Sektion Plastische und Handchirurgie
Zentrum für Schwerbrandverletzte
Universität zu Lübeck
Leiter: Prof. Dr. med. P. Mailänder

Use of human mesenchymal cells to bioactivate scaffolds for dermal regeneration *in vitro* and *in vivo*

Inauguraldissertation
zur
Erlangung der Doktorwürde
der Universität zu Lübeck
- Aus der Medizinischen Fakultät-

Vorgelegt von
José Tomás Egaña Erazo
aus Santiago, Chile.
Lübeck 2007

1. Berichterstatter: Prof. Dr. med. Hans-Günther Machens

2. Berichterstatter: Priv.-Doz. Dr. med. Wulf Ito

Tag der mündlichen Prüfung: 06.06.2008

zum Druck genehmigt. Lübeck, den 06.06.2008

gez. Prof. Dr. med. Werner Solbach

- Dekan der Medizinischen Fakultät -

Einverständniserklärung

Name, Vorname: Egaña-Erazo, José-Tomás
Adresse: Kreittmayerstrasse 15/ 80335/ Munich
Fakultät: Medizin
Titel der Dissertation: Use of human mesenchymal cells to bioactivate scaffolds for dermal regeneration in vitro and in vivo

Ich erkläre mich damit einverstanden, dass die Zentrale Hochschulbibliothek Lübeck die elektronische Dissertation und die dazugehörigen Daten in Datennetzen zur öffentlichen Nutzung bereitstellt. Die Zentrale Hochschulbibliothek ist berechtigt, die elektronische Dissertation und die dazugehörigen Daten an die Deutsche Nationalbibliothek weiterzugeben.

Falls persönliche Daten (Lebenslauf) in meiner Dissertation enthalten sind, bin ich damit einverstanden, dass diese von der Zentralen Hochschulbibliothek Lübeck und der Deutschen Nationalbibliothek maschinell gespeichert und zur öffentlichen Nutzung bereitgestellt werden.

.....
Unterschrift

Contents

List of abbreviations

bFGF	Basic Fibroblast Growth Factor
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
ECM	Extracelular Matrix
EDTA	Etilendiaminotetraacetic acido
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPC	Endothelial Progenitor Cells
EPO	Erythropoietin
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
FCS	Fetal Calf Serum
HIFs	Hypoxia-Inducible Factors
IM	Integra matrix
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
PBS	Phosphate Buffer Saline
PDGF	Platelet Derived Growth Factor
PFA	Paraformaldehyde
SDR	Scaffolds for Dermal Regeneration
SDF-1α	Stromal Derived Factor 1α
SV40	Simian Virus 40
VEGF	Vascular Endothelial Growth factor

List of figures

1. Introduction

1.1. Skin structure and functions

Skin is the largest organ in the body being responsible of several vital functions. Such are blood flow distribution, control of the internal temperature, serves as barrier to protect the body from external pathogens and connecting sensorial information from the environment. All these functions are performed by the coordination of two different structures; epidermis and dermis. The epidermis is the outer layer of skin which is mainly composed of keratinocytes, melanocytes and Langerhans cells. Epidermis is a thick layer of elastic and fibrous tissues that gives the skin its flexibility and strength and contains many specialized cells and structures. Such as nerve endings, sweat and sebaceous glands, hair follicles and blood vessels. Nerves transmit the sensation of temperature, pain, touch and pressure; sweat glands produce sweat in response to heat helping to modulate the body's temperature; sebaceous glands produce sebum which maintains the moisture of the skin and protects the body against foreign substances; hair follicles produce hair and contain a stem cell pool which produces keratinocytes. Finally, all the dermal structures are supported by the blood vessel network which provides oxygen and nutrients and, by dilatation or contraction plays an important role in the regulation of the internal temperature of the body, enhancing or decreasing the heat interchange between the body and the environment. The scheme in figure 1 represents the general aspect of the skin and some of its different structures.

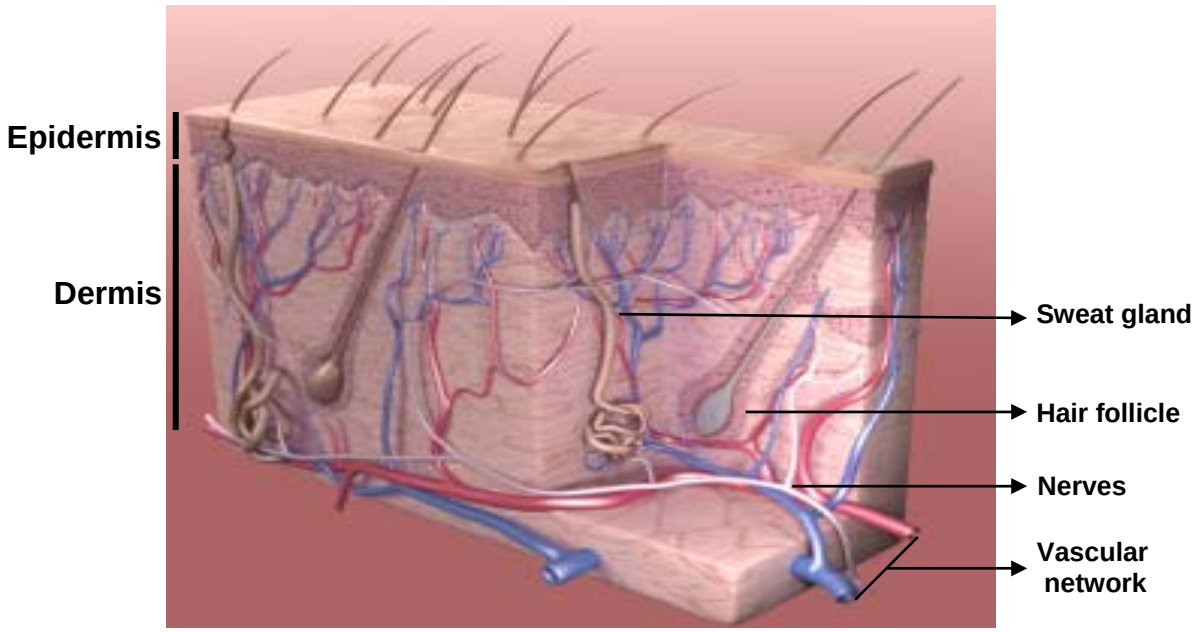


Figure 1: General structure of the skin. Skin is a complex organ composed by two layers: Epidermis and dermis. Different cells and organs, composing this tissue, work together performing several vital functions in the body. (Modified from http://www.turbosquid.com)

Due to the important role of skin in the body, skin disease or skin defects cause the death of thousands of people per year and tremendous cost for the health system. For this reason, several efforts have been made to developed new therapeutic approaches which could improve wound healing and skin regeneration.

1.2. Vascular regeneration.

The functional homeostasis of tissue depends on its adequate blood perfusion. By several conditions blood flow could be pathologically altered, thus activating cellular and molecular regulatory mechanisms. Until now, 2 main mechanisms have been described to explain the formation of new blood vessels in adult tissues: Angiogenesis and vasculogenesis. Angiogenesis occurs by ramification of preexisting vascular structures which can grow in direction to the tissue. In contrast, vasculogenesis is a process where the formation of new vascular

structures involves other cells than preexisting endothelial cells. Figure 2 shows the differences between both mechanisms.

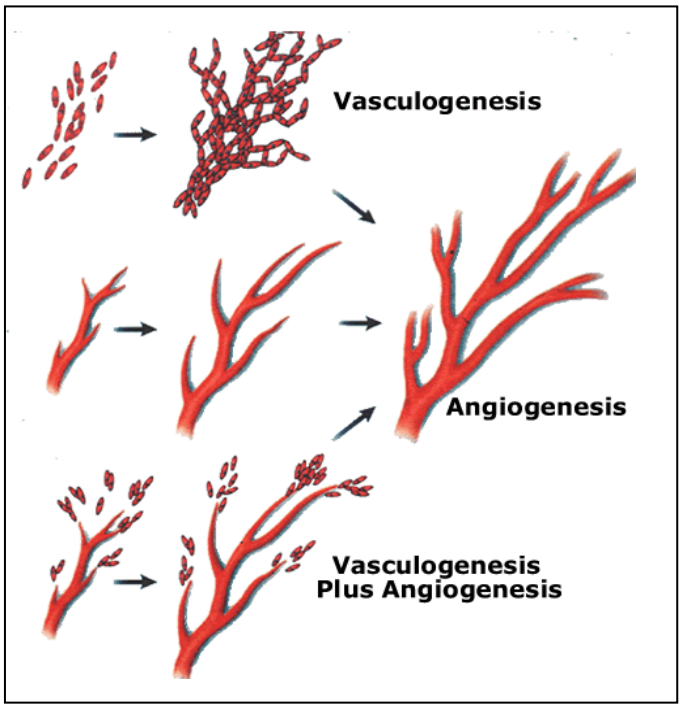


Figure 2: Main mechanism of tissue vascularization. Vasculogenesis is a process where blood vessels are developed *de novo* by the differentiation and assembling of endothelial progenitor cells. In contrast, angiogenesis is referred to the grow of preexisting blood vessels. Both processes may act in parallel to induce vascular tissue regeneration. (From Harvey and Rosenthal, 1999)

Angiogenesis is divided in three different overlapping processes: Invasion, stabilization and regression of the newly formed vessels. These steps are mainly modulated by proangiogenic growth factors such as VEGF, bFGF, PDGF and angiopoietins. These molecules can trigger a direct response in the endothelial cells and also affect the interaction between those cells and others or its interaction with the extracelular matrix (ECM). Angiogenic growth factors are released from the ECM by proteinases or secreted from cells in affected tissues. The most important stimulus to induce gene expression of angiogenic growth factor and further secretion is hypoxia, which induces activation of hypoxia-inducible factors (HIFs). HIFs are the master transcription factors of several key genes, including VEGF, EPO and other 60 genes, which beside angiogenesis are involved in the modulation of cell survival and proliferation and the metabolism of

glucose and iron. All processes are closely related to tissue survival. (Lee et al., 2004)

In the last years, angiogenesis has gained importance since insufficient or excessive angiogenesis is related to several diseases. Due to that, efforts have been taken to inhibit or to induce therapeutic angiogenesis. Excessive or abnormal angiogenesis is involved in the etiopathology of cancer, obesity, psoriasis, retinopaty and arthritis among others. On the other hand, insufficient angiogenesis is linked to several disorders such as Alzheimer disease, amyotrophic lateral sclerosis, stroke, atherosclerosis and hypertension (Carmeliet P. 2003).

1.3 Scaffolds for dermal regeneration and vascularization.

Skin defects can occur due to several conditions such as traumatic accidents, surgical wounds, ulcers or burns, representing major functional and psychological problems for patients including even death. This is the case for deep burn accidents; when they are not fatal they can generate major psychological, economical and functional problems along a patient's life. Traditional strategies for skin replacement includes autologous, heterologous or cadaveric skin transplantation which present several problems related to disponibility and mobidity of donor sites in autologous transplantation and possible disease transmissions in heterologous transplantation procedures. This fact has triggered research efforts devoted to develop new therapeutic alternatives based on the induction and stimulation of the intrinsic capacity of the body to regenerate its structures.

In this context, the development of scaffolds for dermal regeneration (SDR) has been the first and one of the most successful examples of tissue engineering in human medicine. These scaffolds are bioartificial three dimensional matrices, composed mainly of collagen, which serves as a backbone for cell infiltration from the wound bed, followed by neovascularization and tissue regeneration (Moiemen et al., 2006). Figure 3 shows the porous microscopical aspect of Integra matrix (IM), a commonly used SDR.

Nowadays, the use of biodegradable scaffolds is one of the most common procedures to treat critically burned patients with more than 70 % total body surface involved. Major full skin burns (3° degree) require epifascial necrectomy including the subcutaneous tissue to create a wound bed with sufficient vascularisation. Over this region, the scaffold is surgically fixed to serve as a matrix for the infiltration of fibroblasts, lymphocytes, macrophages and especially blood vessels. Parallel to the infiltration, the scaffold is degraded and 2-3 weeks later it is possible to see new dermal tissue. Figure 4 shows a successful example of the clinical use of SDR.

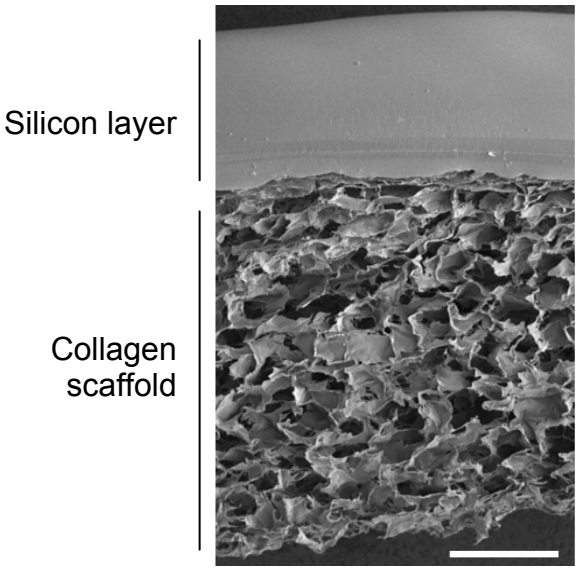


Figure 3: General structure of a SDR. Integra matrix is a commonly used SDR, composed by a silicon layer which act as temporary epidermis and a porous collagen scaffold which allows cell infiltration and dermal regeneration. Scale bar represents 500µm.

Unfortunately, clinical use of SDR is still limited, due its low regenerative capacity and high infection risk for the patients (Heimbach et al., 1988; Machens et al., 2000). These problems are mainly related to the low vascularization capacity of these scaffolds which limits the presence of immune cells, oxygen and nutrients in the wound area. Establishment of successful protocols for tissue engineered skin will require appropriate capability of the scaffold to induce vascularization (Kannan et al., 2005).

Unfortunately, molecular and cellular mechanisms involved in the neovascularization of SDR are poorly understood. However, several efforts have been made to induce vascularization in different tissue engineered structures, including cellular and acellular technologies.

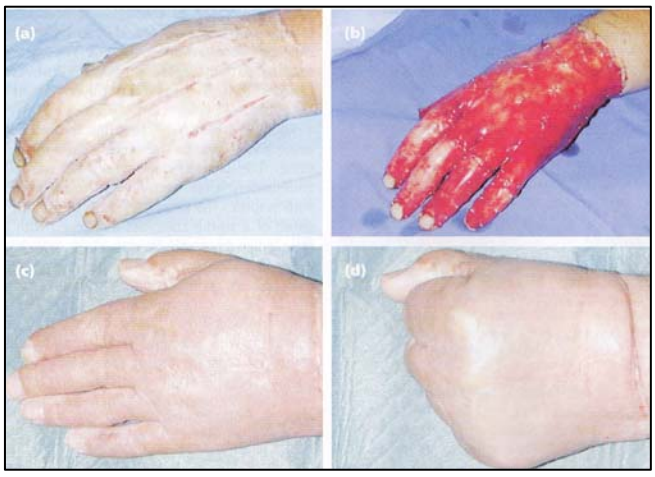


Figure 4: Clinical use of SDR. Damaged skin can be removed and replaced by SDR which, after some weeks, induces dermal regeneration. (Obtained from Dantzer et al., 2003)

A tissue engineered skin substitute that would promote vascularization would be a valuable tool for plastic and reconstructive surgery. Previously, we have shown that preseeding of genetically modified cells, transduced to secrete VEGF and bFGF or the direct addition of those recombinant growth factors improves vascular regeneration *in vivo* in a SDR-based process (Xie et al 2005, Wilcke et al 2007).

The use of transduced cells is an excellent experimental model; however, clinical translation is difficult since *ex vivo* genetic modification of autologous cells requires extensive safety protocols. At the same time the direct application of growth factors in the target area is expensive and requires production, purification and repetitive *in vivo* application of recombinant proteins with a serum half-life of a few minutes. Certain well defined cell populations could act as angiogenic pumps by continous releasing of proangiogenic growth factors. *In vitro* bioactivation of SDR by preseeding of such autologous cells may be an ideal way to promote angiogenesis *in vivo*.

1.4. Integra matrix (IM)

In 1996, IM was approved by the FDA to be used in the treatment of patients with deep burns where it is not possible to apply autografts. However, these patients must tolerate bovine collagen and the wound must not be infected. IM is formed by a porous structure composed by bovine collagen fibers cross linked with glycosaminoglycans which form a structure as scaffold for cell infiltration. The porous structure is covered with a removable silicon layer, which acts as a temporal epidermis, lowering the risk of infection and dehydration during the time of dermal regeneration. Once the dermis has been regenerated, this silicon layer is removed and is replaced by an autologous keratinocyte layer, which is able to establish a definitive epidermis.

For more than 10 years, IM has been used for burned patients with more than 70% of the surface body affected. However, this scaffold can present several problems such as hematoma formation, high infection rate and loss of adherence (Burke et al., 1981; Heimbach et al., 1988; Stern et al., 1990; Machens et al., 2000). All of these problems are related to an insufficient vascular network in the developing dermis.

IM is a useful substrate to perform cellular studies both *in vitro* and *in vivo.* On this aspect, both, keratinocytes and fibroblasts can be seeded *in vitro* and develop a cellular scaffold to be engrafted *in vivo* that aims to improve some clinical aspects of the scaffold (Jones et al., 2003; Wisser and Steffes, 2003).

1.5 Aim of the study and experimental approach

Similar to other fields of tissue engineering, the combined use of bioartificial scaffolds and autologous cells is a rational way to develop skin substitutes with future clinical applications. Moreover, incorporation of autologous cells into SDR may also serve to improve our knowledge about the physiological mechanisms involved in the dermal vascular regeneration process. Aiming to improve vascularization during matrix-based dermal regeneration, we studied whether or not seeding of mesenchymal cells in a scaffold could induce *in vitro* bioactivation, thus enhancing neodermal vascularization *in vivo*. The primary objective of this work was to analyze the behavior of mesenchymal cells *in vitro* and *in vivo* seeded in SDR, focusing on pro-angiogenic processes. Our experiments were performed in order to answer the following next questions:

1. Are mesenchymal cells viable in SDR, bioactivating the dermal scaffold?
2. Can cell-based boactivated SDR improve vascular dermal regeneration *in vivo*?
3. Is the presence of angiongenic proteins secreted from cell-bioactivated SDR sufficient to improve vascular dermal regeneration?

A general overview of the experimental approach is summarized in figure 5.

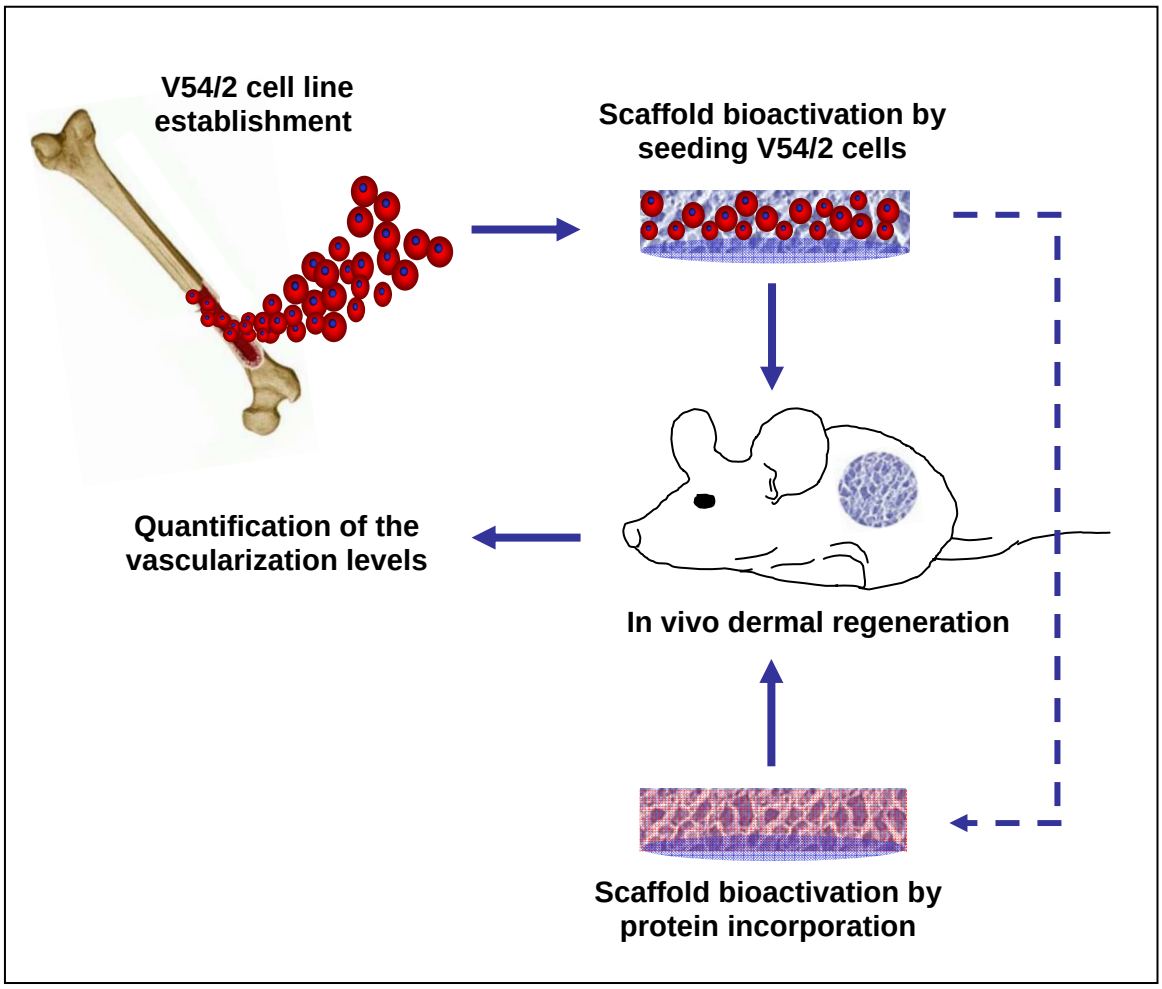


Figure 5: Experimental approach. Human bone marrow derived cells were used for the establishment of the V54/2 cell line. After cell line characterization, cells were seeded and cultured in the SDR and bioactivation of the scaffold was studied *in vitro*. Next, SDR bioactivated with cells were used to induce dermal regeneration *in vivo* and quantification of the vascularization levels was analyzed. In order to analyze the possible mechanism involved in the cell mediated bioactivation process, recombinant growth factors were incorporated in the SDR and induction of

vascularization was evaluated *in vivo*. The discontinuous line represents the possible mechanisms of action of the V54/2 cells.

2. Material and methods

2.1. Cell line

Human $CD34^-$ bone marrow derived cells were immortalized by SV40 Large-T antigen expression. Clone V54/2 was selected and used for the cell line establishment as described by Conrad et al, 2002. V54/2 cells were cultured in DMEM supplemented with 10% or 2% fetal calf serum (FCS) depending on the experiment.

2.2. FACS analysis

V54/2 cells were detached by 5 minutes incubation with trypsin-EDTA solution and washed once with PBS. After blockade of unspecific immunoglobulin binding by incubation for 1 hour with PBS containing 2% FCS, samples were incubated for 1 hour with fluorophore-conjugated antibodies raised against the following cell markers: CD73, CDw90, CD105, CD166, CD34, CD45, CD117 and CD123. As isotype controls, IgG-FITC and IgG-PE were used (all antibodies from BD Biosciences, CA, USA). Samples were examined with a FACScalibur flow cytometer (BD Biosciences, CA, USA).

2.3. VEGF metabolic proliferation assay

$1x10^4$ V54/2 cells were seeded in a 24 well plate and cultured in DMEM 2% FCS during 24 hours. (Gibco, Karlsruhe, Germany). Next, cells were cultured for 4 days in medium supplemented with varying concentrations of VEGF (R&D system, Mineapolis; USA). For cell quantification, MTT assay was performed as described below. Results of three independent experiments were expressed as folds of increase compared to control (cells without exogenous VEGF).

2.4. Scaffold for dermal regeneration

Integra matrix (IM) (Integra LifeScience Corporation, NJ, USA) is a scaffold based on bovine collagen fibers cross linked with glycosaminoglycans which forms a porous biodegradable structure. On top, the structure is covered with a removable silicon layer, which acts as a temporal epidermis.

2.5. Quantification of metabolic activity in the scaffold by MTT assay

$2,5 \times 10^5$ cells were seeded in 1,8 cm^2 scaffolds in 1 ml DMEM 10% FCS. At different time points, medium was removed and scaffolds were incubated for 3 hours in fresh medium containing 5ng/ml of MTT (Sigma-Aldrich, Turkirchen, Germany). Next, medium was removed and replaced by 300µl DMSO. In order to quantify metabolic activity, absorbance at 570nm was measured in the DMSO

containing soluble formazan blue. Scaffolds without cells were used as negative control.

2.6. Cell visualization in the scaffold

At different time points, scaffolds containing cells were fixed in 3% PFA for 1 hour and embedded in paraffin. 15 µm sections were deparaffinized, stained for 20 minutes with DAPI (Sigma-Aldrich, Turkirchen, Germany) and washed twice with PBS. Finally, samples were mounted and analyzed by fluorescence microscopy (Zeiss Axioskop 2, Germany)

2.7. Growth factor release from scaffold- containing cells

2,5 x 10^5 cells were seeded and cultured in scaffold (1,8 cm^2) during two weeks in standard conditions. After 48 hours of cell seeding, medium was replaced for DMEM 2% FCS. Every 48 hours mediums were removed and replaced for fresh medium. VEGF and bFGF concentrations were measured by ELISA according to manufacturer instructions (quantikine ELISA kits, R&D systems, Mineapolis, USA). Scaffolds without cells were used as negative controls.

2.8. Scaffold transplantation

6-8 weeks old athymic nude mice (Body weight 20-25g, Takomi, Copenhague, Denmark) were anesthetized with a mix of ketamine (10 mg/kg of body weight) and xylazin (2,4 mg/Kg body weight) via intraperitoneal injection. Under general

anesthesia, a bilateral full skin defect was created (15 mm of diameter) and scaffolds with or without 2,5 x 10^5 V54/2 cells (12 per group) or preincubated with growth factors or saline (6 per group) were used to induce dermal regeneration in the defects. Scaffolds were fixed to the animal by using non-absorbable sutures and wounds were bandaged (Varihesive®, Convatec, Deeside, UK). All procedures in vivo were approved by the respective local ethical committee authorities (27J04).

2.9. Blood vessel quantification in scaffold

Blood vessel quantification was performed as previously described (Egaña et al, 2007. Submitted). Briefly, after 2 weeks animals were sacrificed and whole skin from the back, including scaffolds, was removed. For vessel visualization tissues were quickly placed on a transiluminator (Hama, LP 5000K, Germany) and standardized digital pictures acquired (Olympus camera, C-5060). For vessel quantification, digital pictures were analyzed with a software developed to perform digital segmentations*. Vascularization levels were calculated as percentage of white pixels from the total. Normal skin of the back of the same animal was used for normalization and results were expressed as a percentage of vascularization in the scaffold related to normal skin. After visualization of the vessels, scaffolds were frozen for further analysis.

* The vessel segmentation program can be free downloaded from www.isip.uni-luebeck.de

2.10. Histological analysis

After 2 weeks of transplantation, animals were sacrificed, scaffolds harvested, fixed with 3% PFA for 30 minutes and embedded in paraffin. 5 µm coronal sections were stained with anti-HLA-class 1 (Clone A18) antibody diluted 1:50. (Santa Cruz Biotechnology, CA, USA).

2.11. Scaffold modification by direct incorporation of growth factors

Scaffolds were incubated overnight in 500µl PBS containing 1µg VEGF and 0.5µg bFGF (both from BD Science, mineapolis, USA) and washed once for 5 minutes with PBS. After that, eight scaffolds per group were used to induce dermal regeneration in vivo. As controls, scaffolds were preincubated overnight in PBS without growth factors.

2.12. Statistical analysis

All assays were repeated at least in three independent experiments. t-test was used to compare samples. $p \leq 0.05$ was considered as significant.

3. Results

3.1. Cell characterization

V54/2 is a $CD34^-$ human bone marrow derived cell line, which has not been fully characterized before. (Conrad et al., 2002). Under normal culture conditions V54/2 cells were strongly adherent, exhibiting a homogeneous fibroblast-like morphology (Fig. 6a). Immunophenotypical characterization of the cells by FACS analysis showed a typical mesenchymal expression profile being strongly positive for the markers CD73, CDw90, CD105 and CD166 and negative for the hematopoietic markers CD34, CD45, CD117 and CD123 (Fig 6b). In order to evaluate the potential of V54/2 cells to respond to external stimuli, cells were cultivated under increasing concentrations of recombinant human VEGF (25, 50 and 100 ng/ml) for 4 days. Results of MTT metabolic assay show that in the presence of VEGF cell number was significantly higher ($p< 0.05$) (Fig. 6c).

3.2. Cell viability and proliferation in scaffold

Next, we evaluated the growth potential of V54/2 after culturing in 3D-scaffolds. As seen in Figures 3 and 7 A-B, Integra matrix (IM) confers enormous surfaces for cell adhesion, enabling a presumably different grow condition as compared to two-dimensional culture plates. To test long term viability of cells in the scaffolds, samples were incubated with MTT. In presence of MTT, scaffolds containing cells turn to dark blue color in contrast to control scaffolds where no color appeared. Microscopically, cells were clearly visualized as blue structures in the scaffold (Fig. 7B upper panel). After 8 and 15 days of V54/2 cells culturing in the scaffolds,

formazan blue formation was significantly increased (3.14 ± 0.44 and 4.46 ± 0.05 folds respectively) as compared to 1 day after cell seeding (Fig. 7B). Cell visualization by nuclear staining with DAPI was also increased after 8 and 15 days of cultivation in the scaffold (Fig. 7A).

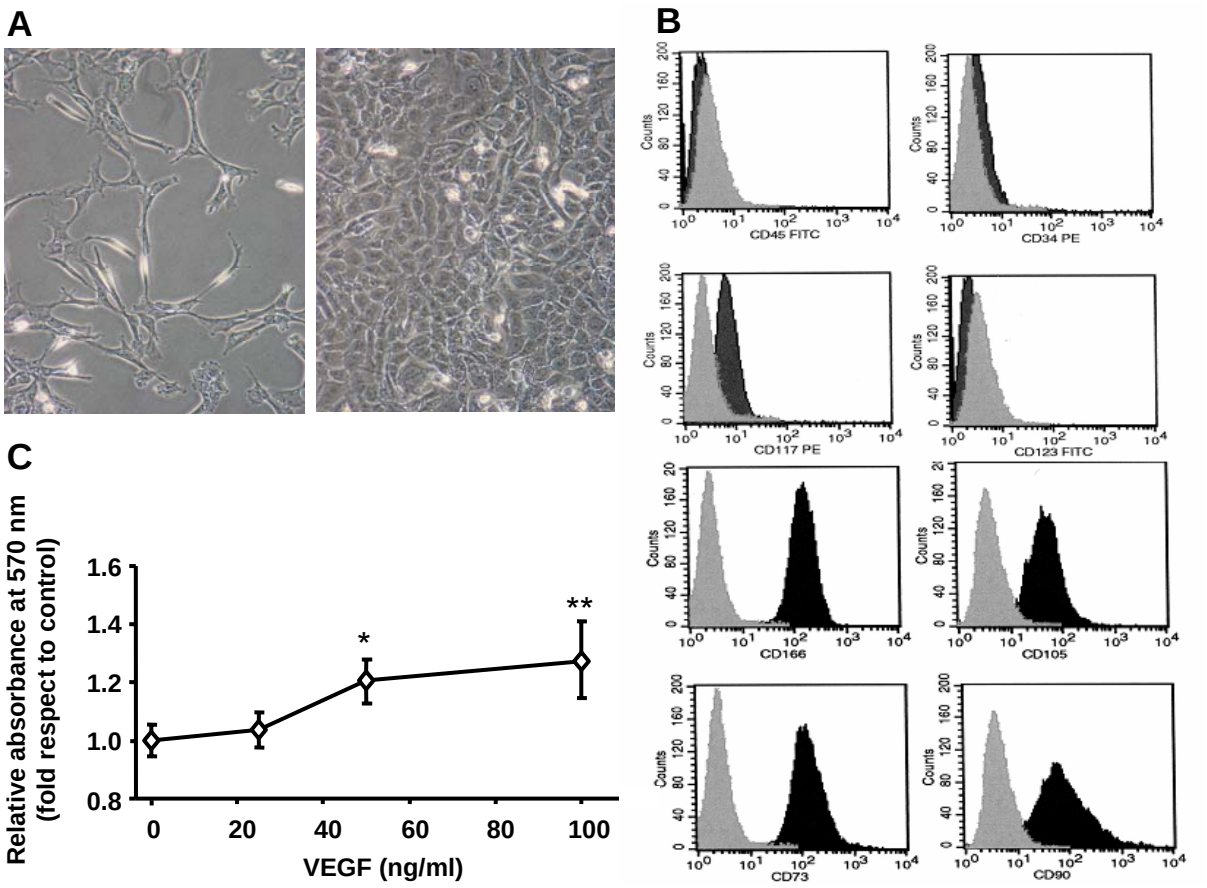


Figure 6: Characterization of V54/2 cell line. (A) Cell morphology. Cells were seeded at confluent and sub confluent densities and observed under 40x phase contrast microscopy. (B) FACS analysis. Mesenchymal immunophenotype of the cell line was confirmed by FACS analysis using the markers CD73, CDw90, CD105, CD166, CD34, CD45, CD117 and CD123. The black and gray curves show the intensity of each specific marker and the corresponding isotype control, respectively. (C) MTT metabolic activity assay. Cells were plated and stimulated with VEGF (0-100 ng/ml) for 72 hours. Metabolic activity was assayed by the formation of formazan blue and determined by spectrophotometry. Values are mean ± SEM, n = 3. *p<0.05 and **p<0.001 in respect to non-stimulated cells.

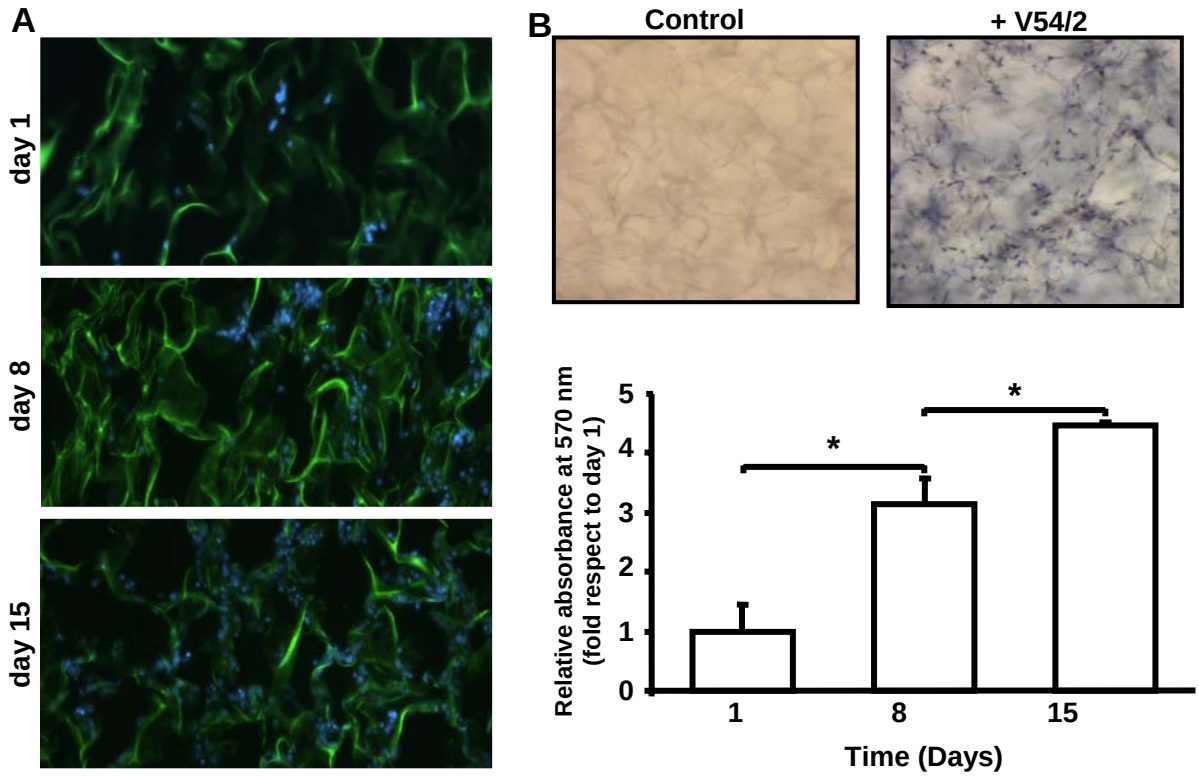


Figure 7: Viability and proliferation of the V54/2 cells in the scaffold. Visualization of cells (blue) in scaffold (green) by DAPI staining suggest cell proliferation by increasing cells number in the scaffold, comparing days 1, 8 and 15 after seeding (A). Cells were seeded in scaffolds and viability was analyzed by MTT assay after 1, 8 and 15 days. Scaffold with cells showed a strong metabolic activity compared to controls (empty scaffold) (B upper panel). Quantification of the formazan blue formation showed significant increase at 8 days compared to 1 day (p= 0.004) and after 15 days compared to 8 days (p= 0.006) (B) confirming the results suggested in A.

3.3. Growth factor release from scaffolds containing cells

Once we confirmed viability of V54/2 in the scaffold, we evaluated their potential to bioactivate IM by releasing proangiogenic growth factors to the environment. Medium from scaffolds containing cells was replaced every 48 hours during two weeks and analyzed by ELISA. Results showed continuing secretion of VEGF and bFGF. Concentration of VEGF in mediums was stable during 2 weeks with values ranging from 1.5 to 2.1 ng/ml. After 48 hours, the concentration of bFGF was close to 0 rising up to 0.35 ng/ml with a stabilization of values around 0.2 ng/ml (Fig. 8). No growth factor secretion was observed in control scaffolds.

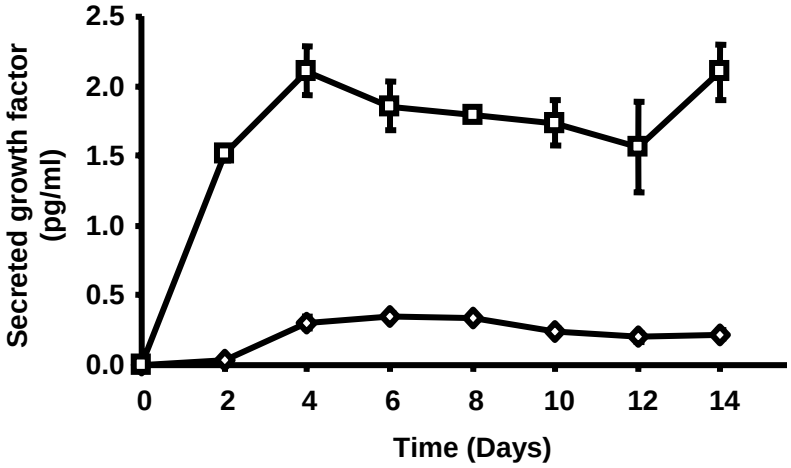


Figure 8: Growth factor secretion from scaffolds containing cells. Mediums from scaffolds containing cells were collected every 48 hours during two weeks and analyzed by ELISA. VEGF (squares, upper curve) and bFGF (rhombus, lower curve).

3.4. *In vivo* dermal regeneration model

Under sterile condition scaffolds were transplanted in a bilateral full skin defect nude mice model (Fig. 9). No major complications were observed during the operation procedure or postoperatively. After 2 weeks, no signs of infection or contraction were observed in the wound area (Fig. 10, left)

In order to evaluate the contribution of V54/2 cells to the neovascularization process, scaffolds were analyzed by a novel method, developed in our laboratory, based on visualization of vessels by tissue transillumination and quantification of vascular networks by digital segmentation. Figure 10 shows an example of this technique.

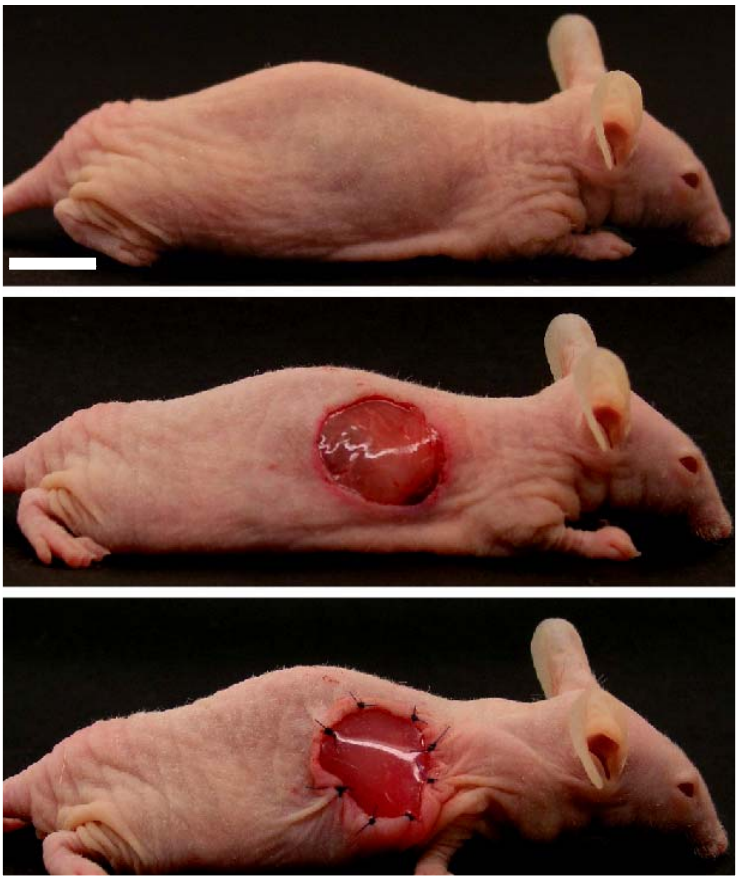


Fig. 9. Full skin defect model for dermal regeneration. 1,8 cm^2 skin were surgically removed in each side and replaced by a scaffold to create a bilateral full skin defect model for dermal regeneration. Scale bar represents 1 cm.

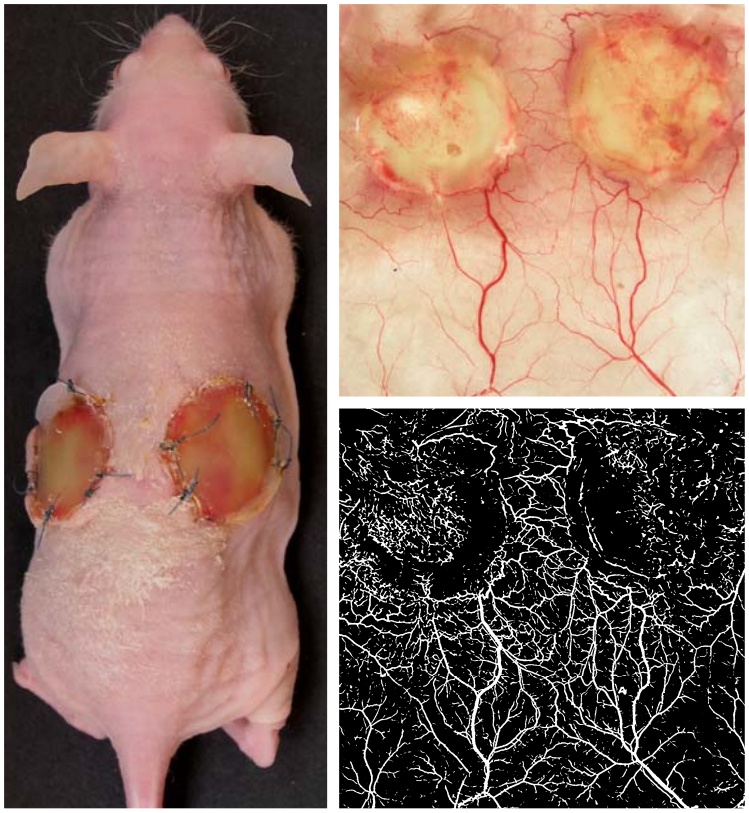


Figure 10: Tissue transillumination and digital segmentation. After two weeks of dermal regeneration (left) the whole skin, including scaffolds, was removed and placed over a transiluminator The entire vascular network was visualized in normal tissue and scaffolds (upper panel right). For vessel quantification, digital segmentation of the whole tissue was performed (lower panel right). Results were expressed as percentage of white pixels in the scaffold compared to normal.

After two weeks of transplantation, vascularization of 12 scaffolds per group (6 animals) was analyzed by transillumination and digital segmentation. Results showed that the presence of V54/2 cells significantly improved vascular regeneration during the scaffold based dermal regeneration process *in vivo* (p= 0.001) (Fig. 11).

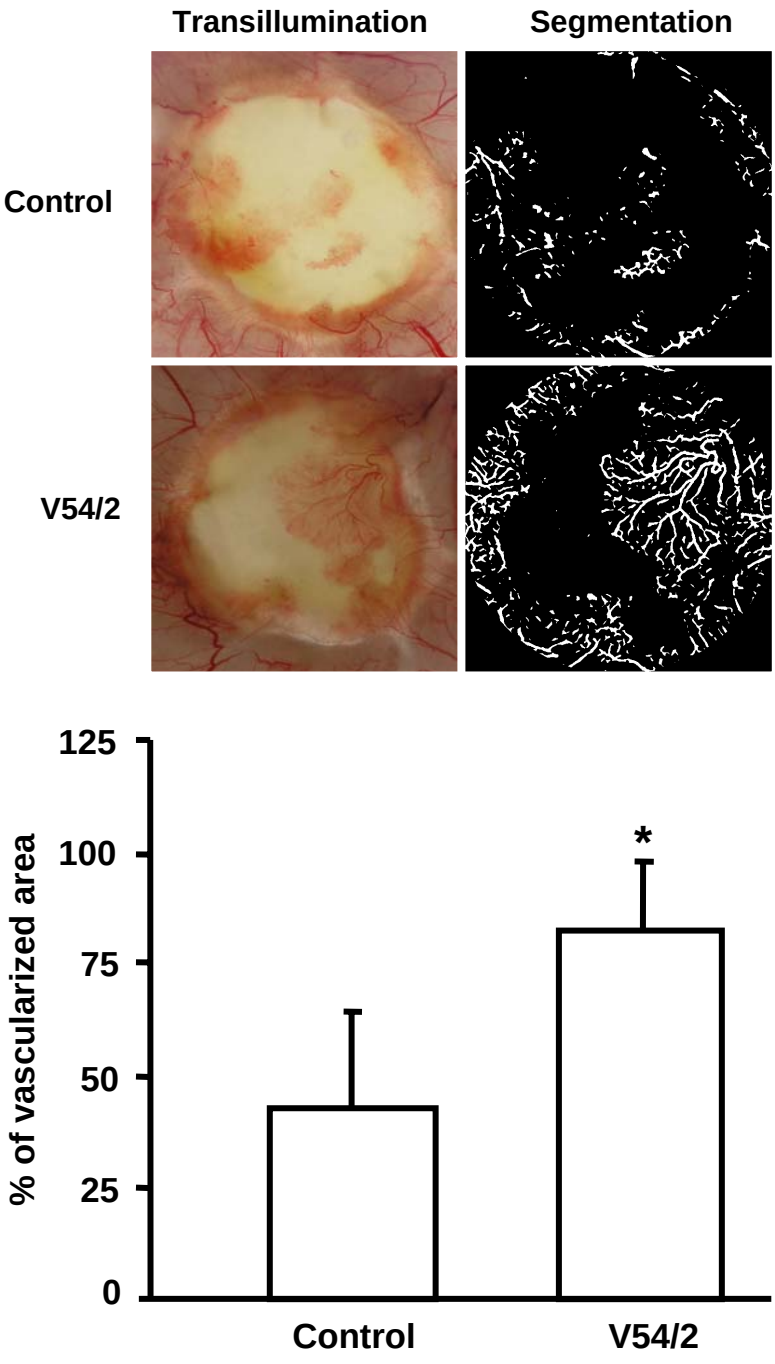
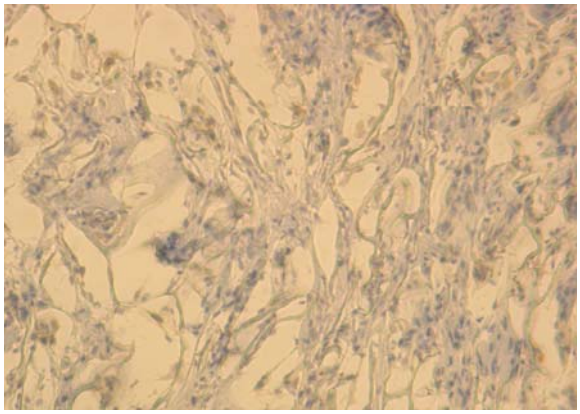


Figure 11. Blood vessel visualization and quantification. After two weeks of dermal regeneration animals were sacrificed and skin was removed to analyze scaffold neovascularization. A representative picture of transillumination and digital segmentation of one scaffold is shown (upper panel). Quantification of digital segmentation showed significatively higher vascularization levels in animals with scaffolds containing cells (lower panel) (* p= 0.001).

Immunohistological analysis of tissue sections (4 scaffolds per group), showed high levels of cellularization in both groups by eosin stain. Presence of human cells was detected by the use of HLA-class 1 antibody. After two weeks of transplantation, positive reaction was observed only in the group of animals transplanted with scaffolds containing V54/2 cells (Fig. 12).

Control



V54/2

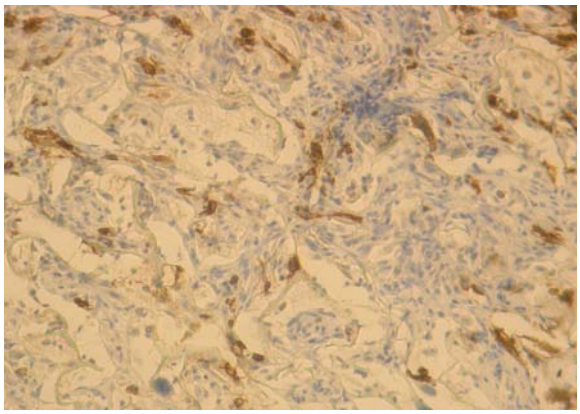


Figure 12: Immunohistological analysis. Tissues were analyzed after two weeks of dermal regeneration. In both conditions high cellularization levels were observed by eosin staining. Presence of V54/2 cells was visualized by HLA immunostaining (brown).

3.5 Presence of angiogenic growth factors in scaffold induces vascular regeneration.

Finally, we tested if the secretion of proangiogenic growth factors from the V54/2 cells line in the scaffold could be sufficient to improve vascular regeneration. Scaffolds preincubated with VEGF and bFGF were transplanted in the full skin defect model. After 2 weeks, transillumination and digital segmentation of the tissue in regeneration was performed and levels of vascularization between

scaffolds preincubated with VEGF/bFGF or with PBS were compared. Quantitative analysis of 6 scaffolds per group (3 animals), showed that presence of growth factors significantly improved the levels of vascularization in the scaffold (p= 0.036) (Fig. 13).

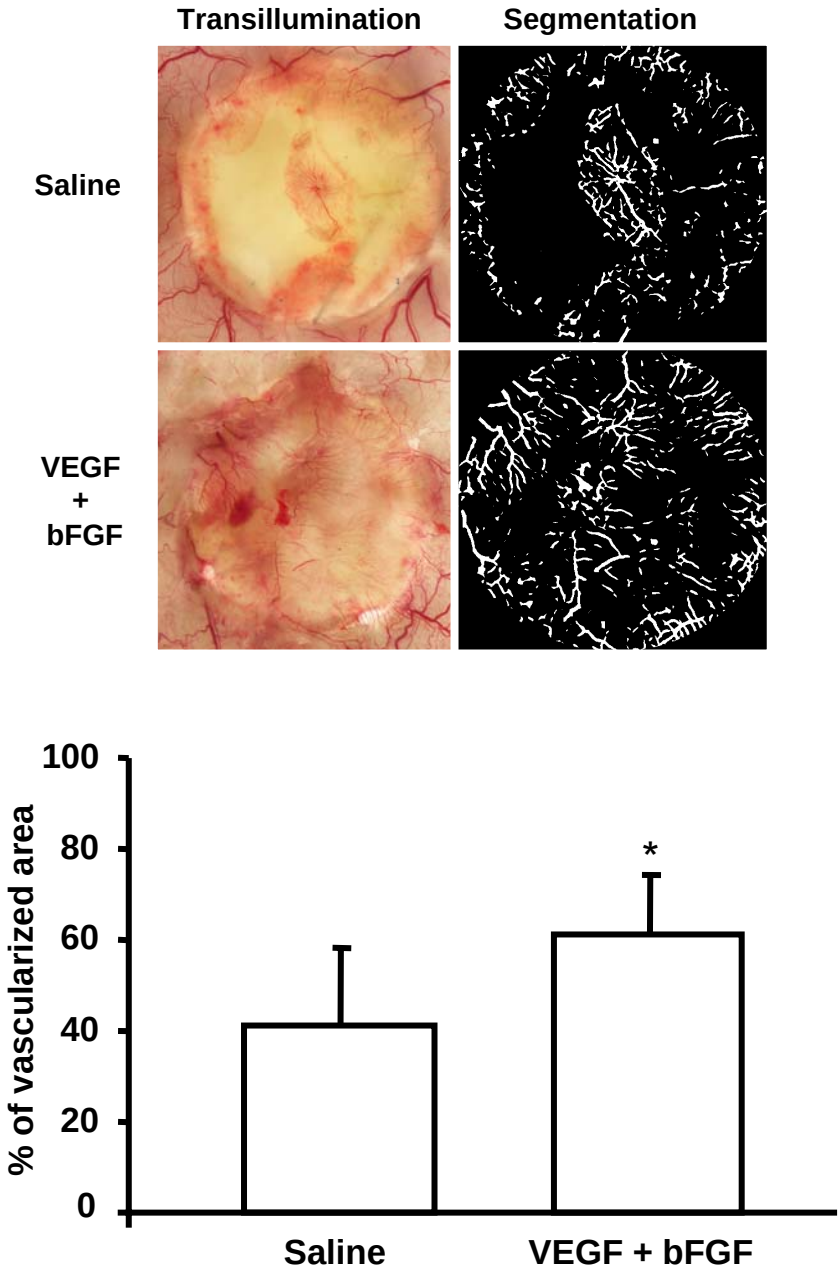


Figure 13: Blood vessel visualization and quantification. Scaffolds were incubated over night with 500 ul PBS containing 1 µg VEGF and 0.5 µg bFGF. After two weeks of dermal regeneration tissue was harvested. Transillumination and digital segmentation of a representative scaffold of each group is shown in the upper panel. Lower panel shows that presence of growth factors in the scaffold induce angiogenesis compared to scaffolds preincubated in PBS. (p= 0.036)

4. Discussion

Three overlapped stages are involved in normal wound healing: (1) Inflammation, (2) proliferation and, (3) remodeling (Eppley et al., 2006). The initial stage is characterized by infiltration of neutrophils, monocytes and T-lymphocytes which, in summary, protect from tissue infection, remove tissue debris and provide the elements to start with the angiogenic process. At the end of the inflammatory phase, a transient well-vascularized tissue, called granulation tissue, is formed in the wound area. This tissue is replaced during the proliferative stage, for a specific tissue of the area (e.g. bone, cartilage, etc). Here, factors such as cytokines, pH, oxygen tension, and growth factors play the main role creating local environments to promote cell migration, survival and differentiation. Finally, during the remodeling, the new tissue is reorganized and reshapes to regenerate a tissue similar to the original. Unfortunately, skin is repaired but not regenerated. Scar tissue formation is the mechanism by which the body heals skin wounds and is composed mainly by fibroblast and extracelular matrix (ECM). This tissue may restore integrity but not functionality. Scar formation is critical in massive (e.g. burns) or specific (e.g. neck, hands, elbows) skin injuries where a functional tissue is required. In this context, efforts in tissue engineering are focused to reach skin regeneration by mimicking the normal physiological regeneration processes described before. For that propose, SDR has been used as template where inflammatory, proliferation and remodeling could take place. Although the idea is good, clinical success has been poorer than expected.

It is commonly accepted that the lack of vascularization is one of the most important sources of complications in the scaffold-based dermal regeneration

process. In this work, we show evidence supporting the idea that this problem is, at least, partially solved by cell-mediated scaffold bioativation, enhancing vascularization *in vivo*. Two different mechanisms mediate adult tissue vascularization: vasculogenesis and angiogenesis. In the first, blood vessels are developed *de novo* by mobilization, recruitment and differentiation of endothelial progenitor cells (EPC) (Kawamoto et al., 2003). This is in contrast to angiogenesis where vascularization occurs by ramification of preexisting vascular structures that grow into ischemic areas (Folkmann et al., 1992).

Here, we investigated the improvement of vascular regeneration by angiogenesis, a process mainly regulated by growth factors such as VEGF, angiopoietins and bFGF (Carmeliet P, 2003; Fiedler and Austin, 2006; Angelo and Kurzrock, 2007) which are released to tissues from different cell types or by degradation of ECM. Such ECM, same as IM, is mainly composed of collagen which can directly interact with VEGF and bFGF (Kanematsu et al., 2004). VEGF plays a key role in angiogenesis during wound repair since its vasopermeability actions increase hydraulic conductivity and fenestration (Lie et al., 2003). Moreover, VEGF is a potent mitogen for endothelial cells and induces endothelial cell migration, sprouting and survival. Expression of VEGF inhibitor has been shown to be reduced in the dermal layer of acute wounds compared to chronic wounds or normal skin (Conway et al., 2007). Similarly, bFGF promotes endothelial cell proliferation, differentiation and migration into the wound area (Lie et al., 2003) and has been previously used, in combination with SDR, to improve angiogenesis during matrix-based dermal regeneration in a diabetic full skin defect model. (Nagato et al., 2006). V54/2 cells seeded in IM release VEGF and bFGF (Fig. 8). As shown in fig. 6C, VEGF induce cell proliferation in these cells suggesting that

this fact enhances the V54/2 cell pool by autocrine signaling and promotes angiogenesis during skin regeneration. Scaffold bioactivation by cell seeding or by direct incorporation of recombinant growth factors could enhance angiogenesis by local growth factor release and also by blocking the growth factors interaction sites presented in the scaffold, which could inhibit angiogenesis by sequestration of the growth factor released by the tissue during wound healing. Brem et al showed expression, *in vitro* and *in vivo,* of 15 different growth factors in a scaffold seeded with keratinocytes and fibroblasts. Those growth factors are known to promote wound healing, which could modulate the scaffold dependent skin regeneration process (Brem et al., 2003).

Several efforts have been made to induce vascularization in different tissue engineered structures. Prefabrication of free flaps *in vivo* is a modern approach to harbor prevascularized scaffolds using the animal as bioreactor (Kian et al., 2007; Safak et al., 2000). Similarly, the introduction of an arteriovenous loop in a matrix allows autologous cellularization and vascularization of scaffolds (Arkudas et al., 2007). Afterwards, scaffolds can be re-implanted in the defect. Direct modifications in the biochemical structure of the scaffold by cross linking proangiogenic molecules allows improved angiogenesis by local release of growth factors (Zisch et al., 2003). In order to improve *in vivo* vascularization, we have modified the scaffold by incorporation of recombinant growth factors in a fibrin pellet or as soluble proteins (Wilcke et al., 2007). Other approaches to enhance angiogenesis can be achieved by direct incorporation of DNA in scaffolds (Bonadio et al., 2000) or by incorporation of *ex vivo* genetically modified cells overexpressing angiogenic target genes, as we and others have previously showed (Xie et al., 2005, Bleiziffer et al., 2007). Finally, co-culture of smooth muscle cells with endothelial cells in

bioartificial (e.g. collagen) or polymeric (e.g. polyglicolic acid) scaffolds may allow formation of *in vitro* structures with vessel features (Kakisis et al., 2005). Such technologies may in the future help to perfuse larger tissue engineered structures.

In order to enhance angiogenesis, we selected a cellular technology based on the intrinsic capacity of certain cell types to secrete proangiogenic growth factors. Since no genetic manipulation is required, this approach could present advantages compared to others, where clinical translation could be difficult. Autologous cells have been shown to be more effective than allogenic cells for scaffold bioactivation in skin substitutes, showing that autologous treatments induce less scar formation, contraction and immunoreaction and better epidermalization of the graft *in vivo* (Lamme et al., 2002, Marimoto et al., 2005).

In our study, the V54/2 cells line was characterized (Fig. 6) and seeded in IM. After seeding, cells were able to interact with the scaffold (Fig. 7A). Cell attachment could be mediated by both $\alpha_2ß_1$ and Arg-Gly-Asp binding integrins, as was previously demonstrated seeding different cell types in IM (Grzesiak et al., 1997). Previous evidence showed that keratinocytes and fibroblast can survive and proliferate when they are seeded in IM (Ojeh et al., 2003). Similarily, here we show cell survival and proliferation of V54/2 cells seeded in IM *in vitro* (Fig. 7). Presence of human cells in the neodermal mouse tissue was confirmed *in vivo* by immunohistology (Fig. 12). Survival *in vitro* (Fig. 7), presence of the cells *in vivo* (Fig. 12) and constant growth factor secretion *in vitro* (Fig. 8), suggest that scaffolds containing such cells were constantly bioactivated.

VEGF and bFGF levels released from bioactivated scaffolds were rather stable in time and probably represent only a part of the total amount of growth factors secreted. Another important fraction could be interacting with the scaffold and inside the cells. Moreover, due to the lack of vascularization *in vivo*, scaffolds containing cells were primarily under hypoxic conditions which could enhance the production of proangiogenic growth factors during the dermal regeneration process. Continuous growth factors release from these cells in the matrix (Fig. 8) ensures prolonged bioactivity of the molecules in the targeted area as compared to the addition of recombinant proteins.

After two weeks of transplantation, in a bilateral full skin defect model (Fig. 9), bioactivated scaffolds were able to improve vascularization levels (Fig. 11). Those levels were evaluated with a novel method which presents advantages compared to traditional techniques (Egaña et al., 2007) (Fig. 10). Using this technology, a broad target area can be analyzed at once, allowing visualization and quantification of the whole vascular network during the scaffold-based dermal regeneration process. Similarily, incorporation of recombinant VEGF and bFGF in the scaffold also improves vascularization during matrix based dermal regeneration as shown in Fig. 13.

Although our results suggest a possible angiogenic contribution of V54/2 cells, we cannot exclude the possibility of vascularization enhancement mediated by vasculogenesis, where V54/2 cells could act as EPC, differentiating into endothelial cells or facilitating recruitment of those cells in the scaffold by releasing chemoattractant molecules such as SDF-1α (Lapidot et al., 2005). However, here we showed that cells were able to secrete proangiogenic growth factors (Fig. 8)

and that local presence of them improves vascularization (Fig. 13). No statistical differences were found between V54/2- and VEGF/bFGF-containing scaffolds. However, in order to properly compare, both methods should be optimized in experiments of time and dose response.

The use of cells pre-cultured in scaffolds to improve regeneration has been used in several fields of tissue engineering including bone, cartilage and nerve regeneration, being successfully applied in experimental models and clinical trials (Hurtado et al., 2006; Yoon et al., 2007; Shangkai et al., 2007). In skin tissue engineering, clinical reports have shown that good results can be obtained in patients with different skin related problems when they are treated with a combination of autologous cells and SDR. Hollander et al showed that in three patients autologous keratinocytes preseeded in a hyaluronic acid scaffold can be used to heal full thickness ulcers. Similarly, Wisser et al treated a 19 year old patient with 76% of her body surface burned, with a combination of autologous keratinociytes and fibroblasts preseeded in IM, improving wound healing and skin regeneration. In this study they found a correlation between the time of wound closure and the number of fibroblasts applied.

Cell survival *in vivo* is limited by the primary lack of vascularization. In order to improve cell survival *in vivo*, in this work cells were immortalized by transduction with the SV40 large antigen. Expression of this protein allows improved cell viability and proliferation *in vivo*. SV40 large antigen binds to heat shock chaperone 70, the retinoblastoma family of tumor suppressors and the transcription factor p53. All those interactions result in alterations including apoptotic resistance (Ahuja et al., 2005). After two weeks *in vivo*, presence of

V54/2 cells was confirmed by histology, showing a broad general distribution of human cells in the new dermal mouse tissue (Fig. 12).

The results of our investigation clearly demonstrate that immortalized human mesenchymal cells can be seeded and cultured in SDR being able to proliferate and release proangiogenic growth factors *in vitro*. *In vivo*, cells were stably engrafted in the neodermis. Bioactivated scaffolds with V54/2 cells or recombinant proangiogenic growth factors were able to improve vascularization in a bilateral full skin defect model. Our study provides promising results for the use of cell technologies to enhance angiogenic capacity in dermal tissue engineering strategies. However, further studies should be performed to cover several aspects non included in this work such as the optimum density for cell seeding *in vitro,* comparing results with different types of autologous primary or immortalized cells. Also larger animal models (eg. pigs) with major skin defects and long term analysis experiments should be performed. Finally, further analysis focused on relevant clinical aspects such as quality of the regenerated dermis or infection rates would be extremely important.

5. Abstract

All engineered bioartificial structures developed for tissue regeneration or organ replacement require oxygen and nutriments in order to establish proper physiological functions. Aiming to improve vascularization during dermal regeneration process, in the present work, we combined the use of a bioartificial collagen scaffold (Integra matrix™) and a defined human mesenchymal cell line. This cell line, termed V54/2, exhibits typical morphology and immunophenotype of mesenchymal cells and cell proliferation responded to VEGF ($p<0.05$). V54/2 cells seeded in the scaffold were able to survive, proliferate and release significant amounts of VEGF and bFGF during at least two weeks. Scaffolds with or without cells were transplanted to induce dermal regeneration in a nude mouse full skin defect model. After two weeks of transplantation we found that scaffolds with V54/2 cells significantly improved vascularization during the dermal regeneration process ($p=0.001$) as compared to controls (empty scaffolds). Presence of human cells in the tissue in regeneration was detected by immunohistochemistry. In order to confirm if local presence of angiogenic growth factors is enough to induce neovascularization, scaffolds were loaded with VEGF and bFGF and used to induce dermal regeneration *in vivo*. Results show that after two weeks, scaffolds supplemented with growth factor were significatively more vascularized than controls ($p<0.05$). The present work suggests that combined use of autologous mesenchymal cells and bioartificial scaffolds could be useful to induce therapeutic angiogenesis during the scaffold based dermal regeneration process, contributing to improve clinical success in tissue engineered skin strategies.

6. Zusammenfassung

Alle künstlich hergestellten biologischen Strukturen, die zur Gewebe-regeneration oder als Organersatz hergestellt werden, benötigen Sauerstoff und Nährstoffe, um regelrechte physiologische Funktionen aufnehmen zu können. Mit dem Ziel, die Vaskularisierung während dermaler Regenerationsprozesse zu verbessern, kombinierten wir in der vorliegenden Arbeit den Gebrauch eines bioartifiziellen Kollagen-Gerüsts (Integra Matrix) und einer bestimmten humanen mesenchymalen Zelllinie. Diese Zelllinie mit dem Namen V54/2 zeigt die typische Morphologie und den Immunphänotyp mesenchymaler Zellen und ihre Proliferation reagiert positiv auf VEGF ($p<0,05$). V45/2-Zellen, die auf dem Kollagen-Gerüst ausgesät wurden, waren nicht nur in der Lage mindestens zwei Wochen lang zu überleben, sondern auch zu proliferieren und signifikante Mengen von VEGF und bFGF freizusetzen. Um eine Hautregeneration zu induzieren, wurden die Gerüste mit und ohne Zellbesiedelung in ein Modell nackter Mäuse mit komplettem Hautdefekt transplantiert. Zwei Wochen nach der Transplantation zeigte sich, dass Gerüste mit V54/2-Zellen die Vaskularisierung während des Regenerationsprozesses im Vergleich zu den Kontrollen (leere Gerüste) deutlich verbesserten ($p=0,001$). Die Präsenz der humanen Zellen im regenerierenden Gewebe wurde mit Immunhistochemie nachgewiesen. Um festzustellen, ob die lokale Anwesenheit von angiogenetischen Wachstumsfaktoren ausreichend ist, um die Neovaskularisierung zu induzieren, wurden die Gerüste mit VEGF und bFGF beladen und zur Induktion dermaler Regeneration in vivo eingesetzt. Die Ergebnisse zeigten, dass nach zwei Wochen die Gerüste mit Wachstumsfaktoren signifikant mehr Gefäßbildung aufwiesen als die Kontrollen ($p<0,05$). Die vorliegende Arbeit weist darauf hin, dass die kombinierte Verwendung von

autologen mesenchymalen Zellen und bioartifiziellen Gerüsten sich als nützlich erweisen könnte, um eine therapeutische Angiogenese während des matrixbasierten dermalen Regenerationsprozess zu verstärken. Dies könnte dazu beitragen, den klinischen Erfolg der Tissue-Engineering-Methoden im Hinblick auf die Haut zu verbessern.

7. References

Ahuja D, Sáenz-Robles MT, Pipas JM.SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. Oncogene. 2005; 24: 7729-7745.

Angelo LS, Kurzrock R. Vascular endothelial growth factor and its relationship to inflammatory mediators. Clin Cancer Res. 2007; 13: 2825-2830.

Arkudas A, Beier JP, Heidner K, Tjiawi J, Polykandriotis E, Srour S, Sturzl M, Horch RE, Kneser. U.Axial prevascularization of porous matrices using an arteriovenous loop promotes survival and differentiation of transplanted autologous osteoblasts. Tissue Eng. 2007; 13:1549-1560.

Bleiziffer O, Eriksson E, Yao F, Horch RE, Kneser U. Gene transfer strategies in tissue engineering. J Cell Mol Med. 2007; 11: 206-223.

Bonadio J. Tissue engineering via local gene delivery: update and future prospects for enhancing the technology. Adv Drug Deliv Rev. 2000: 44; 185-194.

Brem H, Young J, Tomic-Canic M, Isaacs C, Ehrlich HP. Clinical efficacy and mechanism of bilayered living human skin equivalent (HSE) in treatment of diabetic foot ulcers. Surg Technol Int. 2003; 11: 23-31.

Burke JF, Yannas IV, Quinby WC Jr, Bondoc CC, Jung WK. Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. Ann Surg 194: 413-428, 1981.

Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003; 9: 653-60.

Conrad C, Gottgens B, Kinston S, Ellwart J, Huss R. GATA transcription in a small rhodamine 123(low)CD34(+) subpopulation of a peripheral blood-derived CD34(-)CD105(+) mesenchymal cell line Exp Hematol. 2002; 30: 887-895.

Conway KP, Price P, Harding KG, Jiang WG. The role of vascular endothelial growth inhibitor in wound healing. Int Wound J. 2007; 4: 55-64.

Dantzer E, Queruel P, Salinier L, Palmier B, Quinot JF. Dermal regeneration template for deep hand burns: clinical utility for both early grafting and reconstructive surgery. Br J Plast Surg. 2003; 56: 764-774.

Egaña JT, Condurache A, Lavandero S, Machens HG. Ex vivo method to visualize and quantify vascular networks in native and tissue engineered skin Accepted for review in Langenbecks Archives of Surgery.

Eppley BL, Pietrzak WS, Blanton M.Platelet-rich plasma: a review of biology and applications in plastic surgery. Plast Reconstr Surg. 2006; 118: 147-159.

Fiedler U, Augustin HG. Angiopoietins: a link between angiogenesis and inflammation. Trends Immunol. 2006; 27: 552-558.

Folkman J, Shing Y. Angiogenesis. J Biol Chem. 1992; 267: 10931-10934.

Grzesiak JJ, Pierschbacher MD, Amodeo MF, Malaney TI, Glass JR. Enhancement of cell interactions with collagen/glycosaminoglycan matrices by RGD derivatization. Biomaterials. 1997; 18:1625-1632.

Harvey, R.P. and Rosenthal, N. Heart Development. Academic Press, San Diego, p. 228. 1999.

Heimbach D, Luterman A, Burke J, Cram A, Herndon D, Hunt J, Jordan M, McManus W, Solem L, Warden G, Zawacki B. Artificial dermis for major burns. A multi-center randomized clinical trial. Ann Surg. 1988; 208: 313-320.

Hollander D, Stein M, Bernd A, Windolf J, Pannike A. Autologous keratinocytes cultured on benzylester hyaluronic acid membranes in the treatment of chronic full-thickness ulcers. J Wound Care. 1999; 8: 351-355.

Hurtado A, Moon LD, Maquet V, Blits B, Jérome R, Oudega. Poly (D,L-lactic acid) macroporous guidance scaffolds seeded with Schwann cells genetically modified to secrete a bi-functional neurotrophin implanted in the completely transected adult rat thoracic spinal cord. Biomaterials. 2006; 27: 430-442.

Jones I, James SE, Rubin P, Martin R. Upward migration of cultured autologous keratinocytes in Integra artificial skin: a preliminary report. Wound Repair Regen 11: 132-138, 2003.

Kakisis JD, Liapis CD, Breuer C, Sumpio BE. Artificial blood vessel: the Holy Grail of peripheral vascular surgery. J Vasc Surg. 2005; 2: 349-354.

Kanematsu A, Yamamoto S, Ozeki M, Noguchi T, Kanatani I, Ogawa O, Tabata Y. Collagenous matrices as release carriers of exogenous growth factors. Biomaterials. 2004; 25: 4513-4520.

Kannan RY, Salacinski HJ, Sales K, Butler P, Seifalian AM. The roles of tissue engineering and vascularisation in the development of micro-vascular networks: a review. Biomaterials. 2005; 26: 1857-1875.

Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, Uchida S, Masuo O, Iwaguro H, Ma H, Hanley A, Silver M, Kearney M, Losordo DW, Isner JM, Asahara T. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation. 2003; 107: 461-468.

Kian Kwan Oo K, Chen Ong W, Hui Chi Ang A, Hutmacher DW, Kim Siang Tan L.Tissue engineered prefabricated vascularized flaps. Head Neck. 2007; 29: 458-464.

Lamme EN, van Leeuwen RT, Mekkes JR, Middelkoop E. Allogeneic fibroblasts in dermal substitutes induce inflammation and scar formation. Wound Repair Regen. 2002; 10: 152-160.

Lapidot T, Dar A, Kollet O. How the stem cells find their way home? Blood. 2005; 106: 1901-1910.

Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW. Hypoxia-inducible factor (HIF-1) alpha: its protein stability and biological functions. Exp Mol Med. 2004;36:1-12.

Li J, Zhang YP, Kirsner RS. Angiogenesis in wound repair: angiogenic growth factors and the extracellular scaffold. Microsc Res Tech. 2003; 60: 107- 114.

Machens HG, Berger AC, Mailaender P. Bioartificial skin. Cells Tissues Organs. 2000; 167: 88-94.

Moiemen NS, Vlachou E, Staiano JJ, Thawy Y, Frame JD. Reconstructive surgery with Integra dermal regeneration template: histologic study, clinical evaluation, and current practice. Plast Reconstr Surg. 2006; 117: 160-174

Morimoto N, Saso Y, Tomihata K, Taira T, Takahashi Y, Ohta M, Suzuki S. Viability and function of autologous and allogeneic fibroblasts seeded in dermal substitutes after implantation. J Surg Res. 2005 May 1;125(1):56-67.

Nagato H, Umebayashi Y, Wako M, Tabata Y, Manabe M. Collagen-poly glycolic acid hybrid matrix with basic fibroblast growth factor accelerated angiogenesis and granulation tissue formation in diabetic mice. J Dermatol. 2006; 33: 670-675.

Ojeh NO, Frame JD, Navsaria HA. In vitro characterization of an artificial dermal scaffold. Tissue Eng. 2001; 7: 457-472.

Safak T, Akyurek M, Ozcan G, Kecik A, Aydin M. Osteocutaneous flap prefabrication based on the principle of vascular induction: an experimental and clinical study. Plast Reconstr Surg. 2000; 105: 1304-1313.

Shangkai C, Naohide T, Koji Y, Yasuji H, Masaaki N, Tomohiro T, Yasushi T. Transplantation of allogeneic chondrocytes cultured in fibroin sponge and stirring chamber to promote cartilage regeneration. Tissue Eng. 2007; 13: 483-492.

Stern R, McPherson M, Longaker MT. Histologic study of artificial skin used in the treatment of full-thickness thermal injury. J Burn Care Rehabil 11: 7-13, 1990.

Wilcke I, Lohmeyer JA, Liu S, Condurache A, Krüger S, Mailänder P, Machens HG. VEGF(165) and bFGF protein-based therapy in a slow release system to improve angiogenesis in a bioartificial dermal substitute in vitro and in vivo. Langenbecks Arch Surg. 2007; 392: 305-314.

Wisser D, Steffes J. Skin replacement with a collagen based dermal substitute, autologous keratinocytes and fibroblasts in burn trauma. Burns. 2003; 29: 375-380.

Xie WG, Lindenmaier W, Gryzybowski S, Machens HG. Influence of vascular endothelial growth factor gene modification on skin substitute grafted on nude mice. Zhonghua Shao Shang Za Zhi. 2005; 21:203-206.

Yoon SJ, Park KS, Kim MS, Rhee JM, Khang G, Lee HB. Repair of diaphyseal bone defects with calcitriol-loaded PLGA scaffolds and marrow stromal cells. Tissue Eng. 2007; 13: 1125-1133.

Zisch AH, Lutolf MP, Ehrbar M, Raeber GP, Rizzi SC, Davies N, Schmokel H, Bezuidenhout D, Djonov V, Zilla P, Hubbell JA. Cell-demanded release of VEGF from synthetic, biointeractive cell ingrowth matrices for vascularized tissue growth. FASEB J. 2003; 17: 2260-2272.

8.1. Curriculum vitae

I. Personal data

Name: José Tomás Egaña Erazo
Date of Birth: 9 of July 1978
Place of birth: Santiago de Chile
Citizenship: Chilean
Passport number: 13.441869-9
Phone: 0049-451-5002710 (Office)
Email address: tomasega@gmail.com

II. Education

1997-2001: B.Sc. on Engineering in Molecular Biotechnology, Faculty of Sciences, University of Chile.

2001-2003: Professional degree of Engineer in Molecular Biotechnology, Faculty of Sciences, University of Chile. (Equivalent to master in USA)

Since 2004: PhD student at the Dept. of Plastic Surgery. Faculty of Medicine, University of Luebeck. Germany.

III. Publications

Egaña JT, Zambrano C, Gonzalez-Billault C, Núñez MT, Maccioni, RB (2003) “Iron-induced oxidative stress modify tau phosphorylation patterns in hippocampal cell cultures” Biometals 16: 215-223.

Zambrano C, **Egaña JT**, Núñez MT, Maccioni RB, Gonzalez-Billault C. (2004) "Oxidative stress promotes tau dephosphorylation in neuronal cells. Role of Cdk5 and PP1" Free Rad Biol & Med 36:1393-1402.

Machens HG, Grzybowski S, Bucsky B, Stöckelhuber B, Condurache A, Liu F**,** **Egaña JT**, Kaun M, Mailänder P, Aach T (2006). "A technique to detect and to quantify fasciocutaneous blood vessels in small laboratory animals ex vivo" J Surg Res. 131: 91-96.

Egaña JT, Condurache A, Lohmeyer JA, Kremer M, Stoeckelhuber B, Lavandero S, Machens HG. Ex vivo method to visualize and quantify vascular networks in native and tissue engineered skin. Langenbecks Arch Surg. In press

Egaña JT, Fierro FA, Krueger S, Huss R, Bornhauser M, Lavandero S, Machens HG. Use of Human Mesenchymal Cells to Improve Vascularization in a Mouse Model for Scaffold-based Dermal Regeneration. Tissue Engineering. In press

IV. Congress presentations (Selected as first author only)

Egaña JT, Zambrano C, Quintanilla R, Nuñez M.T and Maccioni R.B. Oxidative stress, iron and inflamation in the patogenesis of Alzheimer´s disease. Second International Workshop on Iron and Copper Homeostasis (SIWICH), Pucón, Chile. November, 2001. (Poster presentation)

JT Egaña, C Zambrano, JP Muñoz, C Otth, I Concha, R Quintanilla, A Mendoza, C Gonzalez, MT Nuñes and RB Maccioni. "Tau modification: a central paradigm in Alzheimer's type neurodegeneration".Second International Forum on Cytoskeleton. Valdivia, Chile. January, 2002. (Oral presentation)

JT Egaña, Liu F, Krengel S, Krüger S, Lindenmaier W, Machens HG. "Construction of tissue-engineered skin by genetically modified human keratinocytes and fibroblast in a collagen-GAG matrix" 123. Kongress der Deutschen Gesellschaft für Chirurgie. May 2006, Berlin, Germany. (Oral presentation)

Egaña JT, Liu F, Fierro FA, Krüger S, Lavandero S, Huss R, Stöckelhuber B, Condurache A, Machens HG. Stable engraftment of human MSC in an artificial scaffold improves vascularization in a nude mouse full skin defect model. 41 Congress of the European society for surgical research. May 2006, Rostock, Germany. (Oral presentation)

Egaña JT, Liu F, Fierro FA, Krüger S, Lavandero S, Huss R, Stöckelhuber B, Condurache A, Machens HG Stable engraftment of human MSC in an artificial scaffold improves vascularization in a nude mouse full skin defect model. 10 ECSAPS Congres. September 2006, London, England. (Oral presentation)

Egaña JT, Condurache A, Lavandero S, Machens HG. A reliable ex vivo technique to detect and to quantify small blood vessels in laboratory animals. 10th ECSAPS Congress. September 2006, London, England. (Poster presentation)

Egaña JT, Fierro FA, Butzal M, Bauer N, Lavandero S, Krüger S, Schlencke P, Machens HG. Use of bone marrow derived stem cells to improve vascularization in an in vivo matrix based dermal regeneration model. 1th Conference of the German Society for Stem Cell Research. November 2006, Cologne, Germany. (Poster presentation)

Egaña JT, Lavandero S, Butzal M, Fierro F, Bauer N, Krüger S, Condurache A, Machens HG. Cell therapy to induce vascular regeneration in skin tissue engineering. 49th meeting of the Chilean society of biology. November 2006. Pucón, Chile. (Oral presentation)

Egaña JT, Fierro FA, Butzal M, Bauer N, Lavandero S, Krüger S, Schlencke P, Machens HG. Use of bone marrow derived stem cells to improve vascularization in an in vivo matrix based dermal regeneration model. 124. Kongress der Deutschen Gesellschaft für Chirurgie. May 2007, Munich, Germany. (Oral presentation)

Egaña JT , Fierro FA, Butzal M, Bauer N, Lavandero S, Krüger S, Schlencke P, Machens HG. Human Endothelial Progenitor Cells improve vascularization during matrix based dermal regeneration in an in vivo model. International congress of stem cells and tissue engineering in plastic, reconstructive and aesthetic surgery. May 2007, Samsun, Turkey. (Oral presentation)

Egaña JT, Ugarte F, Fierro FF, Bornhaeuser M, Lavandero S, Machens HG. Use of primary MSC to induce scaffold bioactivation in vitro. 11th ECSAPS Congress. September 2007, Aachen, Germany. (Oral presentation)

Egaña JT, Fierro FA, Butzal M, Bauer N, Lavandero S, Krüger S, Schlencke P, Machens HG. Use of EPC to improve vascularization in an in vivo matrix based dermal regeneration model. 11th ECSAPS Congress. September 2007, Aachen, Germany. (Oral presentation)

Egaña JT, Fierro FA, Krüger S, Bornhaeuser M, Huss R, Lavandero S, Machens HG. Use of immortalized mesenchimal cells to induce in vivo angiogenesis during matrix based dermal regeneration 3rd World Congress of Regenerative Medicine. October 2007, Leipzig, Germany. (Oral presentation)

8.2. Acknowledgment

First of all, I would like to thank Prof. Dr. Machens for his trust, his constant supervision and support during the performance of this thesis. I also would like to thank at all who, during these three years, are or were part of our lab. Especially to Mathias Kremer, Fang Liu, Shuhua Liu, Jorn Lohmeyer, Haitham Salem, Insa Wilcke and Karen Witting for their friendship, help and smart advices. Also I would like to acknowledge at our neighbors in KEF: AG Klein, AG Hiort and AG Ito for their help, patience and constant German translation. I am very grateful to my friend Dr. Ralf Werner, for his friendship and his important scientific discussion during the experimental performance and data analysis and, especially, for the tons of coffees and biers that we have drunk in this years. Also I would like to thank at my old friends from the scientific Chilean “mafia” in Europe which were very actives in the discussion, planification and analysis of the results obtained in this work. Specially at Drs. Fernando Fierro and Fernando Ugarte from the MPI-CBG in Dresden; Paula Martinez from the MPI in Leipzig; Rodrigo Hess and Eduardo Villablanca from the San Raffaelle Institute in Milan and, Emilio Erazo, Natalia Mackenzie and Felipe Opazo from The MPI in Goettingen. Once more I have to thank at Drs. Presley and Sinatra for providing me inspiration and help during long hours of experiments and data analysis. Finally, I have to thank to my family in Chile, for their constant support in this adventure.