in the in the Collagen Matrix Study ASVF as well as the ASC were restrained to develop their full regenerative potential. Considering the arguments mentioned above the limited regenerative potential might be due to the lack of a proper niche.

Facing qualitative aspects of reepithelialization and wound healing three inconsistent observations have been made further on in the Collagen Matrix Study: First, the macroscopic appearance of the ASVF/CMF group showed in comparison to the ASC/CMF and the control (no treatment) group a circumferential region of hyperemic, erythematous tissue at the wound's margin of epithelialization at post operative day 6 in series I, representing a proper wound healing environment. Second, in series III of the Collagen Matrix Study this difference has also been observed between the ASC/CMF and the CMF groups. Third, in series II the macroscopic observation for the ASVF/CMF group in comparison to the acellular CMF and control group was not made.

These differing observations with respect to wound morphology leads to the possible explanation that the cell pool itself originated from different donors has different abilities. Indeed Hong et al. reported for autologous cells as a cellular component of tissue engineered skin substitutes a high variability of the therapeutic agent based on the donors biology[71]. The uncontrolled application

regarding donor's biology depicts the vulnerability of this Collagen Matrix Study design because it may lead to a high variability in therapeutic outcome.

As a suitable measurement for new vessel formation in vivo the microvascular density has been used to investigate the contribution of stem cells to angiogenesis[59]. Measurement of microvascular density in this study did not point out any significant effect neither in the ASVF group nor in the ASC group of the Collagen Matrix Study. This is contradictive to earlier findings by Altman et al. They showed that ASC delivered via a silk chitosan scaffold significantly increased microvascular density[59]. A possible explanation of this difference might be the lack of a matrix. Silk chitosan for a so far unidentified reason might enhance the ability of ASC to either directly or indirectly contribute to vascularization during healing processes.

Seeded ADM Study

The second in vivo study (ADM seeded Study) dealt with the efficiency of an ASVF loaded ADM to improve wound healing. Using ADM, a well known ECM had been proven and shown its benefits in clinical trials before[26,29,30].

Interestingly, one key result in macroscopic observation was that no treatment at the wound site (=control group) compared to the treatment with either ADM or ASVF/ADM seems to result in a comparable reepithelialization after 7 days. This would indicate that there was no beneficial effect of neither ADM nor ADM/ ASVF. However, it appeared that the untreated control had more wrinkles than the other groups. Thus, the wound closure might have been primarily mediated by wound contraction rather than reepithelialization.

Indeed in rodents wound site contraction is the dominant mechanism of wound closure whereas formation of granulation tissue and reepithelialization are main forces in human wound healing[72,73]. In theory, placing any rigid matrix into the wound bed as done in this study might keep the function of an artificial spacer that diminishes the adequate wound contraction. The question needs to be addressed on what conditions regular wound healing models in rodents reflect an effective experimental setting to evaluate ASVF reepithelialization properties. It might be useful for future settings to apply e.g. a silicon splint to prevent

wounds from contraction to facilitate granulation and reepithelialization like successfully done before by Lin et al. in a diabetic wound model[74] based on the work of Galiano et al.[72]. Another useful design might be to mark the original wound margin by tattooing. By that any contraction of the wound's margin would be detected easily by wound margin shriveling. This method has been successfully used before by Lamme et al.[75].

Likewise Blanton et al. using ASCs did not realize any significant difference in wound size reduction. Still the group observed a benefit of administered ASC in wound healing especially facing cosmetic and angiogenic properties[76]. On the other hand Kim et al., Wu et al. and Altman et al. successfully enhanced wound healing rates and increased wound closure in their settings [1,2,77].

However, using ASC and ASVF in impaired wound healing models, e.g. systemic illness as diabetes mellitus, a significant increase of reepithelialization has been reported [68,78]. This leads to the conclusion that the benefit of ASC and ASVF needs to be further investigated: The studies by Nambu et al. confirm that ASC and ASVF contribute to reepithelialization. It is common sense that reepithelialization in impaired models is less sufficient than in a healthy one. Therefore it should be worth to further prove under which circumstances ASC and ASVF exhibit an effect in general.

Another key result throughout the postoperative period was a less embossed graft in the cell loaded ASVF/ADM group and less scabbing on post operative day 7 and post operative day 12. Furthermore, the connection between ADM and the adjacent wound margins appeared more homogenous in the ASVF/ADM group in comparison to the ADM group. Besides the less embossed graft, fewer wrinkles were present (see above). A hyperemic erythematous reepithelialized area visibly separated the remaining granulation tissue from the unwounded skin in the ASVF/ADM group, which might indicate a better engraftment. These results are supported by other's findings: Facing the effect of cells transplanted to wound sites Erdag et al. recognized that the addition of human fibroblasts to a cultured skin substitute (CSS) forms a thicker epidermis, prevents graft contraction and enhances the vascularization of the graft finally supporting epidermal regeneration[55]. Additionally, Fuchs et al. demonstrated

that cells seeded on ADM improved mechanical and functional graft properties by organized cellularity and parallel alignment of collagen fibers[56]. In conclusion the positive effect of ASVF on graft incorporation should be further reconfirmed by further studies.

ASC have been identified to secrete multiple factors like platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF) which play a key role in wound healing. For example, in Arronson's et al. studies KGF secretion by ASC occurred[79]. Furthermore KGF, a fibroblast growth factor (FGF) relative, confirmed its important role in wound reepithelialization [79–81]. As well fibroblast proliferation and collagen secretion is induced by ASC [1]. Altman et al. complement this by showing that ASC, delivered via ADM spontaneously differentiate into endothelial, fibroblastic and epidermal lineages and improve wound healing[2]. It needs to be further explored if these underlying mechanisms also clarify the beneficial graft incorporation of ASVF.

Facing cosmetics Lamme et al. showed that addition of fibroblasts to a dermal substitute forms a smoother surface, rather pink than red colored and supple scars as well as less contraction[82]. In the present study the macroscopic impression of less scarring and wrinkles in the ADM seeded graft group endorse this. Kim et al. showed e.g. that ASC alter the pathomechanism of photoaged skin by increasing collagen synthesis and activate human dermal fibroblasts, which play a key role in the pathology of photoaging[1]. Even if the effect in the present study on reepithelialization over time is not striking, the impact of scaring in cosmetic surgery opens up a future key aspect for research in studying the effectiveness of ASVF respectively ASC for aesthetic wound healing.

In a clinical context Treiman et al. described the importance of neovascularization and proper blood supply for wound healing[83]. As one part of angiogenesis VEGF has been identified to play a key role in orchestrating angiogenesis in wound healing[84,85]. ASC and ASVF keep the ability to secrete

chemokines e.g. VEGF to stimulate angiogenesis as well [62,78,86]. Contradictory in the present study by analyzing microvascular density beneath the epithelia the number of microvessels was not significantly different in any treatment group. To find an explanation the following facts should be considered:

The first critical aspect is the cell quantity. It needs to be discussed if the cell quantity was too low to affect vasculogenesis. Other groups have already denied this fact by applying a similar cell amount of ASC or MSC as used in this study ($1x10^6$) to similar wound models in athymic mice. Kim et al. and Wu et al. successfully enhanced wound healing rates and increased the gross wound closure[1,77]. Likewise Altman et al. showed that ASC ($2x10^5$) successfully unfold their potential for soft tissue reconstruction with a cell pool fivefold less than the cell pool used in this study[2].

Second, the in vitro time frame for cell grafting to the dermal substitute needs to be discussed. Facing the adherence capability over time Altman et al. showed that 96% of ASC already stick to ADM in 120 minutes of coculture[87]. Evidently Altman et al. used exactly the same in vitro process for cell adherence to the dermal substitute as in this study. However, in my studies only the cell pool was changed from ASC to AVSF. One explanation could be differences in adherence capacities. Maybe passaged and processed cells (ASC) as Altman et al. applied adhere better than freshly isolated cells (ASVF) as used in this study. It is well known that ASC enrich for a stromal phenotype[5]. Furthermore I suppose that processed stromal cells keep better adherence features than unprocessed cells. As a result for future research projects it needs to be investigated if ASVF differ from ASC regarding their adherence capacity to dermal matrices.

Third, the non significant statistical deviation in microvascular density needs to be discussed. The lack of effect on microvessels could also been due to the dermal matrix used in this study, but ADM has been shown before to get vascularized. Consequently, the provided ECM is well suited for the angiogenic differentiation of ASC[27]. Alike, Altman et al. showed that ADM and ASC have the potential to improve soft tissue reconstruction[2].

Conclusion

As the discussion of the major results of this study in the context of the current scientific literature shows there is no consensus to rule out the benefits of tissue reconstruction by using ASVF. To realize a final explanatory framework for the conditions of ASVF in facilitating wound healing I suggest more fundamental research in respect to the properties and potentials of ASVF. In the present scientific context the fallowing issues sum up this work:

- To predict the effect of stem cells on wound healing other relevant parameters than phenotypic characteristics alone need to be taken into consideration.
- The optimal niche as a suitable carrier vehicle for ASVF as well as ASCs to survive and support wound healing needs to be identified.
- Prior to in vivo stem cell application the cell pools, originated from different donors, need to be defined individually on donor's biology and quality.
- Before transferring the results of animal studies into clinical application of tissues transplants the investigated wound healing models and it's underlying mechanism of wound healing need to be considered respectively identified.
- Facing the effect on wound healing in general, more parameters than the area of reepithelialization and vascularization over time need to be studied. Beside therapeutic benefits, aesthetics properties like scaring and wrinkles as well as functional and mechanical properties like graft incorporation and flexibility are additional aspects to be considered in ASVF research.

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8. Declaration

Eidesstattliche Versicherung

Ich versichere an Eides statt, dass die Dissertation selbstständig und ohne unzulässige fremde Hilfe erstellt worden ist und die hier vorgelegte Dissertation nicht von einer anderen Medizinischen Fakultät abgelehnt worden ist.

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Christoph Beckmann