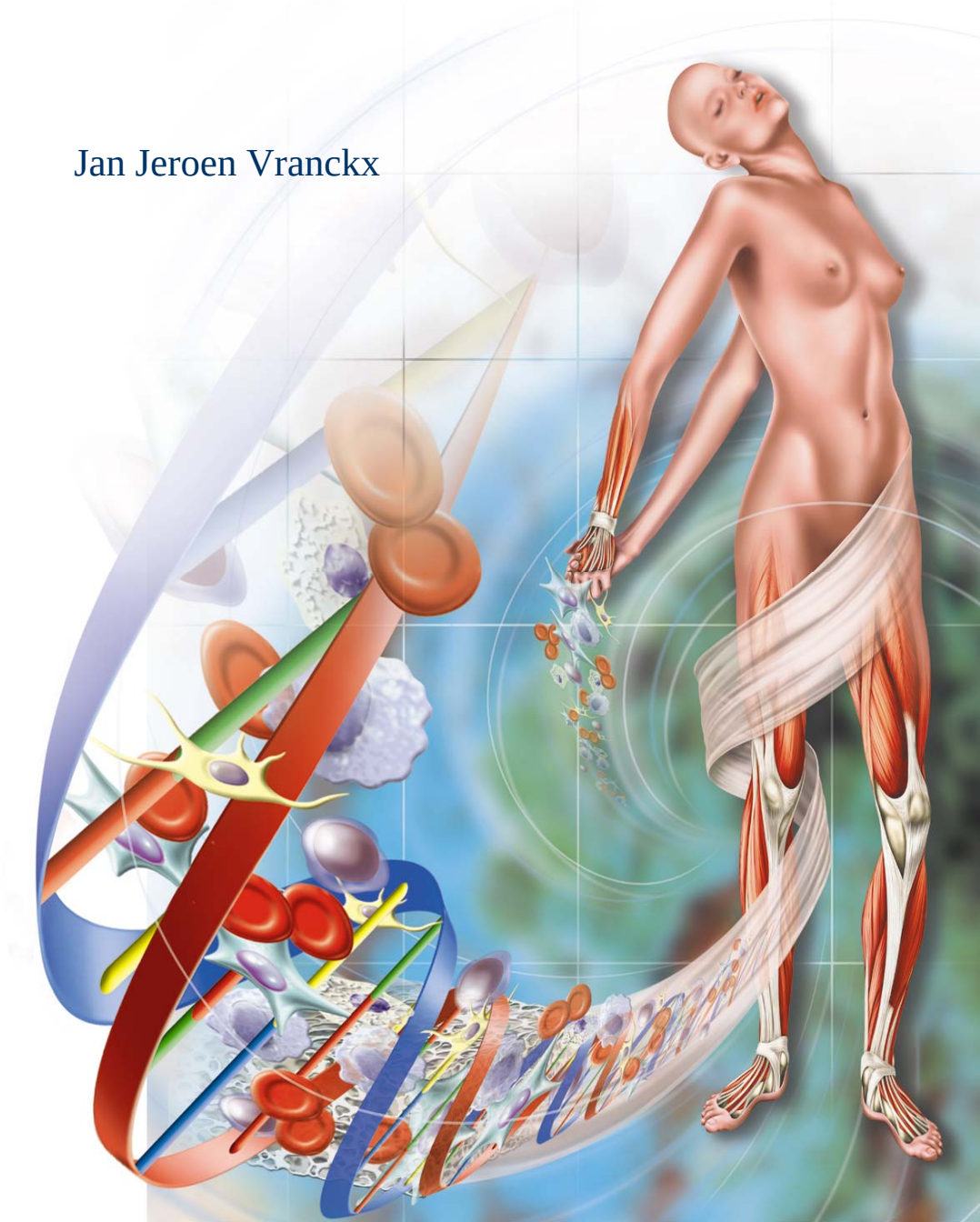
Ex vivo gene transfer to full thickness wounds. A platform for autologous ‘smart’ tissue engineering for tissue repair.

Jan Jeroen Vranckx



Katholieke Universiteit Leuven
Groep Biomedische Wetenschappen
Faculteit Geneeskunde
Dept. Heelkunde
Afd. Plastische & Reconstructieve Heelkunde

Jan Jeroen Vranckx

Ex vivo gene transfer to full thickness wounds.
A platform for autologous '*smart*' tissue engineering for tissue repair.

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Promotores:
Prof. Dr.W. Flameng, KULeuven
Prof. Dr. E. Eriksson, BWH Hospital, Harvard Medical School, Boston

Chairman:
Prof. Dr. P.Broos, KULeuven

Secretary:
Prof. Dr. F. Luyten, KULeuven

Examining Committee:
Prof. Dr. G. Cherry, University of Oxford Medical School, Oxford
Prof. Dr. M. Garmyn, KULeuven
Prof. Dr. G. Opdenacker, KULeuven
Prof. Dr. R. Vanwijck, Université Catholique de Louvain
Prof. Dr. P. Vogt, Hannover Medical School, Hannover

Illustration design and layout:
Jan J.Vranckx

Illustrators:
NV Dream Team, Loonbeek

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In vivo gene delivery of Ad-$VEGF_{121}$ to full thickness wounds in aged pigs results in high VEGF expression .
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J Tissue Viability 15 :16-21,2005

A novel titanium wound chamber for the study of wound infections in pigs.
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A defined porcine basal keratinocyte cell suspension culture system for ex vivo gene transfer under serum-limited conditions".
Vranckx J.J., Dickens S.,Hendrickx B.,Vandenberge S.,Vermeulen P.
Submitted to the J. Tissue Engineering & Regenerative Medicine

Cell suspension cultures of allogenic keratinocytes are efficient carriers for ex vivo gene transfer and accelerate healing of full-thickness skin wounds by overexpression of hEGF.
Vranckx, JJ.,Hoeller D.,Velander, P.,Theopold, C.,Petrie, N.,Takedo, A.,Eriksson, E.,Yao, F.
Accepted in Wound Repair and Regeneration, 2007.

Regulable $VEGF_{165}$ overexpression by *ex vivo* expanded keratinocyte cultures, promotes matrix formation, angiogenesis and healing in porcine full thickness wounds.
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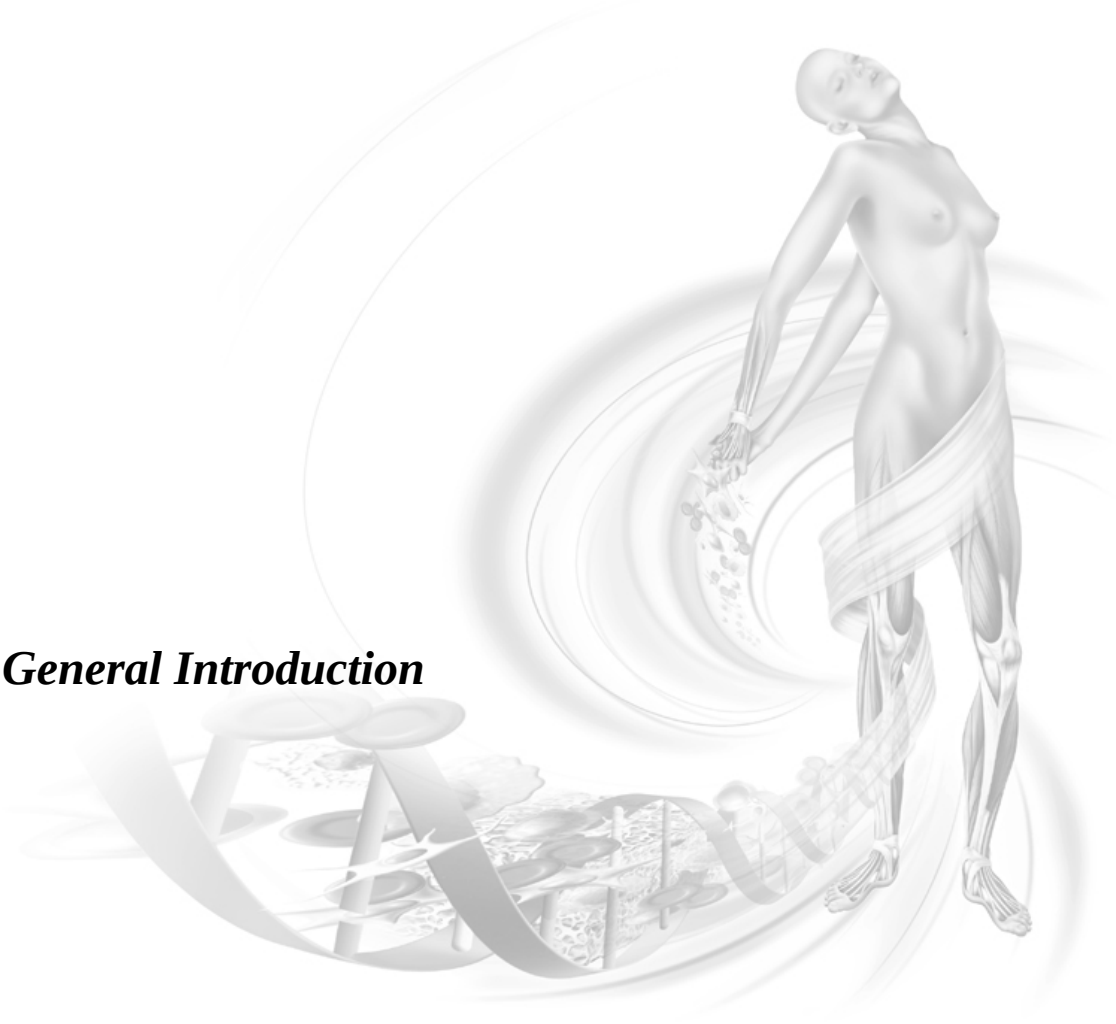
List of Abbreviatons

BCKs	Basal cell keratinocytes
ECs	Endothelial cells
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FBs	Fibroblasts
FTW	Full thickness skin wounds
EGF	Epidermal growth factor
EPCs	Endothelial progenitor cells
H&E	Hematoxylin and eosin
KCs	Keratinocytes
MMP	Matrix metallo proteinases
PDGF	Platelet derived growth factor
PRP	Platelet rich plasma
STG	Split thickness skin graft
TC	Tetracycline
VEGF	Vascular endothelial growth factor

A painting has a life on its own
I only try to let it come through.
Jackson Pollock



Chapter I: General Introduction

1.1. Requirements for the treatment of full thickness wounds: plastic reconstructive surgery and tissue engineering.

Plastic reconstructive surgery focuses on the reconstruction of tissue defects caused by trauma, burns, congenital deformity or as the result of tumor excision. These defects may be located anywhere on the body, from head to toe, and may comprise any type of tissues. Aim is to restore function, volume, and contour of lost tissues. Based on specific features of the defect, the most appropriate strategy is selected in order to reconstruct tissues as authentically as possible. Optimal donor tissues may be available at the loco-regional area around the defect, or may be harvested with their intrinsic vascular pedicle at a distant donor area and transplanted to the defect location where the intrinsic blood vessels are anastomosed to a local vascular network by microsurgical techniques (Fig 1).

These locoregional or remote donor tissues might be most useful for restoring the defect, but their harvest might cause significant donor site morbidity. Therefore, for every single defect, a cost-benefit analysis should be performed in order to select 'best' options. Sometimes less 'authentic' donor tissues are preferred rather than complex donor tissues in order to avoid the donor site morbidity. Otherwise, large and complex defects, for instance in the face, may justify the harvest of complex donor tissues regardless of associated donor site problems (Fig.2). State-of-the art reconstructive strategies should restore function and aesthetics along with minimal donor site morbidity.

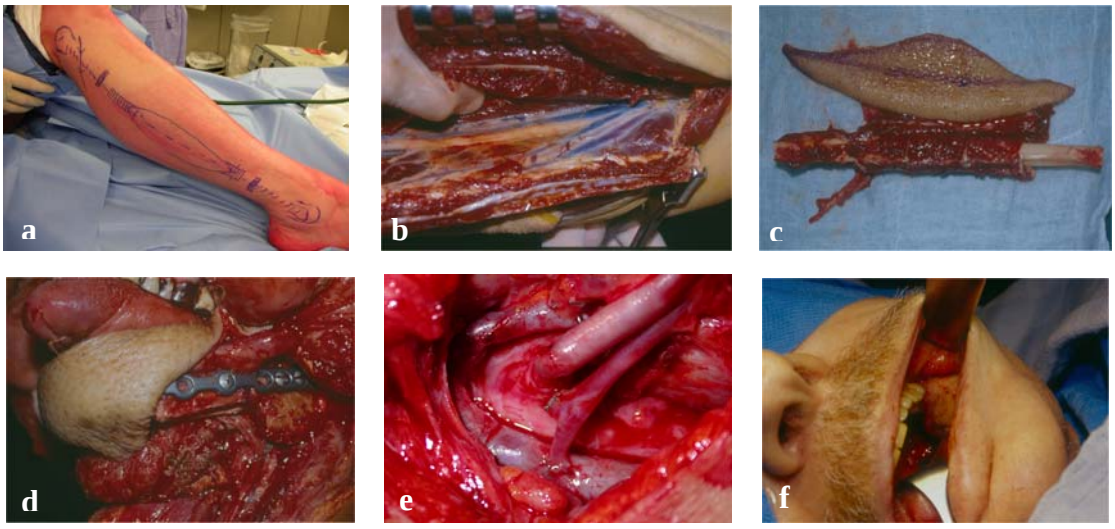


Fig 1 T2N0M0 oral floor tumor with mandibular invasion. En bloc resection and reconstruction with an autologous free osteocutaneous fibula flap transfer. 1.a. donor site at the non-dominant lower leg. 1.b. fibula free flap harvest. Dissection of its vascular peroneal pedicle. Junction zone of the peroneal vessels and the tibial neurovascular pedicle. 1.c. free flap specimen before osteotomies and transfer to the defect. 1.d. Donor tissue in place. A titanium plate is screwed on the fibular bone to reconstruct the mandibula. The fibular skin island is used to cover the floor of the mouth. 1.e. status after free flap transfer and microsurgical anastomosis. End-to-end to the Superior Thyroid Artery and end-to-side to the Internal Jugular Vein. 1.f. post-operative status. View on the healed skin island in the mouth.

Reconstructive surgery of the 21st century focuses on this trilogy. Virtually in all subdisciplines of plastic and reconstructive surgery, fine-tuning of surgical techniques and a renaissance in applied tissue anatomy have accompanied this trilogy[1-3]. Donor site morbidity nowadays takes a primordial place in the cost-benefit analysis and preoperative planning of reconstructive procedures.

It is in this context that in the mid 80s *tissue engineering* was heralded as an ultimately appealing and promising novel strategy for tissue repair. Large hope and expectation accompanied its world wide media coverage; the concept of growing 3-dimensional tissues derived from the patients' own cells was considered to be of enormous value in many challenging clinical situations such as for the previously mentioned complex defects with significant donor site morbidity, but also for the treatment of chronic non-healing (diabetic, arterial, venous) ulcers that currently demand extensive clinical care and enormous financial efforts of social security.

However, in no other circumstance are the reconstructive needs for optimal donor tissues as large as in extensive and deep burn wounds where we are often confronted with the shortcomings of actual wound treatment modalities.

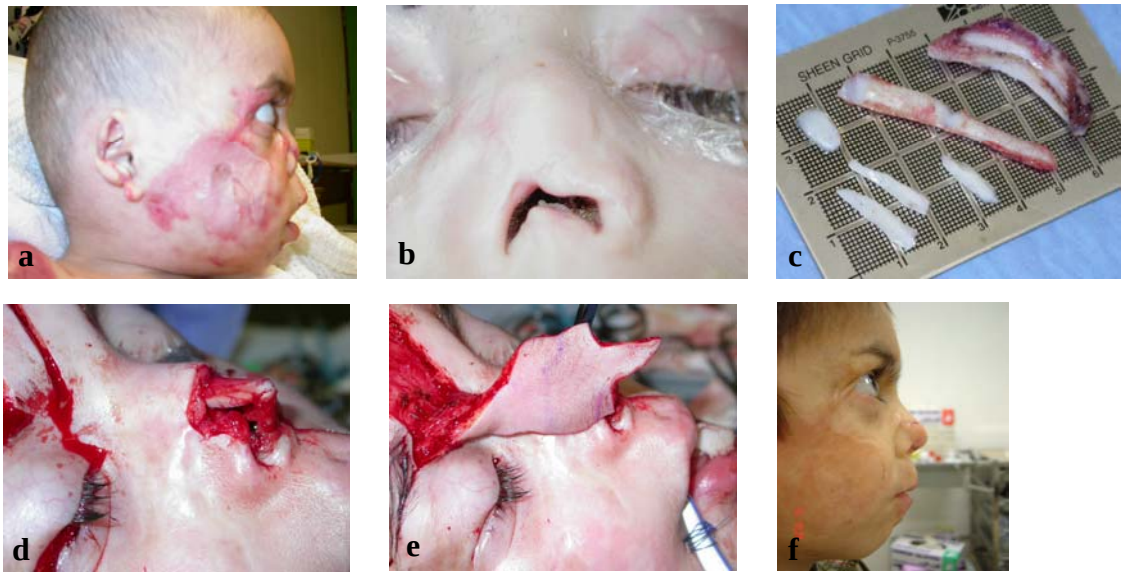


Fig. 2 Three year old kid recovering after Meningococcal sepsis and necrotic skin area's in the face and limbs.(a,b) Nose reconstruction with rib cage cartilage(c,d), which was covered by a frontal flap from the face in two operative steps.(e) Artificial dermis and split thickness skin grafts to treat cheeks and lips.(f)

Over the past two decades, standard ICU treatment of extensive and deep burn injury has been substantially modified. After the enormous fluid requirements of burn patients were recognized in the 30s and the concept of burn resuscitation was refined in the 70s, the advantage of early excision and closure of burn wounds came into the focus of well deserved attention[4,5]. Physiotherapy of scars and attention to functional mobility, aligned with reconstructive procedures to restore extensive tissue destruction, have led to increasingly satisfying long-term outcomes.

However, in terms of wound coverage, there has hardly been a significant evolution the past 20 years. We still use split-thickness skin grafts (STG) as primary means of burn wound coverage. These STG may be meshed for volume expansion which leaves the characteristic waffle-pattern after healing. Resulting scars after skin grafting are rigid, non-flexible and often stigmatic. Moreover, in very extensively burned patients, there even may be a shortage of such skin graft donor sites.

Rheinwald and Green discovered in the late 70s that keratinocytes, when placed in a specified growth medium, could proliferate and migrate. O'Connor et al. used such keratinocyte cell cultures to treat burn wounds in the early 80s. Starting from a 5 cm^2 STG, sufficient number of KCs can be cultivated in 4 weeks time to cover a 75% burned patient. However,the initial euphoria about these cell culture procedures has been tempered drastically. The fragile skin cell cultures feature a low 'take' on the woundbed and regularly do not lead to efficient wound coverage. Nowadays we rather utilize cell cultures as a cover on widely meshed STG or on the donor sites of STG to enhance healing by protein release.

The elementary problem using skin grafts or KC cell cultures as epidermal cover in wound repair is the lack of the deeper dermal layers. Therefore, resulting scars remain rigid and non-elastic and often contract which may lead to significant functional loss.

The actual absence of an ultimately reliable and durable, autologous, full thickness skin equivalent that optimally mimics human skin in its features of elasticity, pliability, texture, and resistance to biochemical and biomechanical aggressors, continues to hinder recovery. Such a construct would represent an enormous improvement in restoration of functional and esthetic outcome, which in turn would have a large impact on social reintegration. Furthermore, since adherent skin substitutes have been reported to decrease pain as compared to occlusive dressings, it is very likely that an autologous, adherent, full thickness skin equivalent will also further improve pain relief and reduce discomfort caused by environmental assaults such as surface dissecation or buildup exsudate which retard healing[6,7].

1.2. Repair and regeneration of full thickness skin defects: cells and scaffolds, growth factors and genes.

The reason why no such autologous skin equivalent has been developed yet is directly related to the complexity of human skin and its requirements for healing; functionally, skin has two highly specialized layers with an effective (dermo-epidermal junction) mechanism of integration. The thin epidermis consists of a basal stem cell layer of highly clonogenic cells that is anchored through the basal lamina components with the papillary dermis. Basal cells give origin to keratinocytes that fully differentiate into corneocytes over a time span of four weeks. In the dermal layer, fibroblasts produce matrix molecules such as fibronectin, elastin, and collagens which shape the extracellular matrix. Endothelial cells line an intrinsic network of fine bloodvessels and a deeper subcutaneous layer of fibrofatty structures delivers elasticity to full thickness skin[8,9]. In full thickness skin defects, whether caused by trauma, burns, or after tumor resection, none of these layers remain; as a consequence, no authentic healing can occur and the wound closes by granulation tissue formation and wound contraction.

In deep and extensive burns, tissue contractions in the face or near articulations may lead to significant functional loss and mutilation. Split thickness skin grafts, which consist of the epidermis and the superficial part of the papillary dermis, leave the deeper dermal layers intact. In these deeper layers skin stem cells reside along with the extra cellular matrix and the vascular network required for healing.

These layers will be able to restore the superficial epidermal defect by providing new keratinocytes to cover the wound on a well vascularised wound bed. The thin donor skin grafts on the other hand cannot replace full thickness defects. As a consequence, if a skin graft is made more substantial (a full-thickness skin graft) by harvest in a deeper plane, the donor site will no longer heal.

Moreover, skin grafts only attach and integrate on a well vascularised wound bed. A skin graft receives its blood supply by diffusion of nutrients from the wound bed. The thicker the graft, the more the nutritional demand. When in a STG one leaves the subcutaneous fat layer underneath the dermis, the STG will fail to survive. Appropriate vascularisation is an elementary condition in wound repair.

To overcome this lack of a dermal layer, several artificial dermal constructs have been developed since the 80s, especially for the treatment of burns in the face, on joint surfaces or on hands[5,10,11]. An ‘artificial dermis’ consists of a dermal layer built up by matrix components such as collagen and glucosaminoglucans. These components are from xenogenic or allogenic origin and therefore will be resorbed or rejected after 3-4 weeks. The dermal scaffold is barely 1 mm thick and is sutured on the wound bed. Within weeks, blood vessels grow into the scaffold. After 3 weeks, the scaffold is vascularised and may be covered safely with a thin STG to provide an epidermis (Fig.3). These dermal scaffolds serve as a temporary matrix in which native cells may migrate and proliferate. Despite the limited thickness of the scaffold, skin elasticity and texture may be significantly improved compared to STG alone.

This temporary beneficial function relies on the synergistic interaction between the dermal and epidermal components at the site of wound repair. However, although artificial scaffolds may improve the morphologic characteristics of the wound bed, dermal regeneration remains limited histologically. This is probably secondary to constraints on fibroblast repopulation and on remodeling of the scaffold, as well as the inflammatory responses secondary to the polymer framework.

Fig. 3. Full thickness burn of the hand. Status after burn scar excision. a. Coverage of the wound with a dermal template (integra). b. Removal of the silicone layer after 3 weeks. c. coverage with a full sheet skin graft. d. long term result: an elastic, pliable skin.

While such dermal scaffolds in conjunction with STGs may significantly improve clinical skin elasticity, they still do not represent the full thickness skin layers which are missing in deep burns (Fig.4).

In such situations we can only restore tissue continuity and aesthetic outcome by using skin balloon expanders in neighboring area's to progressively expand intact full thickness skin.

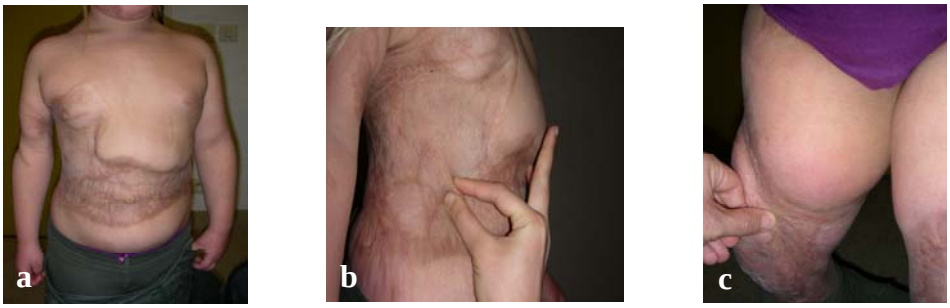


Fig. 4. Status after extensive deep burn treatment in a 6 y/o girl; treatment with Integra dermal template and skin grafts(a-c). Note the elasticity of healed skin(b), but significant loss of volume remains and difference in texture is clear as compared to surrounding intact skin(c).

Unlike the extracellular matrix that represents a millenary natural evolution, artificial biomaterials do not have such a complex structure and chemical composition[12]. In consequence, the trade off to large-scale production and standardization of these 'man-made devices' is the very low bio-information content and the scarce quantity of signals that they transmit to cells, in addition to the potentially immunogenic and significant inflammatory reactions they might elicit[13], which often leads to loss of the dermal equivalent in the clinical setup.

Outcome studies solely based on short-term clinical impact will not be sufficient to predict longterm permanent quality of therapy if molecular biological signs of tissue integration, neoangiogenesis and tissue remodelling are not thoroughly investigated.

For these reasons, engineering of materials that can specifically and molecularly interact with cells in the defect as well as the development of strategies that may influence the quantity and quality of transmitted signals in the repair process, have become emerging fields in tissue repair and regeneration research[14,15].

1.3. Growth factors as directors of the tissue repair and regeneration processes.

The response of tissues to injury forms the foundation of all reconstructive procedures. The intricate wound healing and tissue repair process involves the complex interplay of numerous cells, proteins, and humeral factors[8,9,16]. Growth factors are secreted by all cells orchestrating the several phases of wound repair. These proteins seem to play the role of directors of healing, messengers to signal other cells, and inducers of cellular migration and proliferation. Also, in adult stem cell experiments, the influence of growth factors seems paramount in creating the "niche" microenvironment in which stem cells proliferate, migrate, differentiate, or even de-differentiate[17-19].

Topical administration of recombinant growth factors as proteins has major shortcomings such as short shelf life, low bioavailibility, enzymatic inactivation by proteinases in the wound, and inefficient delivery to target cells[20,21]. Consequently, products derived directly from recombinant growth factors, such as the platelet-derived-growth-factor BB protein-based gel developed to treat neurothropic diabetic wounds, have not been proven to be unequivocally useful.

Gene therapy offers an appealing strategy for direct delivery of growth factor genes into the cells with the intent of altering protein synthesis in the cellular apparatus in order to modify the healing response[23]. Gene therapy can offer targeted local and persistent delivery of de novo synthesized growth factor to the wound environment over many days.

Two major strategies are available for gene transfer to tissues: direct *in vivo* gene transfer delivers the growth factor DNA directly into the wound environment by direct injection of DNA-plasmids or by using innovative techniques such as microparticle bombardment by gene gun[24,25] or microseeding[26]; the other approach is *ex vivo* gene transfer by transplantation of transgene cell cultures into wounds. These cell cultures are grown from (autologous or allogenic) skin grafts after enzymatic digestion with trypsin/dispase solutions. After culturing these cells for several passages, they may be 'transduced' using viral vectors containing the targeted DNA-plasmid, or they may be 'transfected' using non-viral vector strategies such as liposomal techniques or electroporation[23,27]. The advantage of *ex vivo* strategies is the synergistic impact of the cultivated cell substrate and the expressed growth factors on the wound microenvironment.

Such gene transfer techniques may induce repair and regeneration in inert chronic wounds, accelerate healing in FTW and form the backbone of the development of artificial biomaterials by supplying the bio-information content that may induce cell migration and proliferation, angiogenesis, and tissue integration[7,16,27].

1.4. Background on aims and targets

The ultimate tissue engineered skin construct for tissue repair should be autologous in order to optimally integrate without rejection, three-dimensional to bridge deep defects, porous to allow cell migration, bio-inductive for cells to proliferate and to topically produce extra cellular matrix components, bio-inductive for vascular sprouts to develop within the construct from (progenitor) cells, and it should be chemotactic for cells from the wound surroundings to infiltrate and stimulate vasculogenesis and optimize tissue integration[28,29].

Generating cell-matrix constructs to guide tissue regeneration involves isolating appropriate parenchymal cell populations, expanding these in tissue culture, and transferring them to polymer scaffolds. These scaffolds deliver the structural guidance and support for proliferating and migrating cells through their honeycomb structure and they also transmit tissue-specific mechanical forces that cue the behavior of cells within it[6,7,28-30].

Highly proliferative cells with stem cell properties could be seeded into the scaffold in order to obtain the highest proliferation and integration, as well as plasticity, to the wound micro-niche.

Since these constructs should be applicable in burn defects and wounds present in or caused by an unfavorable healing environment such as diabetes, cardiovascular disease, corticosteroid, or cytostatic treatment, these bio-constructs should be populated with cells overexpressing wound-healing mediators, in particular those deficient or lacking in the particular morbidity state. This could be achieved by *in vivo* or *ex vivo* gene transfer strategies[19,22] .
Finally, if we presume that gene therapeutic approaches to cell engineering induce tissue repair and regeneration, we must be able to fully regulate gene expression in this 'smart' construct to coordinate, tune, or just stop growth factor over-expression *in-vivo* based on clinical outcome[31,32].

Tissue engineering combines the molecular biology of cells and genes, the biomaterial sciences of scaffolds and matrices, the clinical sciences of tissue repair and wound healing in an intensive multidisciplinary approach (fig.6). Significant progress will be required in each of the scientific platforms relevant to tissue engineering in order to overcome the actual hurdles to obtain a 3-dimensional well vascularised autogenic tissue engineered construct for tissue repair.

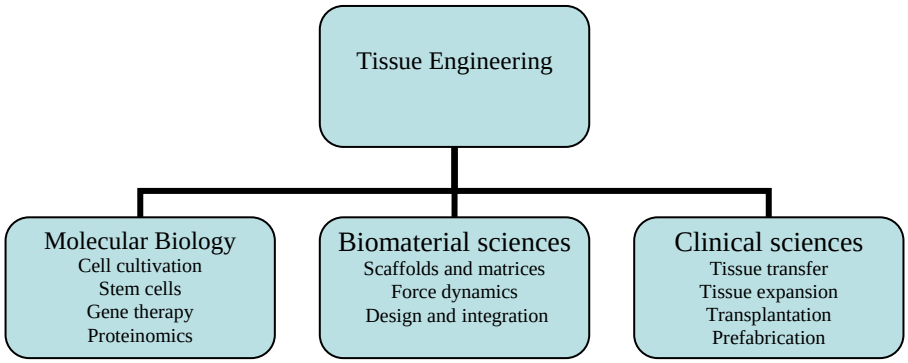
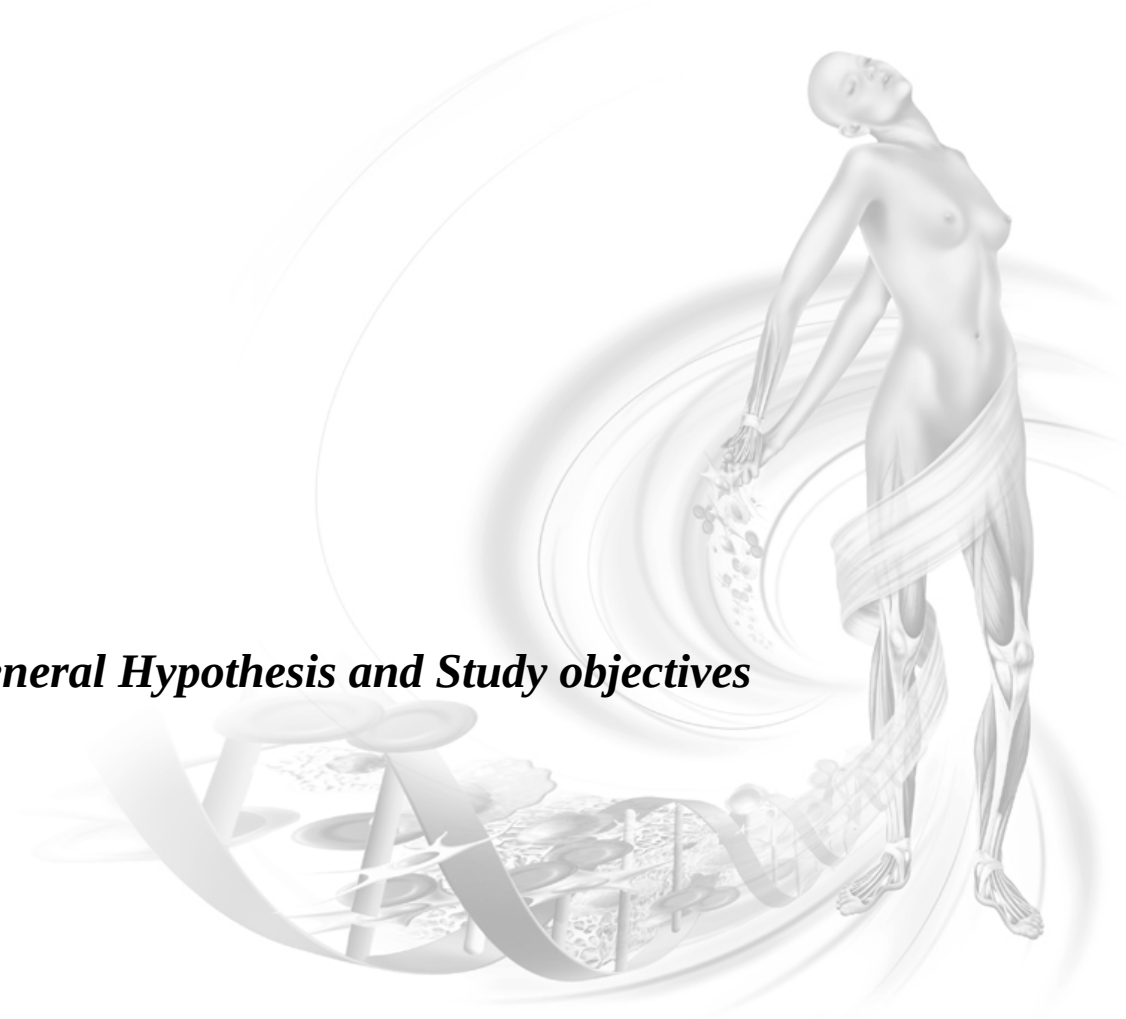


Fig. 6. The multidisciplinary approach in tissue engineering.

This thesis project targets the development of novel strategies for full thickness wound repair using cell engineering protocols and growth factor application by gene therapy.

The scientific platform created may serve as a template for '*smart*' autologous tissue engineering and may be translated to the clinical context of plastic and reconstructive surgery.

Every obstacle yields to stern resolve
He who is fixed to a star does not change his mind.
Leonardo da Vinci



Chapter II: General Hypothesis and Study objectives

1. General Hypothesis:

Cell engineering and gene transfer of growth factors accelerate healing of full thickness skin wounds in a wet wound healing model. This strategy forms the pillar of 'smart autologous three-dimensional full thickness skin engineering'.

2. Aims:

- To induce, facilitate, and accelerate repair of full thickness skin wounds, by transplanting cell suspension cultures that "overexpress" selected growth factors into the wound.
- To accelerate re-epithelialization and vascularization of full thickness skin wounds by ex vivo gene transfer.
- To regulate this gene expression in vitro and in vivo by a tetracycline-inducible gene switch.
- To standardize an ex vivo gene transfer platform for induction of angiogenesis and matrix formation in a 3-D construct.

3. Chronologic workplan:

1. Standardization of an animal wound healing model, based on clinical elements: the clinical and experimental 'wet wound healing model'.

   Paper 1: "Wet Wound Healing" and
   Paper "Wet wound healing: from laboratory to patient to gene therapy."

2. The application of growth factors -as coordinators of wound repair- to accelerate healing of Full thickness wounds in a standardized porcine wet wound healing model;

   - By *in vivo* gene transfer strategies using the "microseeding" approach.

   Paper 2: "In vivo gene delivery of Ad-VEGF$_{121}$ to FTW in aged pigs results in high levels of VEGF expression but not in accelerated healing."

   - By *ex vivo* gene transfer using autologous or allogenic cultured cells which are transfected with DNA-plasmid solutions expressing selected growth factors.

   Paper 3: "A defined porcine basal keratinocyte cell suspension culture system for ex vivo gene transfer under serum-limited conditions."

   Paper 4: "Cell suspension cultures of allogenic keratinocytes are efficient carriers for ex vivo gene transfer and accelerate healing of full-thickness skin wounds by overexpression of hEGF."

3. In vivo regulation of growth factor expression in full thickness wounds by a tetracycline-inducible gene switch.
   Induction of angiogenesis, matrix deposition and accelerated healing by ex vivo gene transfer of $VEGF_{165}$ expressing keratinocyte cell suspensions under the control of a tetracycline inducible gene switch.

   Paper 5: *"Regulable $VEGF_{165}$ expression by ex vivo expanded keratinocyte cultures, promotes matrix formation, angiogenesis and healing in porcine full thickness wounds."*

4. Clinical implications: actual clinical innovative therapies that represent in vivo 'tissue engineering avant la lettre' and pave the way to custom made tissue engineering.
   Which elements do we have. Which ones are missing?

   Appendix: *"The quest for autogenic full thickness skin engineering. Science and fiction."*

Little by little, one travels far.
It's a journey that has never started
that takes the longest to finish.
John Ronald Tolkien

Chapter III: Study Reports

I like pigs. Dogs look up to us.
Cats look down on us
Pigs just treat us as equals.
Sir Winston Churchill

Part 1 - The wound healing model

" Wet Wound Healing".
Jan J.Vranckx,MD., Jaromir Slama, MD.,Stefan Preuss, MD.,Norvin Perez,MD., Tor Svensjö,MD., Scott Visovatti,MD.,Karl Breuing,MD., Richard Bartlett,MD., Julian Pribaz,MD., Denton Weiss,MD.,and Elof Eriksson, MD.,PhD.
Plast.Reconstr.Surg.110; 1680, 2002

" Wet wound healing: from laboratory to patients to gene therapy".
Elof Eriksson,MD.,PhD., Jan J.Vranckx, MD.
Am. J. of Surgery 188; 36S, 2004

Wet Wound Healing

Jan J.Vranckx,MD., Jaromir Slama, MD.,Stefan Preuss, MD.,Norvin Perez,MD.,
Tor Svensjö,MD., Scott Visovatti,MD.,Karl Breuing,MD., Richard Bartlett,MD.,
Julian Pribaz, MD.,Denton Weiss,MD.,and Elof Eriksson, MD.,PhD.

*From the Laboratory of Tissue Repair and Gene Transfer,Division of Plastic Surgery,
Brigham & Women's Hospital, Harvard Medical School, Boston, MA,USA.*

Abstract

Wound treatment in a flexible transparent chamber attached to the perimeter of the wound and containing a liquid has been extensively tested in pre-clinical experiments in pigs and found to offer several advantages. It protects the wound, a liquid medium or saline in the chamber provides in vivo tissue culture like conditions and antibiotics, analgesics, and various molecules can be delivered to the wound through the chamber. The wound chamber has not caused any injury to the wound itself or the surrounding intact skin. Topical delivery of, for instance, antibiotics can provide very high concentrations at the wound site and with a favorable direction of the concentration gradient.

A series of twenty-eight wounds in twenty patients were treated with the wound chamber containing saline and antibiotics. Most patients had significant co-morbidity and had failed conservative or surgical management with debridement and delayed primary closure or skin grafts. Six wounds had foreign bodies present in the wound and four of these were joint prostheses. Seven patients were on corticosteroids for rheumatoid arthritis, lupus or COPD and four patients had diabetes. Most patients were treated with the wound chamber in preparation for delayed primary closure or skin graft or flap procedure, but one was treated with a wound chamber until healed.

Twenty-five of the wounds (89%) healed, five of them (18%) requiring additional conservative management after the initial chamber treatment and grafting procedure. Antibiotic delivery was less than one IV dose daily which avoided the potential for systemic absorption to toxic levels. Antibiotics such as Vancomycin and Gentamicin could be used in concentrations up to 10,000 times MIC. Forty-eight hours after application, twenty percent or more of the original antibiotic concentration was present in the wound chamber fluid. In conclusion, the wound chamber provides a safe, powerful tool in the treatment of difficult wounds.
(Plast.Reconstr.Surg.110;1680,2002)

1. Introduction

Reports on treatment of wounds in a liquid environment date back to the nineteenth century[1]. Burn patients were treated in bathtubs with water for periods of weeks to months. It was noted that pain was reduced and that survival was increased. This treatment preceded the use of skin grafting[2] by more than twenty years and when the patients with major full thickness burns were taken out of the bathtubs, they died.

Bunyan, a British naval surgeon, used what he called the "envelope method" extensively during World War II[3]. The injured site, usually an extremity, was enclosed in the envelope containing water with bleach. The solution was changed frequently, making this an irrigation method. Bunyan noted less pain, less tissue destruction, and fewer infections in the patients subjected to the "envelope" treatment.

Our laboratory has studied healing in a liquid over the past fifteen years[4] .The wound has been enclosed in a sealed, watertight, transparent, vinyl chamber, glued to the periphery of the wound. Conceptually, the chamber has become an in vivo incubator, where the wound can be treated like an in vivo tissue culture. There is rapid exchange over the wound surface allowing both analysis of the wound by examination of the liquid in the chamber and delivery of growth medium, antibiotics, growth factors, cells, genes, etc. by injection into the chamber. Several observations have been made. The liquid has no adverse effects on porcine[4] or human[5] skin, as long as bacteriostatic concentrations of antibiotics are present. There is less tissue necrosis[4], less inflammation[4], and less scar[6]. Porcine partial thickness wet wounds[7] and full thickness wet wounds[8] healed much faster than in a dry environment and wet full thickness wounds healed faster than moist wounds. Autologous keratinocytes transplanted as single cell suspensions to full thickness wounds survived and went through a normal process of proliferation, migration, and differentiation and reconstituted the epidermis[9]. When human epidermal growth factor gene was delivered wounds, which were allowed to heal in the wound chamber, the human epidermal growth factor protein could be detected in the fluid in the chamber and wounds healed faster than controls treated in normal saline[10-12].

This report of twenty-eight wounds treated with a chamber method in twenty patients includes one wound, which has been described earlier[5]. Most of these patients had significant co-morbidity (Table 1) and had failed conservative as well as surgical management with debridement and delayed primary closure or skin grafts. The treatment methods evolved during the time period when these patients were treated and different types of wounds were included in the study.

2. Patients and Methods

The study protocol was approved by the IRB of the Brigham and Women's Hospital. Each patient went through the process of informed consent. Twenty-eight wounds were treated in twenty patients. The diagnoses of the wounds are listed in Table 1. Twenty-two wounds represented chronic leg ulcers and 6 were open, infected, non-healing wounds elsewhere on the body. Six wounds had foreign bodies present in the wound and 4 of these were joint prostheses.

There was significant co-morbidity in most cases; seven patients were on corticosteroids for rheumatoid arthritis, lupus, or COPD. Four patients had diabetes.

The area where the chamber was to be applied was first shaved and cleaned with 70% ethanol. Mastisol R or Tincture of Benzoin was then applied to the skin followed by removal of the adhesive backing and the application of the adhesive base of the chamber to the skin surrounding the wound. A fine needle (27 g) and syringe was used to withdraw all air from and add liquid and antibiotics to the chamber.

The needle puncture hole was then sealed with transparent adhesive tape. A membrane adhesive dressing was sometimes applied to further secure the base. The wound chamber was inspected and the normal saline with antibiotics changed daily (in a few cases, every other day) by needle aspiration and re-injection through the chamber. The aspirated chamber fluid was analyzed intermittently for presence of microorganisms as well as antibiotic concentration.

If the wound contained necrotic tissue it was debrided before application of the wound chamber. If not, debridement was done at the time of delayed primary closure, skin grafting or flap procedure. In most wounds, the chamber with antibiotics was used for two to four days, but in one patient it was used for up to seventy days.

The most common bacteria were Staphylococcus Aureus and Pseudomonas Aeruginosa (Table 1). Based on the resistance pattern of these bacteria, Gentamicin was used alone in 9 cases and in combination with Vancomycin and /or Amphotericin B in 15 other cases. Vancomycin was used alone in 2 cases and in 13 other in combination with other antibiotics. Because of the presence of Candida Albicans or planned treatment beyond three weeks, Amphotericin B was used in six cases (Table 1).

TABLE I
Overview of 28 Wounds Treated with the Wound Chamber

Patient	Age (yrs)	Diagnosis, Comorbidity, Treatment	Local Ulcer Diagnosis*	Wound Culture†	Antibiotics in Chamber‡	Time in Chamber (days)	Treatment Postchamber*	Outcome*
1	78	Obesity, MVA	1: Lower abdominal sinus tract + FB (mesh)	SA, EC, MRSA	V,AB,G	70	None	Healed
		Ventral hernias, small bowel obstruction	2: R subcostal abdominal wound	SA, PA	G,V,AB	45	Delayed primary closure	Healed
			3: Exposed, infected total hip prosthesis	SA, CA	V,G	21	Delayed primary closure	Healed
2	44	95% burn with contractures	Chronic ulcers					
			1: L knee (infrapatellar)	PA	V,G	3	Debrid. + STSG	Healed
			2: R upper thigh	PV	V,G	3	Debrid. + STSG	Healed
			3: R lower thigh	SA	V,G	3	Debrid. + STSG	Healed
3	78	Laparotomy, gastric perforation	Infected midline incision	MRSA	G,AB,V	3	Delayed primary closure	Healed
4	62	Rheumatoid arthritis, steroids, methotrexate	Chronic ulcer L foot	SA, PA	V,G	3	Debrid. + STSG + VAC	STSG 90% take
								Moist dressing, healed
5	86	Chronic hemolytic anemia, pyoderma gangrenosum, smoking	1: Chronic ulcer L lateral ankle	SA, PA, PR	C,G	10	Debrid. + STSG	Healed
			2: Skin ulcer L medial ankle	SA, EB	C	3	Debrid. + STSG	Healed
			Acute open wound knee, lower leg, ankle	PS	G,V,P-T	9	Debrid. + STSG	Healed
6	17	MVA, Salter-Harris II external fixator, degloving lower leg	Acute open wound knee, lower leg, ankle	PS	G,V,P-T	9	Debrid. + STSG	Healed
7	80	RA, CHF, steroids	1: Chronic ulcer L lateral ankle	SA, ST	G	24	Debrid. + STSG	95% take, moist dressing, healed
			2: Chronic ulcer L medial ankle	SA	G	23	Moist dressing	Healed
8	76	CABG, DM	Infected sternal wound	MRSA, PA	V,G	16	Debrid. + flap	Healed
9	45	DM, COPD, steroids	Chronic ulcer L lower leg	SA, PA	G	3	Debrid. + STSG	Healed
10	32	Postpartum SBO, SBN after cesarean delivery	Infected abdominal midline wound	K, SA	G,AB	2	Delayed primary closure	Healed
11	48	RA, amyloidosis renal failure, steroids	1: Chronic ulcer R medial calf	EC	G,V	20	Gastrocn. flap + STSG	90% take, moist dressing, healed
			2: Chronic ulcer R lateral calf	SA	G,V	19	Debrid. + STSG	Healed
12	38	DM	Chronic ulcer lower leg	SA	G	4	Debrid. + STSG	Healed
13	39	SLE pyoderma gangrenosum, steroids	Chronic ulcer BKA stump	MM, SA, BB	G	38	Debrid. + flap + STSG	Small defect, moist dressing, healed
14	36	Multiple craniоplasties, CSF leak	Chronic infected wound L scalp	SA, ST	V,G,AB	3	Debrid. + STSG	Healed
15	70	RA, steroids	Chronic ulcer lower leg	SA, PA	Ct	4	Debrid. + STSG	Healed
16	73	RA, MM excision, steroids	Chronic infected wound after excision MM at L leg	SA	Cp	6	Debrid. + STSG	98% take, moist dressing, healed
17	41	DM neuropathy, femoropopliteal bypass, renal failure	L BKA chronic wound	M	G	16	Debrid. + delayed primary closure	Not healed
18	67	L knee prosthesis Septic knee	1: Open infected knee joint	SA	V	3	Delayed primary closure	Not healed
			2: Open infected knee joint	SA	V,AB	15	Delayed primary closure	Healed
19	38	Open tibia fracture, osteomyelitis, DVT	Chronic venous ulcer ankle	SA, ST	G	7	Debrid. + STSG	Healed
20	41	Open tibia fracture, osteomyelitis, DVT	Lower leg chronic ulcer	SA	G	9	Debrid. + STSG	STSG healed, later breakdown

* Diagnosis/comorbidity/treatment: BKA, below knee amputation; CABG, coronary artery bypass graft; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; CSF, cerebrospinal fluid; debrid., débridement; FB, foreign body; DM, diabetes mellitus; DVT, deep vein thrombosis; L, left; MM, malignant melanoma; MVA, motor vehicle accident; R, right; RA, rheumatoid arthritis; SBO, small bowel obstruction; SBN, small bowel necrosis; SLE, systemic lupus erythematosus; STSG, split-thickness skin graft; VAC, vacuum-assisted closure.
† Wound culture: BB, *Bacteroides bivius;* CA, *Candida albicans;* EC, *Escherichia coli;* EB, *Enterobacter cloacae;* K, *Klebsiella pneumoniae;* M, mixed infection; MM, *Morganella morganii;* MRSA, methicillin-resistant *S. aureus;* PA, *Pseudomonas aeruginosa;* PR, *Providencia rettgeri;* PS, *Pseudomonas stutzeri;* PV, *Proteus vulgaris;* SA, *Staphylococcus aureus.*
‡ Antibiotics: AB, amphotericin B; Cp, Cephalexin; Ct, ceftazidime; G, gentamicin; P, penicillin; P-T, piperacillin-tazobactam; St, streptomycin; V, vancomycin.

3. Results

In twenty (71%) of these wounds, complete healing was achieved during the planned treatment (less than six weeks for twenty-one wounds and ten weeks for one wound). In five wounds (18%), small (less than 10%) areas of non-take of the skin graft were noted. However, this healed within four weeks of treatment with a moist dressing and compression. Three wounds did not heal at all (11%). If the 5 wounds that required less than 4 weeks additional time to heal are included, the healing rate was at 89%.

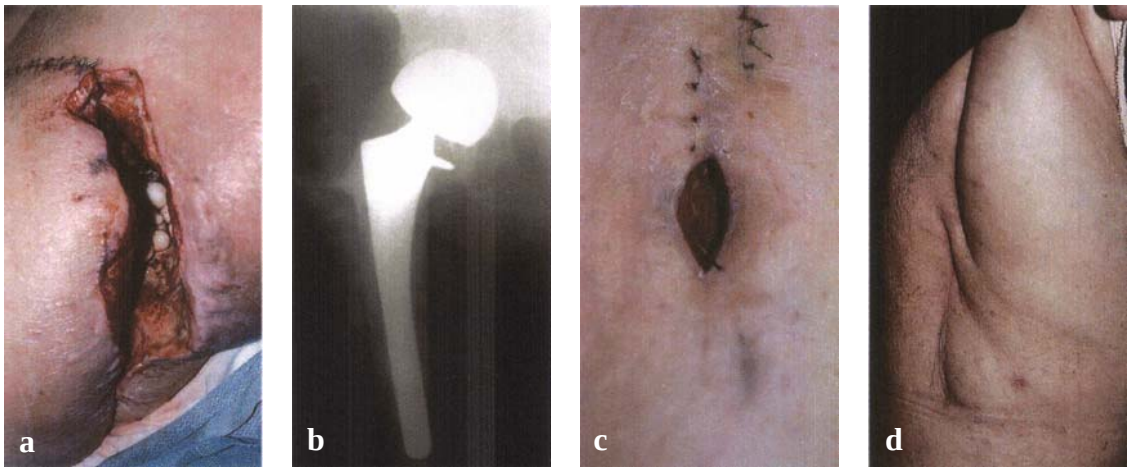


Fig. 1 Patient 1, wound 3.(a) Hip prosthesis infected with Methicilline-resistant Staphylococcus Aureus. (b) Radiograph of the infected hip prosthesis. (c) Before chamber treatment, the wound was debrided and closed, except for the inferior 4 cm, to fit the opening of the wound chamber. A 'Z-plasty' was used to strengthen this partial wound closure. After 21 days of treatment with wound chamber and antibiotics, the skin wound was excised and closed. (d) Healing was achieved without recurrence.

Of the 3 wounds that failed to heal after the initial chamber treatment, one was treated with another debridement and chamber application and finally healed (#18). One wound had a STSG that initially healed well but broke down later (#20). The third patient who failed to heal had a reamputation (#17).

Concentrations of, for instance, Vancomycin and Gentamicin of up to 10,000 times Minimum Inhibitory Concentration (MIC) were used. When the concentration of antibiotics was tested two days later, 0% or more of the original concentration remained in the chamber fluid.

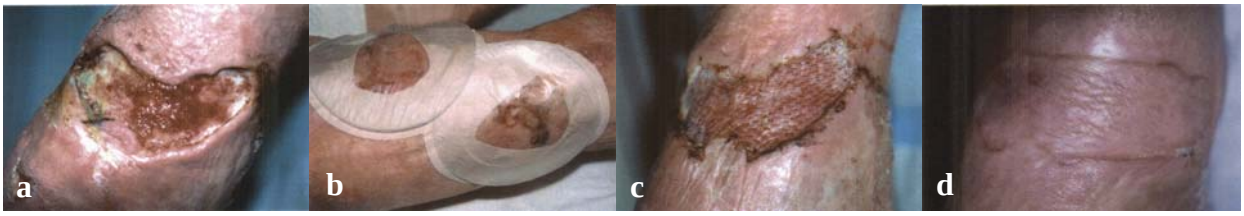


Fig. 2 Patient 2,wound 3.(a) Chronic ulcer at the lower thigh in a patient with a history of 95 % full thickness burn.
(b) Wound chambers applied to the same and a second wound in the thigh area (wound 3 and 2 in Table 1). (c) Five days after debridement and split-thickness skin graft. (d). The healed wound.

When Gentamicin was delivered in high concentration, there was slight pain associated with the injection of the Gentamicin solution into the chamber. However, this could easily be alleviated with a small amount of lidocaine in the chamber.

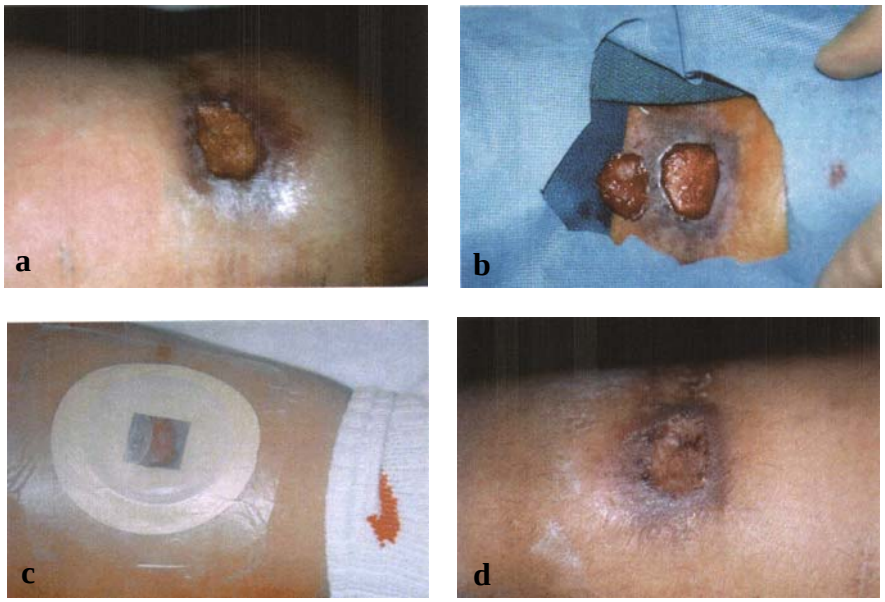


Fig. 3 Patient 19. (a)Chronic venous stasis ulcer after two failed debridements and split-thickness skin graft. (b)The wound chamber with antibiotics covering the wound. (c)The debrided wound 7 days later. (d)The healed wound 3 weeks after debridement and split-thickness skin graft.

4. Discussion

Dry treatment of wounds has one single advantage; it reduces the incidence of certain gram-negative infections[13-15]. It has many disadvantages; it is painful, it promotes progression of early wound necrosis and it delays healing[16].

Winter[17] introduced the moist treatment of wounds in 1963. By using various dressings with defined permeability, pain and necrosis are reduced and the wounds heal faster.

Almost all moist dressings are passive, i.e. no medication is applied and moist dressings have difficulties handling infected or heavily contaminated wounds[18-19].

There are several logical reasons to employ wet healing in a wound chamber. Pain is reduced and progression of tissue necrosis is eliminated[4]. Administration of culture medium, skin cells, analgesics, antibiotics, growth factors, genes and other molecules can easily be carried out. Medications such as antibiotics can be delivered in very high concentrations without risk of systemic toxicity. The direction of the concentration gradient is also favorable when using topical delivery; the concentration is highest where it is needed the most. It provides a liquid medium to the cells in the wound, allowing local growth factors to exert a paracrine effect.

Wet treatment of wounds with the chamber has been validated in a number of experiments in pigs. Being a large animal, which has similar skin to humans, it is well suited for pre-clinical studies of the wound chamber. It has been shown that the wet chamber environment does not damage normal skin[4]. Partial thickness[7] and full thickness[8] wounds heal faster than in a dry environment.

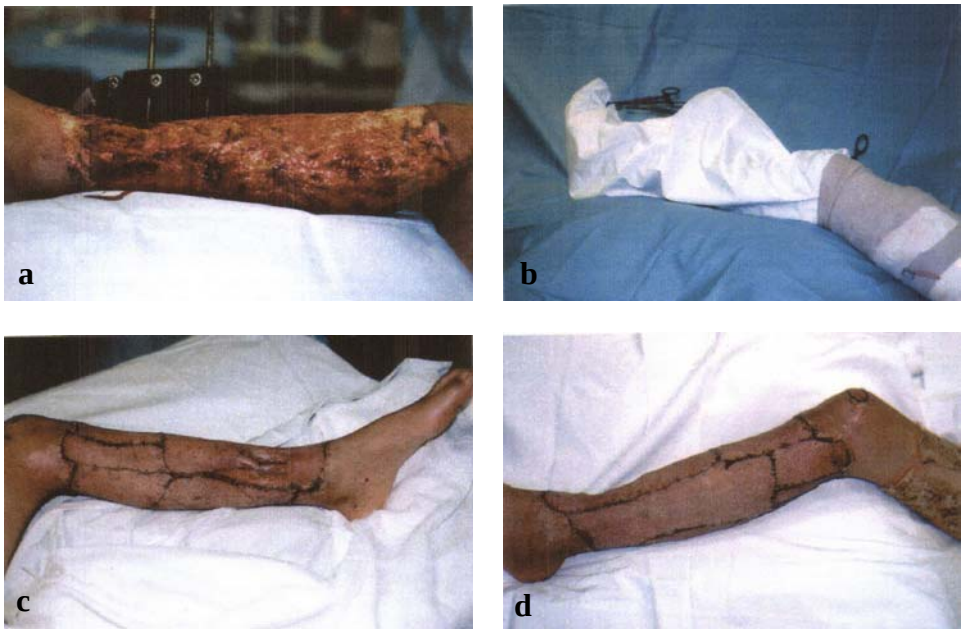


Fig. 4 Patient 6. (a)Infected wound three weeks after crush avulsion trauma to the lower leg. A Salter Harris II fracture is treated with an external fixator. (b)The lower leg was treated in a wound chamber for 9 days. (c)Medial and (d)lateral lower leg 3 weeks after split-thickness skin graft.

Wound chamber treated full thickness wounds also heal faster than the ones treated with a moist dressing[8]. There is also less scarring from healing in a wet environment[4,6]. Growth factors[12,20,21] and genes[9,10,22] can be delivered in precise amounts. The chamber also allows for representative sampling of molecules and microorganisms from the wound fluid. In the first patient similar observations were made. There was no damage to normal tissue and pain was reduced. Twenty-seven additional wounds were therefore treated. They were mainly chronic wounds in patients with systemic or regional vascular problems, but there were also a few patients with infected or contaminated acute wounds and some with exposed joint or other prostheses. The overall rate of healing was 89 %.

5. Summary

Twenty-eight wounds in twenty patients were treated with a wound chamber containing saline and antibiotics. A total of twenty-five of the wounds (89%) healed, five of them (18%) requiring additional conservative management after the initial chamber treatment and grafting procedure. No adverse effects on normal skin or the wound itself were seen. Limiting antibiotic delivery to less than one IV dose per daily application avoided the potential of systemic absorption to toxic levels.

Antibiotics such as Vancomycin and Gentamicin could be used in concentrations up to 10, w000 x MIC. Two days after application, 20% or more of the original antibiotic concentration was present in the wound chamber fluid.

Correspondent author:

Elof Eriksson, M.D, PhD
Laboratory of Tissue Repair and Gene Transfer
Division of Plastic Surgery Brigham & Women's Hospital
Harvard Medical School, 75 Francis St.,Boston, MA 02115

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Learn from yesterday
Live for today
Hope for tomorrow
The important thing is
not to stop questioning.
Albert Einstein

Part 2 - Growth factor delivery to full thickness wounds by in vivo gene transfer

" In-vivo gene delivery of Ad-$VEGF_{121}$ to Full-Thickness Wounds in Aged Pigs results in a high VEGF-expression but not in accelerated healing".
Jan J. Vranckx, MD.,Feng Yao , PhD., Nicola Petrie, MD.,Hanka Augustinova, MD., Daniela Hoeller, MD., Scott Visovatti, Elof Eriksson, MD, PhD.
Wound Rep.Regen.13;51,2005

" Gene Transfer of Growth Factors for Wound Repair".
Jan J. Vranckx, Feng Yao, Elof Eriksson.
In, The Epidermis in Wound Healing 265,2004 Eds. D.Rovee, H.Maibach, CRC Press, Boca Raton, Fl.USA.

In-vivo gene delivery of Ad-VEGF$_{121}$ to Full-Thickness Wounds in Aged Pigs results in a high VEGF-expression but not in accelerated healing.

Jan J. Vranckx*, MD.,Feng Yao , PhD., Nicola Petrie, MD.,Hanka Augustinova, MD., Daniela Hoeller, MD., Scott Visovatti, Elof Eriksson, MD, PhD.

From the Laboratory of Tissue Repair & Gene Transfer, Div. Plastic Surgery, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, USA
**Div. Plastic & Reconstructive Surgery, K.U.Leuven University Hospitals, Leuven, Belgium*

Abstract

Utilizing a swine model developed in this laboratory, we demonstrated that reepithelialization decreased with increasing age. Hence, where investigating the role of Growth Factors in the healing of porcine full-thickness wounds, we found that the endogenous Vasculo-Endothelial Growth Factor (VEGF) concentration in older pigs peaked later and at one-fourth the level of young pigs. These data suggest that VEGF might play a major role in the healing of full-thickness wounds in the aged pig. By in vivo gene transfer using the microseeding technique, we treated full thickness wounds with different doses of VEGF-expressing adenoviral vector (Ad-VEGF) varying from $1x10^7$ to $2.7x\ 10^{11}$ particles per wound (ppw). We found that the VEGF expression in wound fluid followed a dose-response pattern. However, when wounds were microseeded with the highest concentration of Ad-VEGF ($2.7x\ 10^{11}$ ppw) diminished healing rates were found.

Therefore, in a further study, we determined the minimal functional concentrations of Ad-VEGF. We used five aged Yucatan Minipigs, all retired breeders, to analyze the role of overexpression of $1x10^8$ and $1x\ 10^9$ ppw of Ad-VEGF (n=78) in terms of healing of full-thickness wounds, all 2.5 x 2.5 x 1cm in size (n=158). The Ad-VEGF solutions were delivered to the wound floor and borders by 'in vivo' microseeding. Control wounds (n=80) were microseeded with Ad-Lac-Z (n=25), treated with saline (n=49) or treated dry (n=6). All wounds, except for the dry treated ones, were covered with a wound chamber and a wet environment was created by injecting 2.5 cc saline in the chamber. Daily, wound fluid was collected from the chamber and the level of VEGF expression was determined by Elisa-assays. Reepithelialization rates were measured on histology slides from biopsies taken at day 16 and 21. Wound contraction was measured by planimetry of scanned wound tracings. Neoangiogenesis was detected by immunostaining of conglomerates of endothelial cells using the biotynilated lectins BS-1 and RCA. VEGF was expressed in the wound fluid with a rapid onset and a peak of 2300-4000 pg/mL at day 2 or 3. This high expression was measured in each of the aged pigs for all wounds microseeded with $1x10^9$ ppw (n=29) but was not seen in the saline (n=49) or Ad-null (n=25) control groups.

The VEGF expression obtained for the wounds treated with $1x10^8$ and $3x10^8$ particles per wound (n=39), had a slower onset and a lower peak concentration (400-920 pg/mL) that was reached by day 5-7 and dropped to the endogenous VEGF levels (180-250 pg/mL) after day 6-8, as measured in the saline treated wounds. Despite this high expression of VEGF in the wound fluid, we could not demonstrate a significant increase in neovascularization when comparing stained sections.

In terms of healing rates as measured by reepithelialization and wound contraction percentages, we found no significant difference comparing full thickness wounds treated with in-vivo Ad-VEGF gene transfer and Ad-null or saline treated wounds in the aged 'wet wound healing' pig model. These data show that overexpression of VEGF by in vivo gene transfer is not the decisive factor for wound repair in the aged pig model. In contrast, in vivo gene transfer of high Ad-VEGF concentrations led to impaired wound healing.

Keywords
VEGF : Vasculo-Endothelial Growth Factor, Ad-VEGF: VEGF-expressing Adenoviral vector
ELISA: Enzyme-linked Immunosorbent Assay, Microseeding, In-vivo gene transfer.

1. Introduction

A fascinating approach to the study of wound healing involves the correlation of age-related changes to alterations in wound healing. Carrell recognized already in 1916 that an important relation exists between the age of the patient, "the area of wounding and the index of cicatrisation" when he analyzed wounds in World War I victims of various ages[1]. Howes et al. studied stomach wounds in young and aged rats and reported in 1932 that the "velocity" of fibroblast proliferation and wound tensile strength diminished in older animals[2]. Fatah et al. documented a prolonged healing in split thickness skin donor sites in patients over 60 years old while Holt et al. focused on the effect of age on wound healing in healthy human beings[3-4]. Further reports noted delayed angiogenesis and impaired wound repair in incisional wounds in mice[5]. More recently, Ashcroft et al. found that aging altered the inflammatory and endothelial cell adhesion molecule profile and further experiments indicated that a slower rate of collagen production, greater collagenase activity and a greater expression of collagenase mRNA by aging fibroblasts could contribute to decreased wound healing in aged patients[6-7].

Using a pig model developed in our laboratory we investigated the correlation of age-related changes to alterations in wound growth factor concentration[8]. In that study Full-Thickness skin Wounds (FTW) were treated with a wound chamber in a wet wound healing environment and reepithelialization rates, wound contraction and endogenous growth factor presence in swine of various ages were determined. We found that the rate of reepithelialization decreased with increasing age. The endogenous Vasculo-Endothelial Growth Factor (VEGF) concentration in the older pigs peaked later and at one forth the level when compared to the younger age groups.

These data might indicate that a correlation exists between delayed wound healing and decreased VEGF peak and onset concentrations.

VEGF is an angiogenic cytokine with endothelial cell mitogenic and vascular permeability enhancing activities. It promotes extravasation of plasma fibrinogen, leading to fibrin deposition.

The modified extracellular matrix subsequently promotes the migration of macrophages, fibroblasts and endothelial cells[9-11]**.** VEGF might play an essential role in the healing of FTW in aged pigs of 6-7 years old where endogenous concentrations were found to be very low. Overexpression of VEGF during early wound healing could accelerate the repair process in old pigs.In the current study, we used the microseeding approach[12] for *in-vivo* gene transfer of VEGF-expressing adenoviral vector to porcine skin wounds. In a first set of experiments we microseeded different doses of VEGF-expressing adenoviral vector to FTW in young pigs.

A dose response curve was established. In a second set of experiments we used aged pigs to analyze the impact of overexpression of selected VEGF concentrations on healing rates and vasculogenesis. Wound chambers covered all the wounds, creating a wet environment.

The Wound Chambers allow us to retrieve wound fluid and accurately measure VEGF concentrations in the wound microenvironment on a daily basis and without disruption of the healing process[13-15]. The chamber and wet wound healing model on porcine skin wounds also provides us with a standard platform for future experiments of overexpression of one or multiple growth factors that are delivered into the wound microenvironment by *in-vivo* gene transfer but also by *ex-vivo* gene transfer with transfected cultured autologous and allogenic skin cells.

2. Material and methods

We used an Ad-VEGF construct, which encodes VEGF under the control of the hCMV major immediate-early promotor, which was inserted in the E1a region of a replicative defective adenovirus (GenVec, Gaitherburg, MD, USA). In a first set of experiments, we determined a dose-response relationship. We used a total of 4 female Yucatan minipigs (2 to 6 years old) and one female Yorkshire pig (4 months old). Full thickness wounds (total number of wounds: n= 140), each measuring 25 x 25 mm, were created on the back of pigs as further described. Experimental wounds (n=90) were microseeded with Ad-VEGF at concentrations of $1x10^8$ ppw (n=17), $1x10^9$ ppw (n=26), $1x10^{10}$ ppw (n=24) and $1x10^{11}$ ppw (n=23). Control wounds were microseeded with Ad-null mutant (n= 14) or saline (n=36) alone. We performed Elisa assays on daily collected wound fluids. A dose-response curve was established and minimal functional doses determined: Ad-VEGF concentrations between $1x10^8$ ppw and $1x10^9$ ppw were selected for further experiments.In an earlier report we described diminished endogenous VEGF secretion in aged pigs [8]. We subsequently used one 7-year-old and four 6-year old (100-180kg) domesticated female Yucatan Minipigs to elucidate the effect of Ad-VEGF by in vivo gene transfer to FTW. All these pigs were retired breeders (Charles River Laboratories, Wilmington, MA). The pigs were penned individually in the Animal Facility to minimize the risk of chamber disruption. We used two mobile, custom-made, stainless steel cages (Universal Metals, Milford, MA) of which the floor consists of a vertically mobile sling with four holes to fit the pigs' legs.

After premedication, pigs enter easily into this mobile cage that can comfortably carry and transport the large aged Yucatan pigs to the operation room. The sling can be fixed in its highest position and serve as an operation table. Its length matches the physiognomy of these large pigs.

The larynx remains well extended, which is mandatory for intubation and appropriate oxygenation.

These custom-made slings significantly reduced the preoperative and peroperative stress that we encountered in the first set of experiments on aged pigs. All these procedures were performed under general anesthesia and strict cardiopulmonary monitoring. The Harvard Medical Area Standing Committee on animals approved all animal procedures.

Based on the dose response curve of previous experiments, we then used one old Yucatan Minipig to further determine the minimal functional dose using $1x10^7$ and $1x10^8$ ppw Ad-VEGF on 32 full-thickness wounds with saline and dry wounds as controls. In the next aged Yucatan Minipig, Ad-VEGF concentrations of 3 $x10^8$ ppw with saline and Ad-Lac Z as controls (n=32) were microseeded to the FTW. Since in vivo gene transfer of $1x10^7$, $1x10^8$ and $3x10^8$ ppw did not result in significant overexpression ($p<0.05$), we treated the next three 6-year-old Yucatan Minipigs with $1x10^9$ ppw versus $1x10^8$ ppw, Ad-Lac Z and saline as controls. Biopsy dates were selected based on the reepithelialization percentages of previous porcine wound healing experiments and kept as standard for all following procedures : for young pigs a first set of biopsies was harvested at day 11 and a second set at day 15 at which day the wounds were almost healed. These dates were not useful in the aged pigs with slower healing rates. Therefore, day 16 and day 21 were selected as biopsy dates per protocol whenever aged pigs (6 and 7-year-old) were used.

3. Operative procedure

Pigs were premedicated with Telazol (4-6 mg/kg I.M.) Xylazine (2.2 mg/kg) and Atropine Sulfate (0.04 mg/kg). After induction and intubation, Isoflurane and oxygen was administered 1:3. Oxygen saturation and heart rate were measured with ‘pulse-oximeter’ ear sensors (Datex Ohmeda), respiratory rate and rectal temperature were monitored during the procedure. The porcine back was clipped with standard fine tooth animal clippers, smaller hairs with razors and shaving gel. The skin was surgically prepared with successive applications of 10% providone iodine scrub and 70% isopropanol.

Each 2.5 x 2.5 cm wound site was outlined with a template. The outlines of the wounds were re-traced using a tattoo gun and black ink (Special Electric Tattoo Marker, Huck Spaulding

Enterprises, Inc., Voorheesville, NY). The tattoo provided us with an indelible outline of the wounds that allowed us to measure the extent of wound contraction every 3 days. Full-thickness excisional wounds were created using a #11 scalpel blade. Incisions were made immediately interior to the tattoo marks and to a uniform depth of 1 cm.

After haemostasis and cleansing of the skin, the wounds were microseeded with the designated solutions. A linear set of 6 solid micro needles surrounded by a guiding steel cylinder is attached to a piston driven by an electric motor (Power Unit II, Spaulding and Rogers MFG.,Inc.).

The Adenoviral vectors were delivered to the tip of the solid micro needles through silicone tubing connected to a 30 G. needle and a 1 cc syringe controlled by an infusion pump 22 (Harvard Apparatus, Cambridge, MA).

The DNA-solution was 'microseeded' per protocol at a depth of 2 mm into the wound base and borders at a rate of 7500 RPM with the needles set in a 60 degrees angle to the wound surface. The infusion pump speed was set at 60 ul/min and the total amount of virus solution administered was 100 ul per wound [12]. A thin layer of medical adhesive (No. 7730, Hollister, Libertyville, IL) was applied to the skin surrounding the wound, and an adhesive-backed vinyl chamber (No. 689, PA Medical Corporation, Colombia, TN) was placed over each wound. We injected 2.5 mL of saline containing 100 units/mL of penicillin and 100 μg/mL of Streptomycin into each chamber using a 3 cc sterile syringe and 25-gauge needle. Air pockets within each chamber were aspirated, and the injection site was sealed with cellophane tape. After recovering from anesthesia, the pigs were penned separately in custom-made, smooth-sided stainless steel cages and 1 cc buprenorphine (0.005 mg/kg) was given IM twice a day for analgesia.

Daily maintenance.

After each 24-hour interval post-surgery, the pig was sedated and placed in the sling. 3L/min of oxygen and isoflurane was delivered via a nose cone. Pulse oximetry, respiratory rate and rectal temperature were monitored. The pig was examined for signs of wound infection (elevated body temperature, elevated respirations, putrid or cloudy wound fluid), or other illness. Wound fluid from a given chamber was aspirated (5cc syringe with a 22G needle) and deposited into an Eppendorf tube (2 cc Ticrocentrifuge Tube, Fisher Scientific, Pittsburgh, PA) labeled with the pig's number, wound number, and time post-surgery. The sample was immediately frozen to -80 degrees C. Wound fluid from each of the remaining wound chambers was collected and stored in the same manner. Chambers with leaks were removed with a medical adhesive remover (#7731, Hollister, Libertyville, IL), replaced, and sealed with Tegaderm (3M). In addition, every third day post-surgery, all chambers were removed and the tattoo outlines of the wounds were traced using a sterile, clear plastic sheet and ultra-fine-point indelible marker. New chambers were then applied in the manner described above.

We injected 2.5 cc of fresh saline-antibiotic solution into the chambers, air bubbles were aspirated, and the injection site was sealed with cellophane tape. This maintenance schedule was followed until day 11 post-surgery for young Yorkshire pigs and day 16 for the aged Yucatan pigs. At this time a first set of biopsies was harvested : five-millimeter-wide biopsy strips were taken from the middle of the wound in a cephalic-to-caudal direction that spanned the entire wound plus a margin of 5 mm of intact skin (Fig.5 a,b). The biopsy sites were closed with vicryl 3-0 (Ethicon). At day 15 post-surgery for the young swine and day 21 for the aged pigs, a second set of biopsies was taken from the remaining wounds in a similar fashion. Wounds were randomly assigned to the first or second set of biopsies. Finally the pig was euthanized via an intravenous administration of 1 mL/10 kg Euthasol. The biopsies were fixed in formalin Natural buffered solution, cut in the middle along the longitudinal axis, and paraffinized with the interior sides faced up. Sections were cut from these paraffin blocks and stained with Hematoxylin and Eosin or baked overnight at 57 degrees C. for immunohistochemistry (Fig 5).

Immunoassays of the daily collected wound fluid.

A 96 well plate (Falcon) was incubated overnight at 4 degrees C with 300 ng of hVEGF Monoclonal Antibody (R&D, MAB 293) in 100 ul PBS 1x. Next day unbound antibody was removed by incubating the wells for 3 hours with 300 ul of 5 % BSA/PBS in 0.02 % Na-Azide. After washing with 1% BSA/PBS, 200 ul of wound fluid (in serial dilutions) were incubated overnight at 4 degrees C along a row of VEGF standards, ranging from 2000 pg/mL to 0 pg/mL diluted with DMEM + 10% FBS in steps of 2.

At the third day, after washing the wells with 1 % BSA/PBS, 100 ng of goat-anti-hVEGF polyclonal antibody (Endogen) were added to each well in presence of 100 ul of 3% BSA/PBS + 0.05 % Tween and incubated for 3 hours at RT. After washing the wells, 100 ul of 1:3000 diluted HRP-goat-anti-rabbit Polyclonal antibody (Santa Cruz) were incubated for 1.5 h at RT. Finally after meticulously rinsing the wells 4 times with PBS 1x + 0.1 % Tween20, the peroxidase assay was performed filling the wells with 100 ul of Biorad's A+B peroxidase solutions (10:1). After 5-10 minutes, when the high standards turned deep blue, 100 ul of H2SO4 was added and after gently mixing, the plate was read at 450 nm in the microplate reader. End concentrations were plotted against the standard curve and final concentrations calculated regarding the dilutions used (Softmax Pro).

Reepithelialization measurements.

Each Hematoxylin-and-Eosin-stained biopsy slide was scanned (Epson Perfection 636U with slide attachment, Epson America Inc., Long Beach, CA) into a computerized JPEG image. The image was loaded into Paintshop Pro (v 7, Jasc Software, Eden Prairie, MN), which allowed us to measure length using pixels as units. Comparing the reepithelialization tongues, expanding from either side of the transition zone between intact and healed dermis, the overall rate of reepithelialization at the center of the FTW could be measured (Fig. 5 a-d). Several sections per wound were measured and means calculated.

Results were averaged for each age group, and graphical comparisons with statistical significance were generated using Student's T-test.

Wound contraction measurements.

The transparant sheets (Kimberly Clark, sterile pouches) containing the wound tracings were scanned into 24-bit bitmap computer images. At fixed resolution, number of pixels of the total wound and healed wound area were calculated using a dedicated computer subroutine

(Scion Image software, Scion Corporation, Frederick, MD). A contraction percentage was calculated for each wound per day. Results were averaged for each age group, and graphical comparisons and statistical significance were generated.

Immunohistochemical staining.

Paraffinized sections were baked overnight in a 57 degrees C oven, deparaffinized next day in Histoclear (HS-200, National Diagnostics) and rehydrated in ethanol baths. Endogenous peroxidase activity was quenched by using 3% H2O2. Primary biotynilated lectins[16] BS-1 and RCA-1 (Sigma) were used in a 1:100 and 1:500 dilution in PBS containing 10% FBS. The immunoperoxidase solution (Vectastain Elite ABC-kit, Vector Laboratories) was made up in high ionic PBS and the Diaminobenzidine (DAB,Sigma) solution was prepared in Tris buffer and 3% hydrogen peroxide. Counterstaining was performed with haematoxylin. Neovascularization was analyzed by counting clusters of stained endothelial cells per field under 20-40-x magnification (Fig 6).

Statistical Analysis.

Means, standard deviations and Student t-Tests were used to compare the wound contraction data as measured by planimetry every 3rd day and the reepithelialization rates for wounds of all groups at day 11 and 15 for the young pigs and day16 and day 21 for the aged pigs. All tests were two-sided, and a P value of 0.05 or less was considered to indicate statistical significance.

4. Results

4.1. Ad-VEGF microseeding to young pigs: first set of experiments with 4 mo/o and 2 y/o pigs.

We measured high level of VEGF expression by Elisa when the FTW were microseeded with 1x1011 ppw (Fig.1). The peak expression of VEGF occurred at day 3 and reached a maximum of 1000 ng/mL, in strong contrast to the VEGF expression in saline control wounds and FTW microseeded with the Ad-null mutant. We found that the VEGF expression showed a dose-response pattern: at day 3 post-transfection, the concentrations of VEGF detected in the wound fluid dropped from 1000 ng/mL for the wounds treated with 1x1011 Ad-VEGF ppw to 90 ng/mL ppw for the FTW treated with 1x1010 ppw, while Ad-VEGF microseeding of 1x109 ppw gave a maximum VEGF expression of 15 ng/mL and 0.7 ng/mL for the Ad-null mutant treated with 1x1011 ppw. Ad-VEGF microseeding of 1x108 ppw resulted in concentrations at background level.

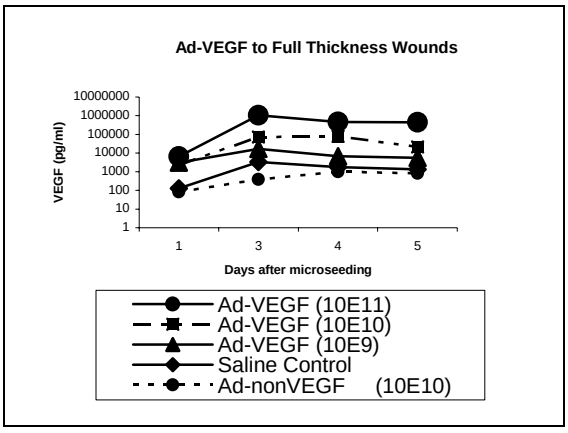

Fig. 1 VEGF concentration in pg/mL as measured by ELISA in wound fluid of full thickness skin wounds microseeded with various amounts of Ad-VEGF particles. Saline and Ad-nonVEGF were used as controls.

Reepithelialization rates of FTW were compared in these 4 month old and 2-year-old Yorkshire pigs microseeded with $1x10^{11}$ ppw with saline as control.

Histology of the FTW at day 11 and day 15 showed significantly lower healing rates in the 2-year-old pigs with a mean of 20% while for the 4-months-old pigs a mean of 40% reepithelialization rate was measured. This lower reepithelialization rate was seen for both the Ad-VEGF $1x10^{11}$ ppw treated wounds as the saline treated wounds (Fig. 2). This difference in healing rate in old versus young pigs was confirmed in all further experiments by measuring mean reepithelialization rate in saline treated wounds. Interestingly we also found that for both age groups, the saline control wounds had a significantly higher reepithelialization rate than the Ad-VEGF microseeded wounds on day 11 and on day 15.

These results suggest that the VEGF-overexpression ($1x10^{11}$ ppw) might have been too high, which resulted in impaired rather than accelerated wound healing. Lower concentrations of the construct were to be microseeded in following experiments to analyze this outcome.

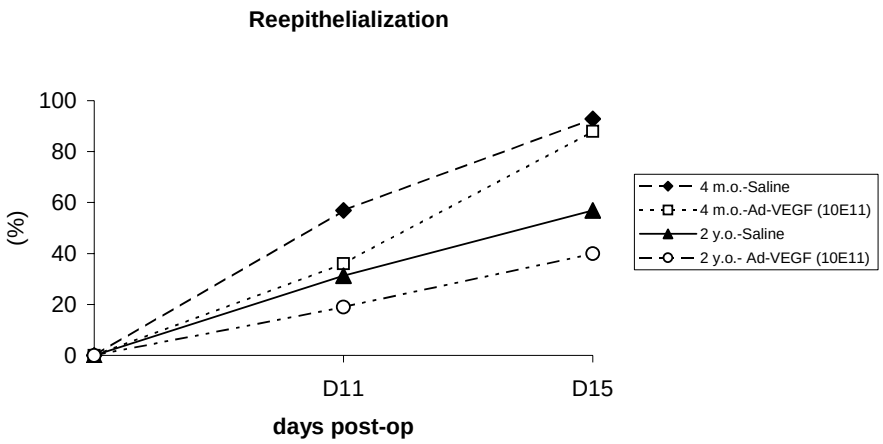

Fig. 2 Reepithelialization rates of FTW were compared in 4 month old and 2-year-old pigs. Full thickness wounds were microseeded with $1x10^{11}$ ppw with saline as control. The histology of the FTW at day 11 and day 15 showed the significantly lower healing rates of the FTW in the 2 year old pigs as well for the Ad-VEGF $1x10^{11}$ ppw treated wounds as for the saline treated wounds.

4.2. Ad-VEGF microseeding to aged Minipigs: second set of experiments using 6 and 7-year-old pigs.

In order to extend the impact of age on the wound healing response and the growth factor release, we used aged retired breeders, 6 to 7-years-old and the minimal functional Ad-VEGF concentrations were determined. All aged pigs sustained the procedures well during the entire experimental setup. Standard full thickness wounds were microseeded with $1x10^{7}$ and $1x10^{8}$ ppw in a 7-year-old Yucatan Minipig. VEGF expression in the wound fluids was low (189 pg/mL at day 3, and 256 pg/mL at day 7) and at no point higher than the endogenous VEGF expression as measured in the saline treated wounds.

The low endogenous concentrations as measured in the saline controls were significantly lower as compared to former experiments in younger pigs, confirming the lower endogenous release of VEGF in the wound fluids.

There was no significant difference measured in reepithelialization or contraction rates comparing the different groups. All wounds had a healing rate around 25 % at day 16 except for the dry wounds that only had a 14 % healing rate at day 16. No impaired healing was seen when

Ad-VEGF microseeded wounds were compared to saline treated wounds using these lower concentrations of Ad-VEGF. Therefore, we microseeded $1x10^9$ and $1x10^8$ ppw of the Ad-VEGF construct with Ad-Lac Z (Ad-null) and saline as controls using three 6-year-old pigs.

In order to define the minimal functional concentration more precisely, we microseeded $3x10^8$ ppw Ad-VEGF versus saline and an Ad-Lac-Z reporter construct in one other 6-year-old pig.

At a VEGF concentration of $1x\ 10^9$ ppw, clear expression of VEGF in the wound fluid was seen with a rapid onset and a peak on day 2 reaching 2.3-4 ng/mL. This significant and high expression was constant in this group for all 3 pigs and was not seen in the $1x10^8$ or the saline group where the maximum expression at day 2 reached 250 pg/mL (Fig. 3).

After 3 days this peak concentration abruptly diminished and was followed by 1 or 2 smaller peaks around 600 pg/mL until day 7. After 7 days, the concentration of VEGF expressed in the $1x\ 10^9$ ppw group dropped to reach the endogenous concentration as measured by the saline treated wounds (368 pg/mL). VEGF expression in the $3x10^8$ ppw treated wounds did not exceed 510 pg/mL by day 2 and further expression followed a comparable course as the $1x10^8$ ppw treated wounds.

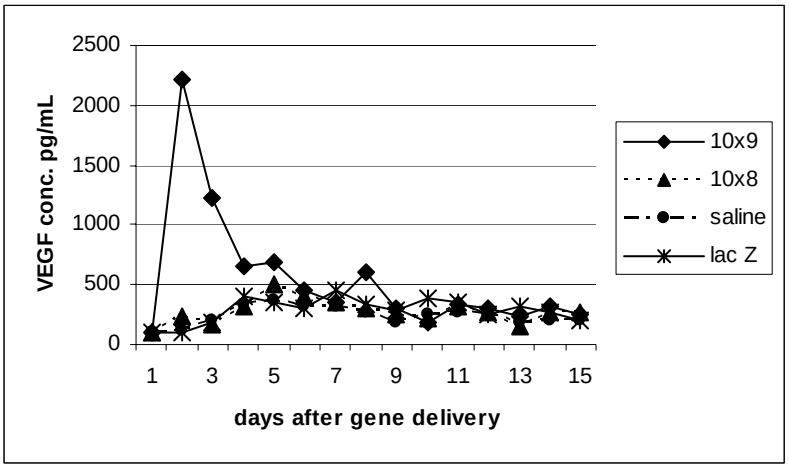

Fig. 3 hVEGF concentration in pg/mL measured by ELISA immunoassay after in vivo gene transfer of Ad-VEGF to full thickness wounds in 6-year old pigs.A peak in VEGF expression in the wound fluid of the wounds treated with $1x10^9$ particles per wound, was seen around day 2-3 for all 4 aged pigs.This peak was not measured in the $1x10^8$ or the saline control group.After day 3 this VEGF overexpression diminished abruptly. After day 10 no more expression higher than the endogenous expression was detected.

For the first two aged pigs (group I), wounds treated with $1x\ 10^9$ ppw, demonstrated a significantly faster healing at day 16 as compared to wounds treated with $1x\ 10^8$ ppw ($p< 0.05$) or saline ($p< 0.05$). By day 21 no significant differences in reepithelialization rates were measured among the microseeded groups. These pigs were treated in a regular pig sling, which was not optimized to their weight or size. Moving the pigs in and out sling always provoked significant pre and postoperative stress.

Also ventilation problems were often seen in these slings during the procedure, likely due to pressure on the larynx. The short frontal part of the standard commercial pig sling bends the head of large pigs into flexion (Group I in Fig.4).

Two new pig slings – custom made for pigs of this size and weight- facilitated the procedures significantly which resulted in easier manipulation of the animals allowing them to walk in and sling spontaneously (Group II in Fig.4).

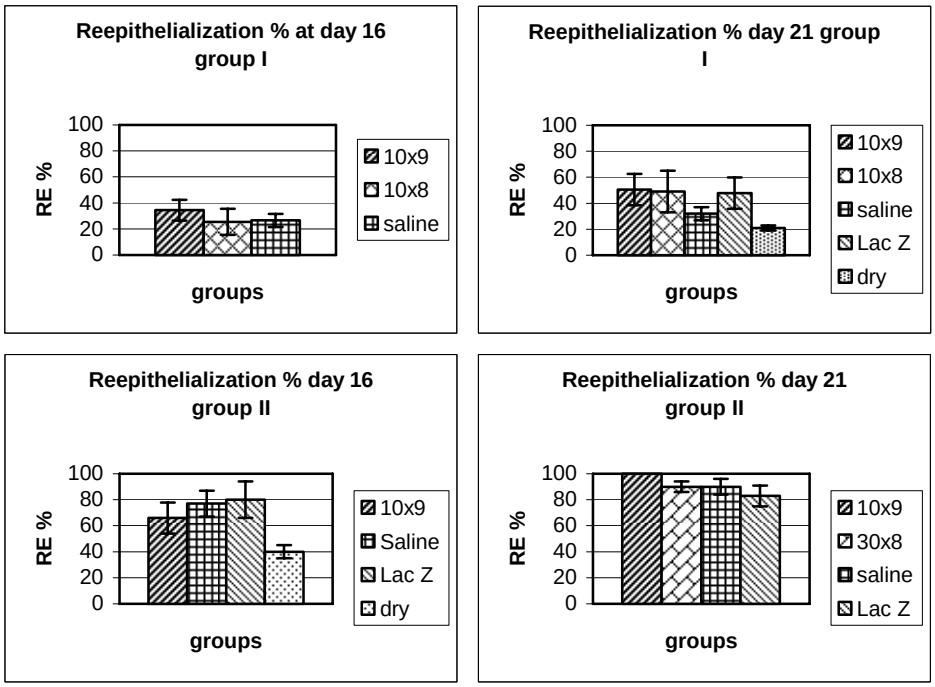

Fig. 4 Mean reepithelialization rates were measured on histological sections of biopsies taken at day 16 and 21. Group I and II each represent 2 pigs in a similar experimental setup. A standard cage was used for Group I while custom made cages and slings were used in group II.

To analyze whether the VEGF microseeded wounds had an accelerated healing at day 16 due to the Ad-VEGF delivery, Ad-Lac Z (= Ad-null) was microseeded into control wounds (Group II).

In this group, we found no significant difference in mean reepithelialization rates of wounds treated with 1x 10^9 ppw Ad-VEGF, Ad-Lac-Z or Saline at day 16 and day 21.

After analysis of all wound contraction parameters in these five aged pigs, no significant difference in contraction rates was found.

Morphology of the newly formed epithelium was investigated under light microscopy (Fig.5a-f). We did not find a difference between the different groups except when wet treated wounds as a group were compared to the dry treated wounds.

Dry treated wounds healed significantly slower (25-40%, $p<0.01$) at day 16 and 21 as compared to wet treated wounds in all 4 pigs where dry wounds (n=6) served as comparison to the wet treated wounds. Dry wounds had less migration of the epidermal layer into the wound.

As a consequence the newly formed epithelium at the wound borders was irregular in shape and counted more keratinocyte layers than the regular shaped epithelium in wet treated wounds.

Immunostaining with the biotinylated lectins BS-1 and RCA-1 did not demonstrate a significant difference in the number of newly formed blood vessels per field versus the control groups that were not treated with Ad-VEGF (Fig.5f). In all groups a similar number of small vessels were present underneath the outgrowing epithelial extensions and within the organizing granulation tissue. A well-organized dermo-epidermal junction with a stained basal cell layer was seen in all sections.

5. Discussion

VEGF is a highly conserved protein that has cross-species activity: between human, rat or bovine VEGF, 84-94 % sequence identity has been observed[11]. The VEGF group of molecules consists of VEGF-A and the VEGF related factors VEGF-B, VEGF-C and PIGF placental growth factor.

VEGF-A is transcribed from a single gene but as a result of alternative splicing, various isoforms exist ranging from 121 to 206 amino-acid residues[9,41,42]. The three isoforms $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are biologically equivalent for in vivo angiogenesis[19].

might also function through a paracrine effect. VEGF possesses multiple biological activities: it induces vascular permeability, more potent than histamine[45], VEGF exerts mitogenic effects on endothelial cells and stimulates the migration and proliferation of endothelial cells. VEGF also promotes macrophage migration and induces the expression of interstitial collagenase[20]. These activities of VEGF might play an essential role in angiogenesis and wound healing.

We used an Adenoviral construct expressing the $VEGF_{121}$ isoform. $VEGF_{121}$ does not contain exons 6 and 7 of the gene and is fully active as an inducer of angiogenesis with endothelial cell specific mitogenic and vascular permeability-enhancing activities[10,19]. $VEGF_{121}$ and $_{165}$ are exported from the cells while $VEGF_{189}$ and $_{206}$ are predominantly cell-associated[17].

Therefore the $VEGF_{121}$ isoform could be useful in our wound healing model: the wound chamber creates a wet environment in which the extracellular VEGF is secreted; in this mini-incubator VEGF

Taub, Padubidri et al. described the enhanced survival of ischemic abdominal flaps in a rat model when these flaps were flushed with $cVEGF_{121}$ and a liposome transfection medium that was injected into the femoral artery[21-23].

Other authors found enhanced wound healing in excisional wounds or in trachea defects by promoting angiogenesis through topically applied Ad-$VEGF_{165}$[21-24].

It is documented that the use of viral vectors leads to higher tranduction efficiency than direct plasmid injection.

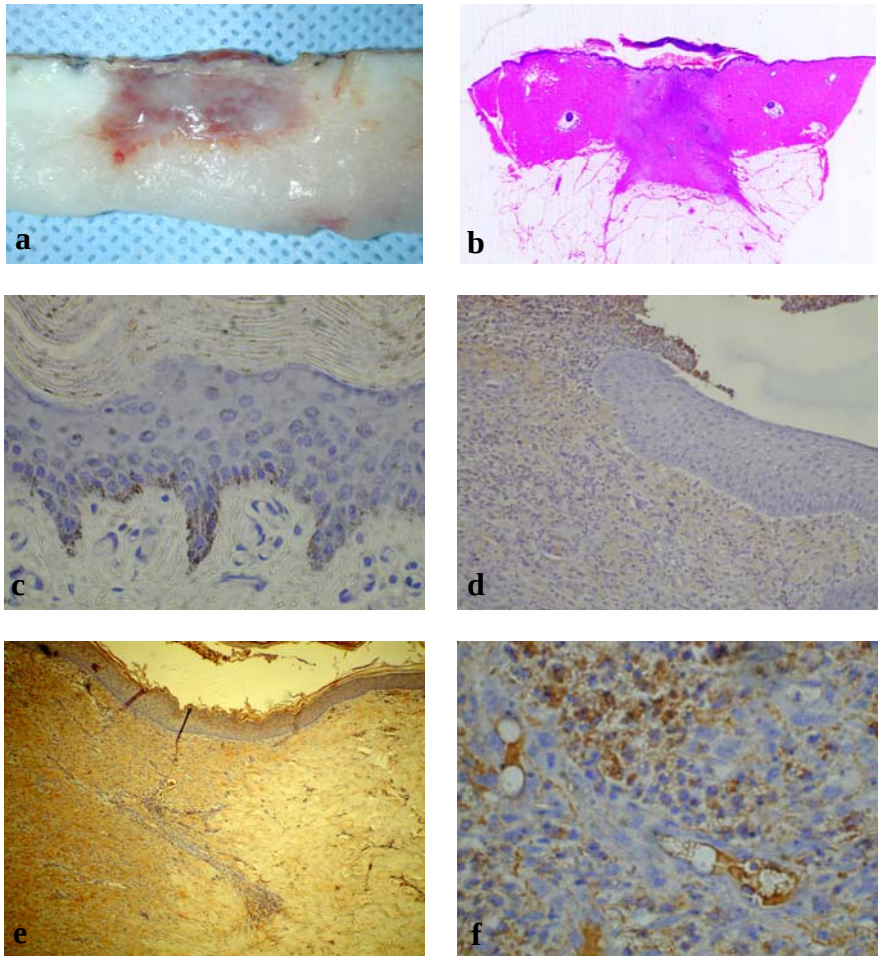

Fig. 5 Histological sections of full thickness skin of aged pigs. (a)sagital full thickness skin biopsy. (b)H&E staining on a full thickness skin section with restored epithelium. (c) Section stained with H&E showing the rete ridges and papillary dermis, similar to human skin. (d)A multilayered epithelial extension of keratinocytes migrates upon the granulation tissue of the wound. (e)The transition zone of wounded (left side) and non-wounded dermis (right side) is used as the arbitrary demarcation line to measure reepithelialization. (f) Small vessels in the granulation tissue stain with biotinylated lectin BS-1.

Deodato et al. demonstrated a significant shortening in healing time of excisional wounds in a rat model using recombinant AAV-vector encoding human $VEGF_{165}$[25] and Ailawadi et al. reported enhanced vascularity and bursting strength of healing abdominal fascia by adenovirus vector-mediated VEGF transfer in a mouse-model[26].

However, all of the above mentioned studies have been performed on rat and mice models.
Healing in these lower mammals results mostly from contraction by a strongly developed panniculus carnosus (‘musculus cutaneus maximus’) and only in smaller extent by granulation and reepithelialization. This healing process stands far from healing in human skin wounds.

The pig model is a useful model to compare wound healing with the clinical wound repair parameters of human skin: the structure of porcine epidermis and dermis, as well as the dermo-epidermal junction, the rete ridges and the papillary dermis, the dermal collagen and the number and distribution of blood vessels are all similar to human skin[36-37].

Also epidermal cell turnover time, type of keratinous proteins and lipid composition of the stratum corneum is similar in both species[38]. We used both Yorkshire and Yucatan Minipigs for our animal experiments.

Adult Yorkshire pigs are large and aggressive and thus difficult to handle. Therefore our aged swine were female Yucatan Minipigs. They are smaller (100-180 kg) and gentle, however hard to get: farmers usually do not keep these retired breeders, due to expensive care and housing. In earlier experiments, we found healing rates and endogenous growth factor concentrations to be similar in young Yorkshire pigs and young Yucatan pigs. For the 'proof-of-principle' experiments and the establishment of a VEGF-dose-response curve, we therefore decided to use 4-month-old female Yorkshire pigs and 2-year-old Yucatan pigs to verify the aging factor on healing and VEGF release. Once these parameters were set, further experiments were performed with aged Yucatan Minipigs.Taking into account the age of fertility (at 6 months) and maximum age (10-12 y/o) a 6-7 year old Yucatan pig represents an age category of more than 80 y/o in humans. In comparison to young Yorkshire pigs and 2 y/o Yucatans, these 6 y/o had a significantly lower mean healing rate. While in the younger pigs reepithelialization is complete around day 13-15, the mean reepithelialization percentage at day 21 for the old pigs is around 50-95%, with a variance among subjects within the same group of age, just as in human wound healing. These considerations determined the time schedule for biopsy harvest in young or aged pigs. We took the first set of biopsies when healing rates appeared clinically around 50%; the second set of biopsies had to analyze progressed healing.This biopsy schedule was used as standard scheme for all further pig experiments, so that control wounds among young Yorkshire pigs could be compared, just as control wounds among aged Yucatan pigs. We demonstrated that the speed of reepithelialization in FTW decreased with increasing age. Furthermore, the endogenous VEGF concentration peaked later and at a four times lower level in aged pigs compared to younger age groups. Rivard et al. stated that angiogenesis responsible for collateral development in limb ischaemia is impaired with aging. Responsible mechanisms include age-related endothelial dysfunction and reduced VEGF expression[32]. These data suggest a relation between VEGF expression, aging and wound healing.

Our hypothesis was that in vivo gene transfer of VEGF-expressing adenoviral vector therefore could accelerate the healing of full thickness wounds in an aged pig model.

Different strategies can be used to apply VEGF to wounds: ex vivo gene transfer e.g. by using VEGF-expressing cultured skin substitutes[27] and in vivo gene transfer by direct injection, particle mediated gene transfer or microseeding[12].

Conversely, transfection with a dominant-negative VEGF receptor inhibited wound angiogenesis and a significant alteration in wound contraction kinetics was found[10,28]. Despite all these fascinating reports, a direct relationship between angiogenesis and improved wound healing hasn't been demonstrated yet.

Takayima and Momose et al. measured high VEGF levels in the cytoplasm of epidermal cells in cutaneous wounds in mice and in human cultured gingival epithelial sheets, which suggests a direct interaction between keratinocytes and VEGF and implies consequences on wound healing and tissue regeneration[29-30]. Other studies showed that basal keratinocytes at the wound edge are the source of hypoxia-inducible-factor-1alfa (HIF-1alfa), a potent transcriptional regulator that binds multiple target genes. One of these target genes is VEGF[31]. We established a dose response curve. Ad-VEGF concentrations below $1x10^8$ ppw did not lead to a significant overexpression in wound fluids when the microseeding procedure was used.

Concentrations of $1x10^9$ ppw led to consistent overexpression in the wound fluids, but not to accelerated healing rates. The highest VEGF concentrations ($1x10^{11}$ ppw) even impaired wound healing. These data suggest that a therapeutic window exists for Ad-VEGF delivery and that concentrations need to be titrated carefully. The success of gene therapy is not solely a function of transfection efficiency or protracted gene expression. Despite relatively low transfection efficiency associated with the use of injection of naked DNA, studies in severely ischaemic limbs of rats with arterial gene transfer of VEGF demonstrated that the angiogenic activity of VEGF is sufficiently potent to achieve a meaningful biological outcome[33-35]. Then why we did not see enhanced vasculogenesis or improved healing in a model with diminished endogenous VEGF concentration?

Skin represents a unique healing-environment with high cell turnover in the differentiated layers. Epidermis does not have a proper circulation and must rely on the subepidermal plexus to receive the necessary metabolites. Wound healing is a temporary process, which implies that only temporary expression of the therapeutic gene is necessary within that limited time frame.

We found a consistent tri-phasic VEGF release in the wound fluids of all wounds treated with $1x10^9$ ppw or higher. An early peak, followed by a dip, and two smaller peaks. Thus, besides growth factor concentrations, timing and duration of the release might be elementary. In our in vivo model, the VEGF overexpression might peak too early in the inflammatory response, since VEGF plays a role especially in the later phase of the wound healing process.

VEGF is an important growth factor in angiogenesis but not the only one. Various reports indicate that also TGF-beta isoforms and PDGF family members play a significant role in neovascularization[39-40].

It is plausible that due to many interactions and synergism between growth factors, neovascularization may take place without VEGF while other factors take a larger share in the angiogenesis process.

We have demonstrated that in the old Yucatan pigs VEGF concentration is strongly diminished, but it is not reduced to zero. What is the threshold concentration of growth factors to allow normal vascularization in the healing of full thickness wounds in an aged pig model? Overexpression of VEGF by the Ad-VEGF vector, which results in a high transfection efficiency, does not result in significant therapeutic results, which might signify that the overexpression is not high enough to make a constant and significant difference. However in our $1x10^{11}$ ppw group, healing rates were less then the controls, while protein expression was 3 to 4 logs higher.

More likely, the threshold of endogenous VEGF still is high enough to induce normal vasculogenesis and wound healing, or other growth factors and cytokines make up for the diminished VEGF concentrations as 'brothers-in-arms'.

Maybe in the pig model another co-factor (growth factor?) might be required to induce vasculogenesis and accelerate wound repair. The synergy of growth factors used in ex-vivo and in-vivo gene transfer procedures must be further investigated since it seems likely that just one growth factor can't change the tide, but the right combination might.

We placed a wound chamber over all wounds, except for the dry treated wounds. The wound chamber creates an incubator-like environment that promotes wound healing[13,43,44] and has been successfully utilized in patients with chronic non-healing ulcers[14-15].The VEGF-treated wounds were compared to a control group that was treated in the same optimal wet wound healing environment. When compared to dry wounds, significantly faster reepithelialization was noted for all wet treated wounds. Maybe these optimal wound healing circumstances dilute the impact of lower endogenous growth factor concentrations in aged pigs and thus the effect of overexpression of growth factors by in vivo gene transfer.

As seen in mice, it is obvious that 'knock-out' pigs would be uttermost beneficial to growth factor research in a clinical setting of wound healing. Unfortunately, this does not represent a current option, and probably it never will.

We report how growth factor concentrations in wound fluids diminish with increasing age. Also reepithelialization rates decrease with increasing age. We postulated that in vivo gene transfer of Ad-VEGF to full thickness wounds in aged pigs therefore could accelerate wound healing. A dose response curve of Ad-VEGF was established in young pigs using concentrations from $1x10^7$ up to $1x10^{11}$ ppw. These cDNA solutions were microseeded into standard full thickness wounds and the wounds were covered with a wound chamber that created a wet wound healing environment. Surprisingly, when the highest concentrations were microseeded in vivo, impaired wound healing resulted. When the lowest concentrations were used, no VEGF-overexpression was detected in the wound fluids collected in the wound chambers over the full thickness wounds.

When $1x10^9$ particles per wound were microseeded to FTW in aged pigs, Elisa assays on the collected wound fluids showed high VEGF protein concentrations with peak values around day 2 for the $1x10^9$ ppw treated wounds. After 2 smaller peaks the VEGF expression reached the endogenous levels at day 7. This high VEGF expression was measured in all wounds treated with 1x109 ppw of the Ad-VEGF. However, no accelerated healing of the full thickness skin wounds or a higher rate of neovascularization was found in these aged pigs. Also wound contraction measurements were similar to control wounds.

When high VEGF concentrations were microseeded (1x 1011 ppw) even diminished wound healing was noted. These data indicate that a therapeutic window exists for VEGF in the swine model. In order to further evaluate the role of in vivo gene transfer and VEGF-overexpression to the healing of full thickness wounds, we need a defined pathogenic pig model where endogenous growth factors play a lesser role and the impact of exogenous in vivo or ex vivo gene transfer of Ad-VEGF might be better determined. The combination of synergistic growth factors also must be further investigated.

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Correspondence

Elof Eriksson, MD.,PhD.
Division of Plastic Surgery**,** Brigham and Women's Hospital
75 Francis Street, Boston, MA 02115. USA.
Tel. 617-7324836 Fax 617-2786918
Eeriksson@partners.org

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They always say time changes things
but you actually have to change them yourself.
Andy Warhol

Part 3 - Growth factor delivery to full thickness wounds by ex vivo gene transfer

- *in vitro cell engineering protocols.*

"A defined porcine basal keratinocyte cell suspension culture system for ex vivo gene transfer under serum-limited conditions".
Vranckx J.J., Dickens S.,Hendrickx B.,Vandenberge S.,Vermeulen P.
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A defined porcine basal keratinocyte cell suspension culture system for ex vivo gene transfer under serum-limited conditions.

Vranckx J.J., Dickens S.,Hendrickx B.,Vandenberge S.,Vermeulen P.

Laboratory of Plastic Surgery & Tissue Engineering Research
Dept. Plastic & Reconstructive Surgery & Burn Centre, KUL Leuven University Hospitals, Leuven, Belgium

Abstract

Ex-vivo gene transfer represents a promising strategy to treat full thickness skin defects by adding cell substrate and using cells as gene transfer vehicles. Before translating such strategies into a clinical setting, standardization of optimal culture conditions and transfection protocols is mandatory. In this study, we first analyzed the impact of different growth media on cultivation parameters of porcine basal cell keratinocyte suspension cultures (BCKs) under serum-reduced conditions. The best medium was then used in ex vivo gene transfer studies to investigate transfection efficiency of six different transfection reagents.

M&M

Primary and cryopreserved porcine BCKs were cultured until fifth passage on collagen I versus fibronectin coated cellware. Cell migration, proliferation, differentiation and clonal capacity were determined. Cell viability was measured by trypan blue exclusion tests and MTT assays. We used KGM II, Optimem and SFM as growth media, enriched with 2.5% calcium depleted FBS, versus KGM enriched with 8% calcium depleted FBS as control medium.

In a second study, we investigated Lipofectin, Lipofectamine 2000 and Fugene as transfection reagents with Lac Z and EGF as reporter genes. We used MTT assays to demonstrate cell viability by optical density at a wavelength of 540 nm and ELISA to determine EGF expression. In a second setting, we used Fugene HD, PEI, and Effectene versus our standard Lipofectin, with Lac Z and GFP as reporter genes. We calculated transfection efficiency by X-gal staining for Lac Z and measured GFP fluorescence by Flow Cytometry.

*Results and Conclusion*s

Cell cultures of BCKs had the highest clonogenic capacity in SFM medium enriched with 2.5% serum. In these serum reduced and low calcium conditions, BCKs migrated with high proliferation rates. Fugene HD with 2 μg of reported DNA led to the highest non-viral transfection efficiency in these serum reduced growth conditions, compared to Effectene, PEI, Fugene 6, Lipofectamine 2000 and previous standard lipofectin. This *ex vivo* gene transfer protocol represents an efficient basic platform for further gene transfer in tissue engineering protocols under serum-reduced conditions.

Keywords

non viral transfection, cell suspension cultures, basal keratinocytes, growth factors, ex vivo gene transfer, wound healing.

1. Introduction

Tissue engineering and gene transfer are fascinating novel approaches for tissue repair. To investigate these promising strategies, we must identify an appropriate animal model which closely approximates human tissue repair. Rodent models are efficient proof-of-principle models, but their outcomes cannot be translated into clinical human wound repair.

Pig skin is comparable to human skin in its histological, immunohistochemical and clinical features[1-4]. However, despite the structural resemblance, porcine keratinocytes (KC) are more fragile than human KC cultures and their overall take is disappointing. Therefore, higher seeding-densities are required and high concentrations of bovine serum (FBS) are used as a rich source of mitogens for KC proliferation[5,6]. Serum contains an high concentration of growth factors, which stimulate cell proliferation and play an integral part in wound repair. Also, serum batches may significantly differ in composition. Eliminating these undefined factors is critical for the investigation of growth factor therapy for FTW repair. Moreover, FBS contains both adhesive (fibronectin and vitronectin) and anti-adhesive proteins (albumin). Therefore, serum-free cell attachment could be at least as good in the absence of serum and feeder layers or coated cellware[7,8].

The first aim of this study was to develop a defined and efficient protocol for culturing cell suspensions of non-differentiated basal cell keratinocytes (BCKs) under serum reduced conditions. These BCKs have high clonal capacity and remain strongly attached to collagen I coated cell ware. In a study by Nakamura et al., 18% of these BCKs were observed to express CD 90, a probable marker of keratinocyte stem cells[9]. Therefore, this BCK cell population represents a promising target cell population for gene transfer and cell engineering.

The second aim of this study was to translate this serum reduced cell culture condition to ex vivo gene transfer protocols, using BCKs as a gene transfer vehicle.

Several transfection and transduction methods exist for ex vivo gene transfer into FTW. Viral vectors are most efficient. The major disadvantages in the use of viral vectors are the possible immunological reactions or the random integration into nuclear DNA with subsequent insertional mutagenesis, which may lead to the activation of cellular oncogenes[10]. Viral vectors also have limited package capacity for the integration of foreign DNA. Highly proliferating KCs can be transfected efficiently with lipid/DNA complexes. We investigated transfection efficiency (TEE) and susbsequent cell cultivation parameters of six non-viral lipid-based reagents under the same serum reduced conditions as observed in the first study.

2. Materials & Methods

2.1. Keratinocyte primary Cultures: the standard protocol.

Three month-old Yorkshire pigs were premedicated with IM Ketamine (50 mg/5mL) and Xylazine (0.4 mg/mL)[4]. For short procedures, we used mask anesthesia with Isoflurane and O2. The skin was prepped with povidone iodine and 75 % isopropanol. We harvested a skin graft with an electrical dermatome set at 0.015 inch. The skin grafts are stored in 20 cc DMEM medium + 1 % Pen/Strep for 1 hour at 4 C. Skin grafts were cleaned with 75% isopropanol for 10 seconds, rinsed for 1 minute in a dish with sterile 1X PBS, cut into squares of 4 cm^2 and incubated for 2 hours at 37 C in a 0.5% neutral dispase II solution of 0.5 U/mg (Roche Diagnostics) to separate dermis from epidermis. After 2 hours, the epidermis was peeled from the dermis. While the dermis was digested with Collagenase IA (Sigma-Aldrich) to grow fibroblasts, the epidermal sheath was incubated in a 0.0625% trypsin solution for 4 minutes at 37 C and subsequently for 3-7 minutes at RT. With the epidermis basal side upward, the basal cell layers were gently scraped. The cell slurry was pipetted to dislodge cell clusters and collected in a sterile cupholder with metal 100mesh sieve (Sigma). The collected cell suspension was centrifuged for 4 minutes at 900 RPM (Beckman CPR centrifuge). The pellet was dissolved into 10cc of medium. Cells are counted on a haemocytometer using the trypan blue exclusion test. Culture dishes are incubated at 37 C, 5% CO2 and 90% humidity. Daily cell culture dishes were checked under light microscope to evaluate morphological changes and digital pictures were taken at 24, 48 and 72 hours until 75-85% confluence. After the first day of seeding the cells, the old medium with floating cells and debris was aspirated and new medium was added every 48 hours.

2.2. Subcultures and viability assessment.

After aspirating the cell culture medium, 5 cc of a 0.25% dispase II neutral protease solution was used for 5-8 minutes to remove the confluent sheet from the collagen I coated dish. The cell suspension was aspirated, gently pipetted with a 10 cc pipet and centrifuged for 4 min. at 1000 RPM. The pellet was dissolved in 8 cc of medium. After each subculture step, viable cells were counted using the trypan blue exclusion test. The solution was plated at a 1:4 expansion ratio on coated dishes. After 3 days of cultivation, we used an MTT assay to demonstrate cell viability in cells between the 2^{nd} and 5^{th} passage. The dehydrogenase activity in the mitochondria of living cells converts the yellow MTT substrate (dimethylthiazol-diphenyltatrazolium bromide-eluted stain assay) into purple formazan. We measured the number of viable cells by optical density at a wavelength of 540 nm. We also compared both dispase II and trypsin 0.0625% in their efficiency to remove BCKs from the coated dishes. Both solutions were used at room temperature. The dispase II was freshly prepared and sterile filtered. The trypsin was stored at 4 C. for less than 2 weeks. We compared the time required to release 50 % and 90 % of the cells from the coated dishes and the growth potential after reseeding the cells after passing at a 1:4 rate. Clonal formation, migration and proliferation as well as cell morphology were compared. The time required to reach confluence was also examined.

2.3. Cultivation of cryopreserved BCKs.

Previously, a split thickness skin graft was harvested from a 3-week-old Yucatan pig and processed for isolation and cultivation of BCK's following above mentioned protocol. The cell suspensions (10x10E6 cells per vial) were solved in 10 % DMSO (Dimethyl sulfoxide, Sigma) and gradually frozen at a rate of 1 C per minute in the –70 C. After 2 hours the tubes were transferred into the liquid nitrogen tank. For recultivation we thawed the tubes gradually and the contents were transferred to a culture flask. Enriched medium was added drop wise. After centrifuging for 2 min. the cell pellet was solved into fresh enriched medium and seeded 1:3.

After 2nd till 5th passage, we determined cell viability by Trypan blue and MTT tests after 2-5days.

2.4. Media.

We analyzed growth parameters of BCKs in 4 different media (Table 1). All cell cultures were passaged 5 times. We compared 4 different growth media. KGM was our standard medium for basal cell keratinocyte cell suspension cultures and was modified to favor KC migration rather than proliferation. KGM II, Optimem and SFM are 3 growth media developed to be used with additives in reduced serum conditions. Since calcium is an important trigger of KC differentiation, we used similar calcium concentrations in all media, except for Optimem, which contains a higher calcium content. We depleted the FBS of Ca2+ ions with Chelex 100, a weak cation chelating 1 % resin (cat.142-2842, Bio-Rad). We allowed the serum to incubate undisturbed for 30 minutes and recovered it by filtration through a 0.22 um filter (Corning 430521), aliquotted and stored the serum at - 20 C. The calcium concentration was determined by mass spectroscopy. All media received FBS derived from the same Chelex 100 treated batch.

We seeded BCKs on both collagen I and fibronectin I coated 60 mm dishes (Becton Dickenson, cell ware).

KGM *(Clonetics/BioWhittaker):*	*KBM + BPE, hEGF, Insulin, Hydrocortisone, GA. 8 % chelex depleted serum was added.*
KGM II *(Clonetics/BioWhittaker):*	*KBM II + BPE, Insulin, Hydrocortisone, Epinephrine, Transferrin, GA-1000. 2.5 % chelex depleted serum was added.*
Keratinocyte-SFM *(Gibco/Invitrogen):*	*basal medium + L-Glutamine, EGF, BPE 2.5 % chelex depleted serum was added.*
Opti-MEM I *(Gibco/Invitrogen):*	*with Glutamax , Hepes, NaHCO3. 2.5 % chelex depleted serum was added.*

BPE: bovine pituitary extract, hEGF: human epidermal growth factor,
GA: gentamycin/ampicilline

KGM	No Ca++	8 % FBS has ~ 0.075 mM	0.0375 mM added → ~ 0.10 mM Ca
KGM II	No Ca++	2.5 % ~ 0.02 mM	0.0750 mM added → ~ 0.10 mM Ca
SFM	< 0.1 mM	2.5 % ~ 0.02 mM	No Ca added → ~ 0.10 mM Ca
Opti-MEM	~ 0.9 mM	2.5 % ~ 0.02 mM	No Ca added → ~ 1.10 mM Ca

Table 1 *Four distinct cell media for the cultivation of porcine basal cell keratinocyte cell suspension cultures.*

2.5. Transfection protocol

All transfection protocols were performed after the 3rd passage (1:3), when cell cultures reached a confluence rate of 50-60 %. For Fugene, Fugene HD and Lipofectamine, transfection was performed at 80 % confluence as recommended by the manufacturer.

2.5.1 -First transfection experiment.

Transfection reagents Fugene (Roche Applied Sciences) was used at room temperature. Lipofectin (Invitrogen) and Lipofectamine 2000 (Invitrogen) were preserved on ice. Plasmid DNA (pcDNA3.1-LacZ) was stored on ice until further use.

Lipofectin transfection.

Keratinocyte cell cultures were transfected following the recommended protocol. We used a 4:1 ratio (µL Lipofectin transfection reagent : µg plasmid DNA). Per 100 µl medium 1 or 2 µg DNA were used. Briefly, plasmid DNA and Lipofectin were solved separately in 200µl Waymouth medium. The 2 mixtures were mixed carefully and incubated at room temperature (RT) for 45 minutes. After gentle mixture, we added the DNA solution to the lipofectin droplet wise and incubated for another 15 minutes. After aspiration of culture medium and rinsing with 1X PBS, we added 1 mL of the DNA:Lipofectin solution per culture dish and added serum and AB-free basal medium to an end volume of 5 cc. After a 6 hour incubation, the medium was replaced with enriched medium and the medium was changed the following day.

Fugene transfection protocol.

Fugene 6 is a non-liposomal multicomponent reagent with very low cytotoxicity. To optimize results, transfection was performed on cells at 80 % confluence, as recommended by the manufacturer for adherent cells. 3 ul of Fugene 6 was added directly to 97 ul of serum-free, AB-free Opti-MEM I , avoiding the tip of the pipette to touch the inside of the tube as Fugene adheres to plastic. After 5 min. of incubation at RT, 1 ug of effector DNA was added to the mixture dropwise. After gently tapping the tube, the DNA/FuGene complex was further incubated for 15 min. After removal of cell supernatant, the Optimem/DNA/FuGene mixture was added to the cells around the dish, swirled well and further incubated overnight at 37°C. After 8 hours, the medium was replaced.

X-gal staining of the Lac Z gene

Medium was removed from the culture plates and rinsed with PBS. BCKs were fixed with fixative solution (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 10 minutes at RT.

For 2.5ml staining solution, 25µl solution A (400mM potassium ferricyanide), 25µl solution B (400mM potassium ferrocyanide), 25µl solution C (200mM magnesium chloride), 125µl 20mg/ml of the chromogenic substrate X-gal in N-N-dimethylformamide was combined in PBS. After fixation, the plates were rinsed twice with PBS, the staining solution was added to the keratinocytes and incubated for 1.5h at 37°C. The KCs were examined under a microscope (20x magnification). Total cells and blue cells were counted in 8 random fields and averaged to estimate TEE.

EGF Elisa of cell supernatant.

We determined hEGF-expression by a hEGF-specific enzyme-linked immunosorbent assay (ELISA) in cell supernatant. Anti-hEGF monoclonal Ab (R&D), anti-hEGF polyclonal Antibody (Santa-Cruz), and an HRP-goat-anti-rabbit polyclonal antibody were used as primary, secondary and tertiary antibodies, respectively. Results were evaluated by a V Max Kinetic Microplate Reader (Moleculare Devices, Sunnyvale, CA) at 450 nm with SoftMax Pro Software (v. 4.0). Results were averaged for each group and compared graphically.

2.5.2 -Second transfection experiment.

Transfection reagents Fugene HD (Roche Applied Sciences) and Effectene (Qiagen) were used at room temperature. Lipofectin (Invitrogen) and PEI (Polysciences Inc.) were preserved on ice. Plasmid DNA (pcDNA3.1-LacZ and pGFP) was stored on ice until further use.

Fugene HD transfection.

FuGENE HD transfection reagent is a non-liposomal multicomponent reagent. Keratinocyte cell cultures at 80 % confluence were transfected following the recommended protocol, using ratios of 5:2 and 8:2 (µl Fugene HD transfection reagent:µg plasmid DNA) and varying concentrations of DNA (1µg and 2µg). Briefly, plasmid DNA was dissolved in 100µl serum-free and AB-free Opti-MEM I. Fugene HD was added, mixed and incubated for 15 minutes at room temperature. The transfection complexes were subsequently added droplet-wise to the cells and left in the transfection medium until the time of analysis.

Effectene transfection.

BCKs were transfected following the recommended protocol, using ratios of 10:1 and 25:1 (µl Effectene transfection reagent:µg plasmid DNA) and varying concentrations of DNA (1µg and 2µg). The kit provides the necessary Effectene transfection reagent, an optimized buffer EC and an Enhancer. Briefly, plasmid DNA was dissolved into 30µl or 60µl buffer EC and a 8-fold higher concentration of Enhancer. The complex was vortexed for 1 second and incubated at room temperature for 4 minutes. Next, Effectene was added to the diluent, mixed for 10 seconds and incubated for 7 minutes at room temperature. 500µl growth medium was added to the transfection complexes, mixed and immediately added in a drop-wise manner to the keratinocytes. We incubated the cells overnight and replaced the growth medium in the morning.

Lipofectin transfection.
Keratinocyte cell cultures were transfected following the recommended protocol, using ratios of 6:2 and 9:2 (µl Lipofectin transfection reagent:µg plasmid DNA) and varying concentrations of DNA (1µg and 2µg). Briefly, plasmid DNA was dissolved in 200µl serum free Waymouth medium. Simultaneously, Lipofectin was dissolved in 200µl Waymouth medium. The 2 mixtures were mixed carefully and incubated at room temperature for 45 minutes. The DNA solution was carefully added to the Lipofectin solution, mixed well and incubated for another 15 minutes. The complexes were added in a drop-wise manner to the keratinocytes, incubated overnight and replace with new growth medium.

PEI transfection (Polyethylenimine).
Keratinocyte cell cultures were transfected following the recommended protocol, using ratios of 4:1 and 10:1 (µl PEI transfection reagent:µg plasmid DNA) and varying DNA concentrations (1µg and 2µg). Briefly, plasmid DNA was dissolved in 400µl serum free Waymouth medium. PEI was added, mixed and incubated for 10 minutes at room temperature. The transfection complexes were subsequently added drop wise to the KCs. Cells were incubated overnight and replaced with new growth medium the following day.
X-gal staining of the Lac Z gene was performed following a previously mentioned protocol.

GFP-FACS analysis.
Keratinocyte growth medium or transfection medium was removed from the culture plates and rinsed with PBS. Keratinocytes were trypsinized for 7 minutes at 37°C. The suspension was centrifuged for 5 minutes at 1500RPM at 4°C. The cell pellet was washed with PBS once and centrifuged. Next, the pellet was dissolved in 500µl PBS and 0.5% BSA and transferred to specific FACS tubes. The cell pellet solution was filtered through a mesh membrane and analyzed by FACS analysis.

3. Results

3.1. Basal cell KC suspension cultures and enriched media (Fig 1-4).

The KGM medium enriched with 8% FBS (Clonetics/Biowhittaker) served as the standard in this study. Smaller FBS amounts reduced proliferation rates. When only 2.5% FBS was added in KGM, the BCKs displayed poor migratory and proliferative capacity. When subculturing BCKs in KGM 8%, good clonal capacity and stable morphology was seen: cells grown into KGM were cuboid in shape with 'cobblestone-morphology', forming regular colonies with strong intercellular connections (Fig 1). A significant number of transient amplifying cells were present in primary- and subcultures. The BCKs grown in KGM were easily released from collagen-1 coated dishes using dispase II in a 0.025% concentration or a short incubation (3-5 min) with trypsin 0.0625%. Longer trypsinization times resulted in a diminished percentage of viable cells. When more than 90% confluent dishes were subcultured using trypsin, cell loss was more than 30%.

KGM-II (Clonetics/Biowhittaker) can be used as a low serum medium, so we added 2.5% depleted FBS to the KGM-II and added CaCl to a final concentration of 0.100 mM.

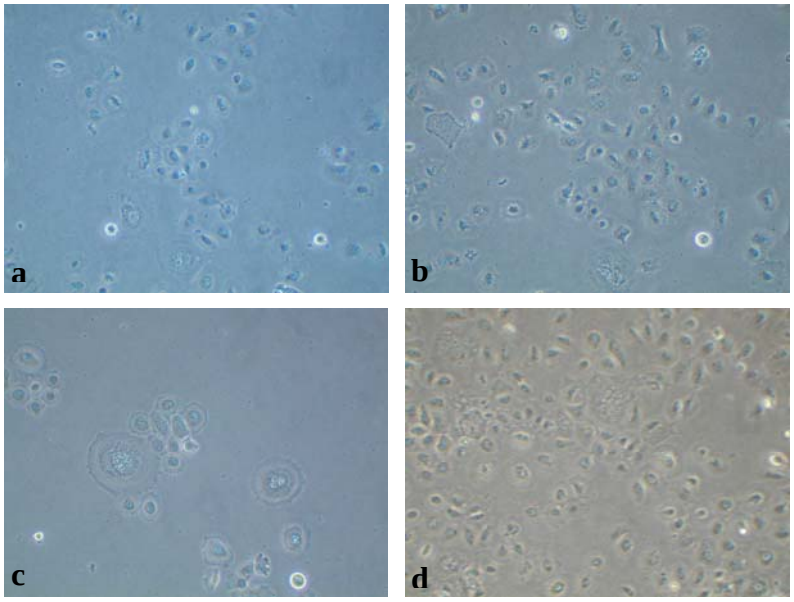

Fig. 1 Subcultures 24 hours post-seeding (1:4) after aspiration of non-attached cells and submersion in: a: KGM 8 % b: KGM-II 2.5 % c: Optimem I 2.5 % d:SFM 2.5 %.

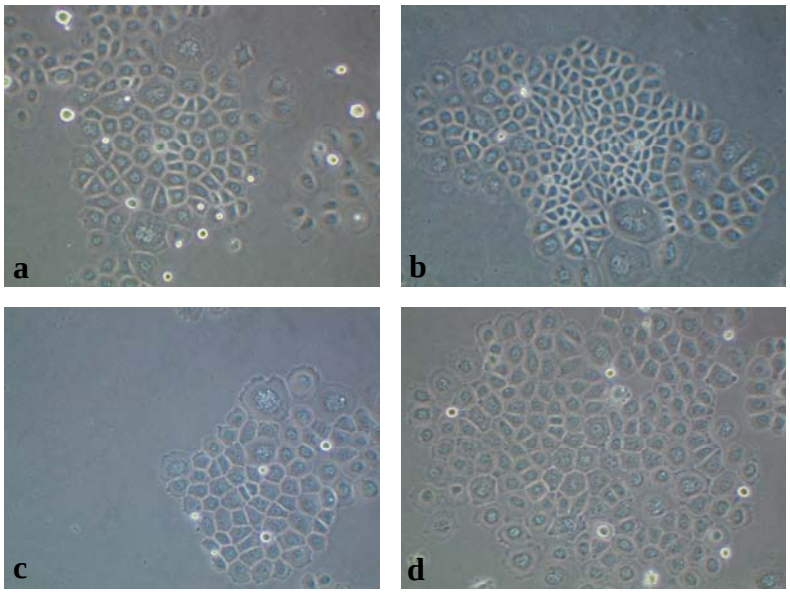

Fig. 2 Subcultures 48 hours post-seeding in: a: KGM 8 % b: KGM-II 2.5 % c: Optimem I 2.5 % d:SFM 2.5

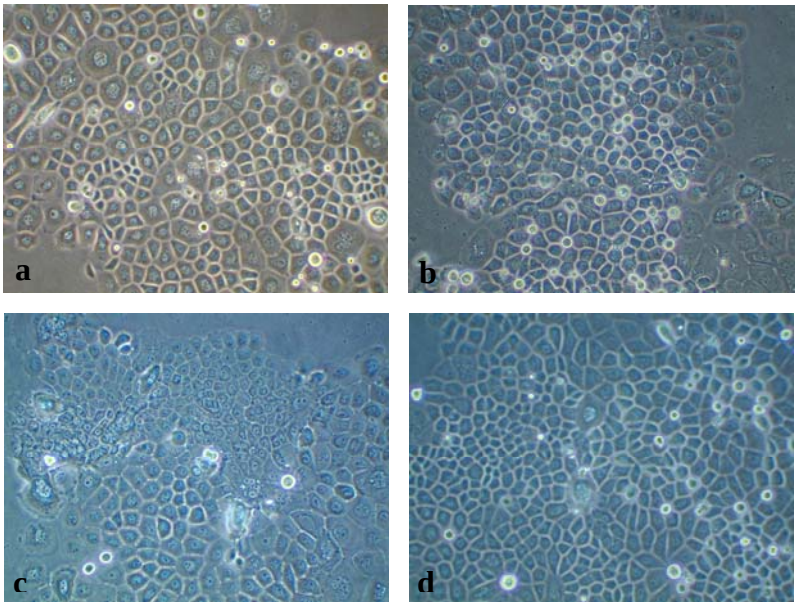

Fig. 3 Subcultures 72 hours post-seeding in:
a: KGM 8 % b: KGM-II 2.5 % c: Optimem I 2.5% d: SFM 2.5%

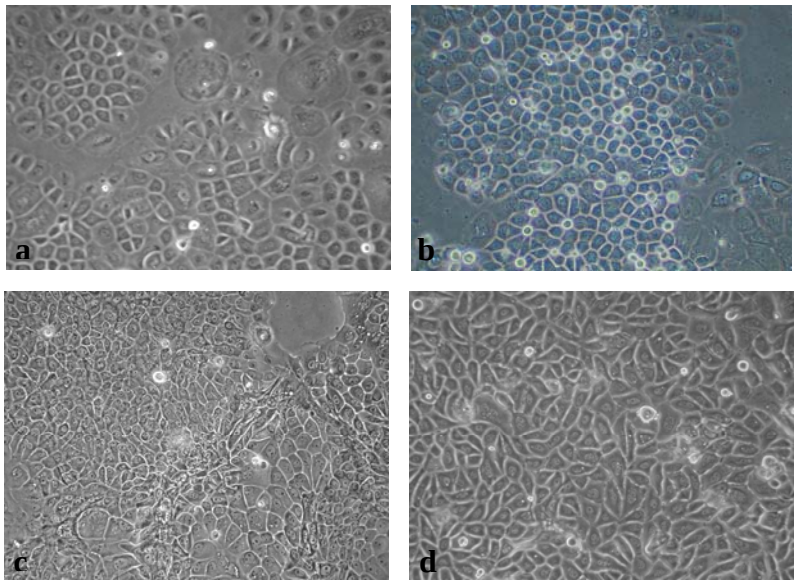

Fig. 4 Subcultures 96 hours post-seeding in:
a: KGM 8% b: KGM-II 2,5% c: Optimem I 2,5% d:SFM 2,5%

Porcine KCs grown in this medium proliferated and migrated well. However, porcine BCKs grown in KGM-II had a tendency to differentiate faster when subcultured, even in lower serum and similar calcium concentrations. The number of transient amplifying cells (TACs) was slightly higher than in KGM ($p<0.05$).

KGM-II grown cells can be easily released from the coated dishes with dispase II in a 0.025% concentration or short trypsin 0.0625 % incubation. A further reduction in FBS diminished the proliferation rate of both young and adult pig originated BCKs.Optimem I (Gibco-Invitrogen) with glutamax, hepes, NaHCO3 is a medium used in some keratinocyte culture protocols as a low-serum medium.

Previously, we used Optimem I as a medium for cationic liposome mediated fibroblast transfection. Optimem I has a high calcium concentration (~ 0.9 mM), which in combination with 2.5% serum, induced differentiation and poor proliferation especially in older BCKs. We discontinued using Optimem as basal serum-reduced medium due to the induced differentiation seen in primary and sub-cultures, which is unwanted in single cell suspensions.

Keratinocyte-SFM (Gibco/Invitrogen) is a basal medium with L-Glutamine, EGF and PBE. Cell migration and proliferation was significantly faster than the other media used in the test. After 3-4 days, primary cultures were consistently confluent when passaged 1:4. After primary culture and subculturing, porcine BCKs grown in SFM 2.5% had high clonogenic and migrative capacities. When FBS was fully omitted, the proliferative capacity of the porcine BKCs was significantly less (P,0.05).We verified cell viability and cell morphology using dispase II 0.025% versus trypsin 0.0625% until all cells were released from the cell ware. For cells grown in SFM medium, it took 32 minutes (mean) for dispase II and 13 minutes (mean) for trypsin to yield 95% cell detachment. Subsequent cell subcultures treated with trypsin had cell survival rates of > 90%, whereas the dispase II-released cells only had a 50% survival rate.

Finally, when comparing fibronectin versus collagen I coated dishes, no significant differences were demonstrated in cell viability, morphology, migration and proliferation, subculturing and regrowth after NO2 freezing.

3.2. Transfection of keratinocyte cultures

3.2.1 -First transfection experiment (Fig 5).

The PcDNA4/TO/Lac Z plasmid was transfected into young cryopreserved BCKs and in primary BCK cultures derived from a 4-month old Yorkshire donor. Both cell groups tolerated the Lipofectin/DNA/KBM mix well with equal amounts of cell survival (87 % and 84 %, $p > 0.05$). X-gal staining demonstrated a TEE of 14-25 % for young BCKs and 8-16 % for the 4-mo-old BCKs. There was no significant difference in TEE between cells cultured in KGM versus SFM; in both media however, young cells had higher TEE than older cells ($P< 0.05$). The visibly damaged cells recovered faster in SFM medium as compared to KGM. The first passage cell cultures after transfection demonstrated faster proliferation and migration in SFM 2.5 % relative to KGM 8 %. In the next step, we investigated different liposomal formulations using SFM 2 % as enriched cell growth medium.

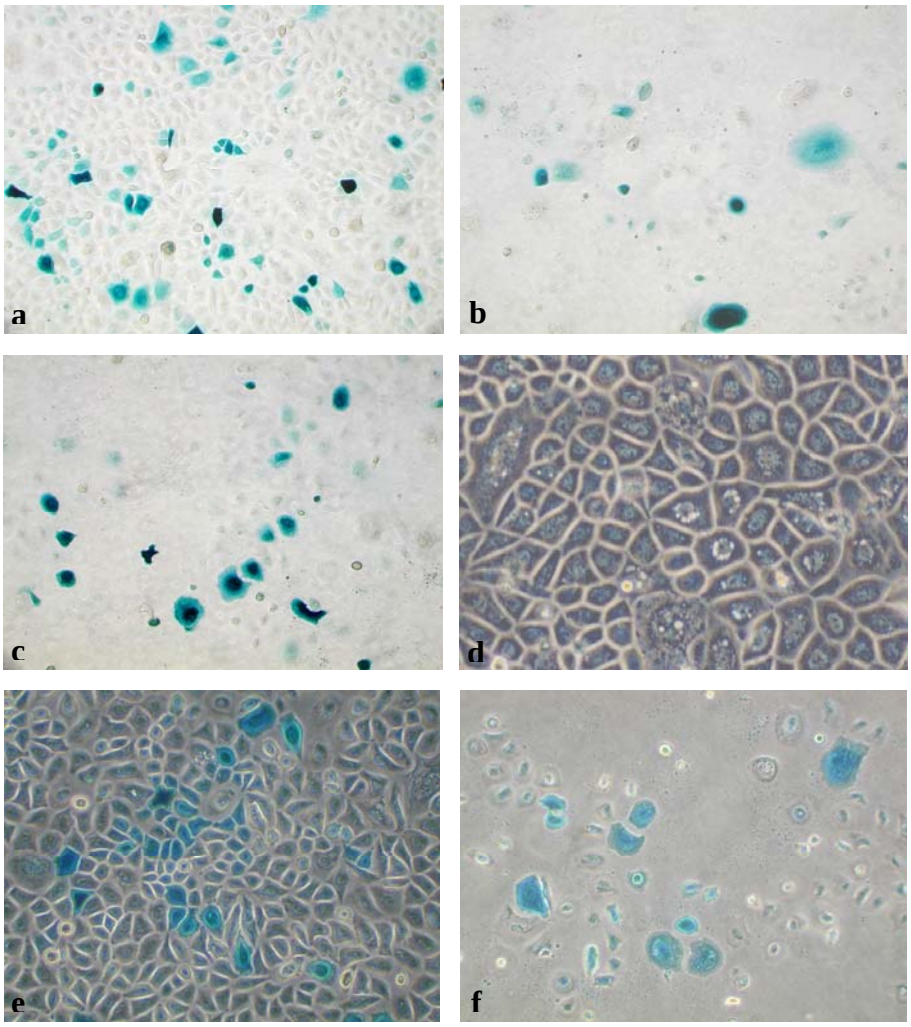

Fig. 5 Transfection of basal cell KC suspension cultures derived from a skin graft of young (a-e) and old Yucatan pigs.Gene transfer of Lac expression plasmid mediated by:a/ Lipofectin b/ Lipofectamine 2000. c/ Fugene.
d-f: d/ non transfected KCs derived from skin graft of baby Yucatan pigs e/ Lipofectin mediated transfer of Lac Z into young BCKs. f/ Lipofectin mediated gene transfer of Lac Z in KCs derived from aged Yucatan pig. Post-transfection stress is hardly seen in baby KCs, while obvious in aged cells.

3.2.2 - Second transfection experiment (Fig.6)

In this experiment, we compared Lipofectin to novel transfection agents Effectene, Fugene HD and PIE. Although some of these novel reagents may also be used under serum enriched conditions, serum-free basal medium was used in all transfection protocols. For each agent, 4 different DNA:lipid ratios were tested, as directed by the manufacturer's instructions. Lac Z and GFP were used as reporter genes. Fugene HD consistently performed best with highest TEE when 1 ug DNA was used in a 2:8 or 2.5 ratio. PEI also demonstrated high transfection rates with 2 ug DNA in a 1:4 ratio. Effectene had significantly lower TEE. Lipofectin had the lowest mean TEE using this cell line.

Reagent	[DNA]	DNA:Lipid	LacZ (%)	GFP (%)
Effectene	0.1 µg	1:10	4.8 [2.8-6.3]	3.40
	0.1 µg	1:25	8.1 [5.3-9.1]	6.00
	0.2 µg	1:10	8.7 [5.4-9.5]	6.80
	0.2 µg	1:25	10.2 [4.5-12.2]	9.05
Fugene HD	1 µg	2:5	14.8 [12.1-16.9]	---
	1 µg	2:8	38.1 [34.1-45.8]	19.30
	2 µg	2:5	27.4 [13.9-32.4]	18.65
	2 µg	2:8	9.9 [6.1-11.0]	8.45
Lipofectin	1 µg	2:6	0.6 [0.3-1.1]	3.50
	1 µg	2:8	1.5 [0.4-2.2]	3.45
	2 µg	2:6	1.4 [0.6-2.4]	5.30
	2 µg	2:8	2.6 [1.7-4.3]	3.65
PEI	1 µg	1:4	14.2 [8.9-18.3]	11.75
	1 µg	1:10	8.4 [6.9-9.7]	9.90
	2 µg	1:4	15.7 [10.6-16.7]	12.15
	2 µg	1:10	9.3 [6.8-12.1]	10.40
No Transfection	---	---	0.0 [0.0-0.0]	0.10

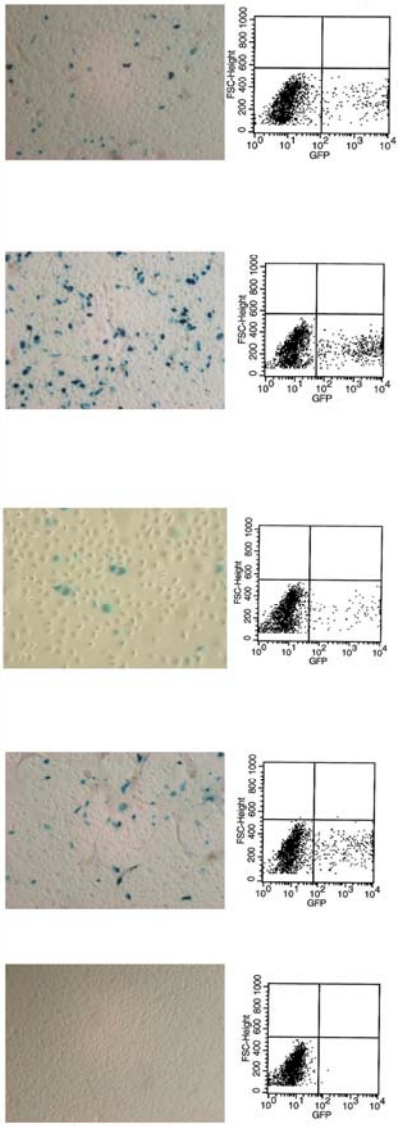

Fig. 6 Transfection efficiency for reagents Effectene, Fugene HD,Lipofectin and PIE under SFM 2.5% serum reduced growth conditions. (Left Column)For each agent, 4 different conditions were tested. Mean and absolute
Lac Z and GFP expression. (Middle Column) Lac Z staining shows best outcome with Fugene HD. (Right Column) FACS analysis of GFP expression confirms the Lac Z outcome.

4. Discussion

4.1. Cultivation Assays of basal cell keratinocytes

FCS (fetal calf serum) or FBS (fetal bovine serum) had originally been reported to be an absolute requirement in all 3T3 models, with an optimal concentration near 10% but with significant batch to batch variation in growth promoting activity[11]. However, reduction or even elimination of serum in cell-media is obligatory in translational research: FCS and FBS have significant physiological variability and contain a wide range of minor components that may have a considerable effect on cell growth. Serum also has a short shelf life and poor consistency and may be contaminated with viruses that interfere with cell growth.

FBS is also banned in several cationic liposome-mediated gene transfer protocols. On the other hand, serum-proteins also have protective and detoxifying actions and the search for serum-free functional media may require different formulations to replace the growth enhancing capacities of serum[12,13]. Although improving steadily, the availability of properly controlled serum-free media is limited and often more expensive than the conventional media[14]. Rosdy et al. demonstrated that the cultivation of cells and development of a reconstructed epidermis could be produced without serum and dermal factors[15]. We tested growth parameters in cell suspension cultures of cryopreserved young and adult BCKs under reduced serum conditions. Human BCK thrive well in serum free SFM (data: Keratinocyten Bank, UZ-Gasthuisberg, Leuven,Belgium), but porcine BCKs thrived best in 2.5% FBS culture conditions during primary culture. After the 3rd passage, porcine BCKs also proliferated well in serum-free culture condition.

MTT assays and trypan blue exclusion tests demonstrated high numbers of attached cells after passage 1, 2 and 3 in all media used. However, the migration rate was highest in SFM 2.5% followed by our previous standard KGM 8%. The larger degree of differentiation in KGM II and Optimem may be the reason for diminished migration rates and lower viability scores after passaging.

The regular cuboid shaped morphology of KGM 8% was mimicked by a slightly flatter morphology of SFM 2.5% cultivated BCKs. In Optimem and KGM II, cells displayed irregular sizes and stages of differentiation.

Epidermal cells have been reported to be extremely sensitive to the toxic effects of antibiotics, particularly gentamycin and fungizone[16]. A 1% Penicilin/Streptomycin solution in the culture medium did not have an influence on colony forming efficiency and cell migration.

Both dispase and trypsin are used to separate the epidermis from the dermis. Dispase II is a thermolysin-like neutral protease. A 0.5 % dispase II solution separates the epidermis from the dermis in 1-2 hours when skin grafts were harvested at 0.015-0.02 inch. Green et al. reported that the detachment of KC sheets by dispase II had virtually no effect on cell viability[17]. Boucher et al. studied the effect of storage of dispase-detached cultures on proliferative potential and adhesion of KCs and demonstrated that with sufficient time and concentration, even neutral proteases such as Dispase II could damage cell surface proteins. Therefore, we prefer a low but efficient 0.5% solution to separate the epidermal from the dermal layer, instead of higher concentrations as presented in literature.

Trypsin is an aggressive serine protease that cleaves peptide bonds between lysine and arginine. A Trypsin/EDTA solution disrupts the intercellular junctions by calcium chelation and provides proteolytic degradation of the intercellular matrix[14]. EDTA (ethylenediaminetetraacetic) is a chelating agent added to Trypsin to enhance its activity by removing calcium and magnesium from the cell surface. The metabolic activity is strongly influenced by temperature. Cell distress by trypsinizing can be avoided by using low trypsin concentrations of 0.05-0.0625% or 'cold trypsinization' at room temperature or 4C and adding EDTA.

Several reports indicate that short incubation with trypsin/EDTA leads to higher post-seeding proliferation than when using trypsin without EDTA, thermolysin or dispase[19,20] . When using SFM 2.5% as basal enriched serum –reduced medium for porcine BCKs, trypsin/EDTA 0.0625% was more efficient, consistent and safer than dispase II in the passaging steps. Shorter incubation times were required than with dispase and significantly higher viable cell rates were measured.

Moreover, short incubation of BCKs by proteases such as trypsin may even induce an episode of proliferation. Meyer-hoffert et al. demonstrated increased KC proliferation by activation of the protease-activated receptor PAR-1, which is expressed by KCs[21]. Trypsin activates PAR-1.

Therefore, we only use dispase to separate the epidermis from the dermis during primary culture.All other cell separation steps for BCKs are performed with tyrpsin/EDTA 0.0625%.

Cultures of irradiated human or mouse cells have been used since the 1970s to promote proliferation, particularly with low-density inocula[22]. High energy irradiation can completely suppress cell division long before general metabolism is affected. Since an irradiated "feeder" cell population continues to metabolize, the non-dividing cells provide diffusible and short-lived growth plus-conditioning factors to the medium. Human KCs can be cultured and serially passaged without a fibroblast feeder layer using a defined low calcium environment and altering seeding density, pH and incubation conditions[23,24] .We used collagen I or fibronectin coated dishes to grow porcine BCKs in a standardized fashion. Fibronectin exists in multimeric form in the ECM and on cell surfaces and plays an elementary role in the cross-talk between fibroblasts and keratinocytes[25,26]. The primary function of fibronectin is cell adhesion to the ECM through an interaction of the cell binding domain with fibronectin-specific cell surface receptors[27]. We found no significant difference in cell migration and colony forming units of cell cultures seeded on fibronectin coated dishes versus the collagen coated dishes. Both collagen and fibronectin are not a requirement for epidermal growth and maintenance, but they may *select* for attachment of basal cells and discourage attachment of differentiated cells[28] .

High calcium and serum concentrations induce differentiation, which results in extensive desmosomal cell attachment and stratification, and are associated with the expression of a variety of differentiation protein markers[29,30]. Ca2+ seemed to be most important in determining whether a colony would form rather than how rapidly a colony would expand. Hennings et al. have shown that lowered medium calcium concentration can significantly prolong the cultured lifespan of epidermal cells by shifting the pattern of growth and differentiation in favor of continued proliferation[31]. The role of calcium in regulating growth is permissive. If no other condition is limiting, calcium is mitogenic below 0.3 mM but it neither stimulates nor inhibits growth above 0.3 mM. The same authors concluded that there is an inverse relationship between growth response (extent of clonal growth) and the differentiation response (extent of differentiation). Factors that activate the differentiation response in growth-arrested cells are ineffective when cells are actively proliferating[32].

4.2. Transfection protocols

Gene therapy can be used to selectively elevate or down regulate expression of a particular growth factor in the wound milieu. This practice is promising in promoting tissue repair, especially for wound healing, which is a local event and requires only transiently high levels of transgene expression[33]. Skin is a useful target for gene therapy not only because of its accessibility and abundant vascularity but also for its regenerative capacity[10,34]. Several methods exist to introduce selected genes into tissues. Viral transduction uses attenuated viruses to achieve a high 'transduction' efficiency[35]. Thus far, non-viral methods attain much lower 'transfection' efficiencies, but there is no risk of virus-related morbidity[36].

Lipofectin is a synthetic cationic lipid polymer, which fuses with mammalian cell membranes. In previous experiments, we found that cytotoxicity is large in cells derived from aged pigs. After a 5 hour incubation, these cells are markedly stressed with high losses during subsequent passaging. In young cells, however, no phenotype changes were observed, even after overnight incubation. In our BCKs under serum reduced conditions, Lipofectamine 2000 and Fugene 6 lead to inferior transfection rates as compared to Lipofectin. We increased the time for complex formation to two hours following the manufacturer's recommendation and -because of low cytotoxicity- we increased the amount of both Fugene 6 reagent and DNA (4 ug). Neither step led to higher transfection rates in these adherent BCKs. Kiefer et al. found minimal TEE for Fugene 6 in serum reduced media, but high efficiency in serum containing medium[36]. However, our aim was to focus on high tranfection efficiency in serum-reduced conditions.

In a subsequent set of experiments, we compared Lipofectin with the newer transfection reagents, Fugene HD, Effectene and PIE, for ex vivo gene transfer of BCKs with LacZ and GFP as reporter genes. The main advantage of GFP expression is the visualization of living cells. However, when the cell cultures are embedded for further analysis, fluorescence is often lost. Lac Z staining is stable upon mounting, but cells must be fixed for staining.

Effectene may be used with serum-free or serum-containing media and only a small amount of DNA is required. Zellmer et al. found a 20-fold higher transfection efficiency of Effectene compared to Lipofectin and Lipofectamine in human keratinocytes[37]. Using our BCKs, TEE was higher than with the previous Lipofectin based standard protocol, but consistently lower than with Fugene HD. PEI is an epithelial and endothelial cell directed transfection reagent (polyethylemimine derivative) with high efficiency and low cytotoxicity and may be used in the presence of serum and antibiotics[38] . In our porcine BCKs, PEI-based transfection resulted in higher efficiency relative to Lipofectin. Cross-linking small PEIs with judiciously designed amide- and ester-bearing linkers may boost their gene delivery efficiency both in vitro and in vivo and might be promising for future applications[38]. Fugene HD is a next generation non-liposomal multicomponent reagent suitable for mammalian cell transfection for protein expression. Fugene HD may also be used with or without serum. Its complexes interact with nucleic acids and promote efficient transfer across cellular membranes with minimal physiological and morphological cell changes and minimal cytotoxicity.

In our BCKs, Fugene HD consistently resulted in the highest TEE using different plasmid concentrations and in different ratio's DNA:Fugene HD. These transfected BCKs kept their viability and proliferation capacity after all passages. We measured GFP expression for 2 weeks. By further optimizing the transfection methods, with respect to time of transfection, ratio of cells to lipid:DNA complexes and the ratio of lipid:DNA, TEE may be further enhanced.

Novel approaches such as Nucleofection, a electroporation-based transfection method that enables the DNA to enter directly the nucleus, are promising for non viral transfection in ex vivo gene transfer protocols in serum reduced conditions[39].

Conclusion

Cell suspension cultures of basal cell keratinocytes (BCKs) had the highest clonogenic capacity in SFM medium enriched with 2.5 % serum. In these serum reduced and low calcium conditions, BCKs migrated with high proliferation rates. Fugene HD with 2 μg of reported DNA led to the highest non-viral transfection efficiency in these serum reduced growth conditions, compared to Effectene ,PEI , Fugene 6, Lipofectamine 2000 and previous standard lipofectin.

This *ex vivo* gene transfer protocol represents an efficient basic platform for further gene transfer in tissue engineering protocols under serum-reduced conditions.

Correspondent author:

Jan Jeroen Vranckx, MD,FCCP.
Dept. Plastic & Reconstructive Surgery
KULeuven University Hospitals
Leuven, Belgium.
32-16-348721 tel 32-16-348723 fax
Jan.vranckx@uz.kuleuven.ac.be

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For a true writer each book should be a new beginning,
where he tries again for something that is beyond attainment.
He should always try for something that has never been done or that others
have tried and failed. Then sometimes, with great luck, he will succeed.
Ernest Hemingway

Part 4 - Growth factor delivery to full thickness wounds by ex vivo gene transfer

- *Ex vivo gene transfer using cells and genes.*

"Cell suspension cultures of allogenic keratinocytes are efficient carriers for ex vivo gene transfer and accelerate healing of full-thickness skin wounds by overexpression of hEGF."
Vranckx, Jan Jeroen, MD; Hoeller, Daniela, MD; Velander, Patrik E.M. MD; Theopold, Christoph F.P., MD; Petrie, Nicola, MD; Takedo, Akira, MD, Eriksson, Elof, MD, PhD and Yao, Feng, PhD
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Cell suspension cultures of allogenic keratinocytes are efficient carriers for ex vivo gene transfer and accelerate healing of full-thickness skin wounds by overexpression of hEGF.

Vranckx, Jan Jeroen, MD[1,2]; Hoeller, Daniela, MD[1,3]; Velander, Patrik E.M. MD[1]; Theopold, Christoph F.P., MD[1]; Petrie, Nicola, MD[1]; Takedo, Akira, MD[1], Eriksson, Elof, MD, PhD[1] and Yao, Feng, PhD[1]

[1] *Laboratory of Wound Repair and Gene Transfer, Div. Plastic Surgery, Brigham & Women's Hospital, Harvard Medical School, Boston 02115, MA, USA.*
[2]*Laboratory of Plastic Surgery & Tissue Engineering Research KUL Leuven University Hospitals, UZ-Gasthuisberg, Leuven 3000, Belgium*
[3]*Dept.of Dermatology, University Clinic of the RWTH University Aachen, Germany*

Abstract

The concept of using growth factor therapy to induce wound repair has been endorsed in studies that show reduced growth factors levels in wound fluid from chronic and aged wounds. In this study, we used cell suspensions of allogenic keratinocytes as gene delivery vehicles for human epidermal growth factor (hEGF) and analyzed their impact on wound repair in a porcine wound healing model.

Full-thickness wounds were created on the back of six Yorkshire pigs and covered with a wound chamber to create a wet wound healing environment. First, 5x10e5 allogenic, autogenic or mixed keratinocytes were transplanted into wounds and healing parameters were analyzed. Secondly, we measured long-term re-epithelialization and contraction rates from day 8 until day 35. In the third experiment, allogenic keratinocytes were transfected with a hEGF-expressing plasmid pCEP4-hEGF and transplanted in full thickness wounds to improve repair.

Wounds treated with autogenic, allogenic or mixed keratinocytes demonstrated a significantly higher rate of re-epithelialization relative to saline-treated control wounds. Repetitive biopsies indicated that the use of allogenic keratinocytes did not lead to long-term wound breakdown. Wounds treated with hEGF-expressing allogenic keratinocytes re-epithelialized faster than wounds treated with allogenic keratinocytes or control wounds. With peak hEGF expression of 920.8 pg/mL, hEGF was detectable until day 5 after transplantation compared to minimal hEGF expression in control wounds.

This study demonstrates that allogenic keratinocytes can serve as efficient gene transfer vehicles for ex vivo growth factor delivery to full-thickness wounds and overexpression of hEGF further improves re-epithelialization rates.

Abbreviations:
PBS = phosphate-buffered saline
BW = body weight
FBS = fetal bovine serum
BSA = bovine serum albumin
hEGF = human epidermal growth factor
KBM = keratinocyte basal medium
SFM = serum-free medium
H&E = hematoxylin and eosin
ELISA = enzyme-linked immunosorbant assay
MHC = major histocompatibility complex

1. Introduction

Growth factors are omnipresent in each phase of the wound healing process. Cells that orchestrate the healing response produce them. Growth factors influence the growth rate of these cells when bound to specific receptors[1,2]. The advent of gene therapy has addressed many of the intrinsic restrictions of topical recombinant growth factor therapy to wounds and has enabled genetic material to be successfully introduced into cells with the intent of altering protein synthesis to modify the healing response[3-5]. Gene therapy can offer targeted local and persistent delivery of de novo synthesized growth factors to the wound microenvironment over many days but also can be used to selectively elevate or downregulate expression of a particular growth factor.

We hypothesized that wound repair can be accelerated by the over expression of targeted exogenous growth factor genes into the wound microenvironment.

In a standardized full-thickness skin wound (FTW) model in young and old pigs, established in our laboratory, we analyzed the impact of *in vivo* and *ex vivo* gene transfer of growth factors such as EGF, KGF, PDGF, VEGF and TGF-ß$_1$ on wound repair[6-10].

In this study, we used *ex vivo* gene transfer mediated by cationic liposomes to transfect cell suspensions of porcine keratinocytes with human epidermal growth factor (hEGF). EGF is produced by platelets, keratinocytes, monocytes, and macrophages and is present in high quantities in the early phase of wound healing. EGF likely increases wound healing by stimulating the proliferation and migration of epithelial cells and mesenchymal cells[11,12]. However, topical application of recombinant growth factors to wounds encounters intrinsic problems such as short half-life of recombinant protein, thus requiring repetitive doses at frequent intervals with subsequent high cost and effort, irregular secretion profiles due to the bioavailability parameters of the utilized vehicle, and lack of proper posttranslational modifications that may impair its biological functions[13,14].

Ex vivo gene transfer of EGF-expressing allogenic keratinoctyes (AKC) may combine cell-based wound healing strategies and gene therapy for coverage of large chronic ulcers or extensive burn wounds, where donor sites are minimal and precious. Such gene-expressing allogenic cells could be cryopreserved in large numbers and stored until required. Once applied to wounds, AKC may contribute to healing by *de novo* expression of growth factors as well as by their direct involvement in epidermal regeneration as a temporary bioscaffold. In this study, we hypothesized that cell suspensions of AKC serve as efficient gene delivery vehicles for hEGF, further accelerate wound healing and lead to long-term repair of porcine full- thickness wounds in a wet wound healing environment.

2. Materials and methods

2.1. Harvest of split thickness skin graft for porcine KC cell cultures.

Consecutively, six 4-month-old female Yorkshire pigs (Parson's Farm, MA) entered the animal facility one week prior to surgery. Pigs were penned individually to minimize the risk of chamber disruption. The environment was set to a temperature range of 20°C, 65% humidity and a 12-hour on-off light cycle. Animals were fed 2 pounds of standard porcine diet per day and clean, fresh water was administered ad libitum. A custom-made mobile sling adjustable in height (Universal Metals, MA) was used to transport and hold animals during the initial operation and during daily maintenance of wound chambers and fluid[10].

Anesthesia was performed following a standard protocol: pigs were premedicated in the sling in the ventral decubitus position with 0.3 mg/kg body weight (BW) of ketamine and 2 mg/kg BW of xylazine through a flexible butterfly needle. Subsequently, the animals were induced with 5% isoflurane for 5 minutes through a snout mask. For procedures longer than 45 minutes, pigs were intubated with 6.5 mm intratracheal tubes in ventral decubitus.

During the operation, pigs received 1.5-2.0% isoflurane and 4 L O2. Pulse oximetry, heart rate (Datex Ohmeda sensors, Andover, MA) and rectal temperature were monitored throughout the procedure.

After hair removal (Nair microwave wax, Church & Dwight, Princeton, NJ), a split thickness skin graft was harvested from the neck of the pig using an electrical dermatome, set on a thickness of 0.0014 inch (Padgett Instruments, Integra Life Sciences, Plainsboro, NJ 08536). The donor site was covered with Tegaderm (3M, St Paul, MN 55144-1000).

After the surgical intervention, pigs were left to awaken in the sling while monitoring of the vital functions continued. After wakening, the pig was transported in the mobile sling to the cage. Twice a day 0.3 mg/kg buprenorphine was injected IM for pain relief. All animal procedures were approved by The Harvard Medical Area Standing Committee on Animals.

2.2. Cell cultures

Split thickness skin grafts were washed once in 70% ethanol and three times in sterile phosphate-buffered saline containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin (Invitrogen, Grand Island, NY) and 0.05 μg/ml amphotericin B (Bristol-Myers Squibb, Princeton, NJ), cut into pieces approximately 2 x 2 cm and left in a dispase solution (2U/ml, Roche, Indianapolis, IN) for 2 hours at 37°C, after which the dermis was peeled gently from the epidermis. Epidermal sheets were treated with 0.0625% trypsin/EDTA (Invitrogen, Grand Island, NY) for 10 minutes at 37°C.

The trypsin was neutralized by addition of one volume of fetal bovine serum (FBS, Sigma, St. Louis, MO). Following centrifugation at 700 rpm for 5 minutes, cells were resuspended in keratinocyte serum-free medium (SFM) Medium (Invitrogen, Grand Island, NY) containing 25 μg/ml bovine pituitary extract, 0.15 ng/ml recombinant EGF, 2% calcium-depleted FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.01 mg/ml gentamycin (Invitrogen, Grand Island, NY) and 0.05 μg/ml amphotericin B (Bristol-Myers Squibb, Princeton, NJ), seeded at a density of 9 x 10^6 keratinocytes per collagen I-coated 100-mm dish, and incubated in a CO_2 incubator (Model 2250, VWR, West Chester, PA) at 37°C in a humidified atmosphere with 5% CO_2. Cells were sub-cultured at 80% confluence.

The porcine keratinocyte cell line 4K3 was used as the source of AKC[15], cultured in SFM and sub-cultured at 80% confluence.

2.3. Transfection Procedure

Keratinocytes at passage 2 or 3 were seeded at a density of 1 x 10e6 cells/100 mm collagen I-coated cell culture dish 24 hours prior to transfection. Transfection was performed with Lipofectin (Invitrogen, Grand Island, NY) at a ratio of 1 μg DNA:4 μl Lipofectin. Each transfection was performed with either 5 μg of pCEP4-hEGF plasmid or 5 μg pCEP4-LacZ plasmid in a final volume of 1 ml of transfection reagent.

pCep4-hEGF is an episomal replicating plasmid that encodes the hEGF gene under the control of hCMV (human cytomegalovirus) major immediate-early promoter[29].

5 μg of DNA and 20 μl of Lipofectin were each incubated in 500 μl serum- and antibiotic free keratinocyte basic medium (KBM, Clonetics/Cambrex,Rockland,MA) for 45 minutes. The DNA solution was added in drops to the Lipofectin solution, incubated for another 15 minutes and added to the cells. After 5 hours of incubation at 37°C, the transfection medium was replaced by serum-free keratinocyte medium (SFM, Gibco/Invitrogen,Grand Island, NY) medium. Cells were incubated for another 60-66 h prior to transplantation. To determine hEGF expression, cell supernatant was collected daily, transferred on ice, and stored at –80 C^o until assayed.

2.4. X-gal Staining

48 hours after transfection, cells transfected with the LacZ-containing plasmid were washed twice with 1x PBS and fixed for 20 minutes with 0.25% glutaraldehyde. After washing with 1x PBS, cells were treated with 0.2% Triton X 100 for 20 minutes, washed again with 1x PBS and stained for 3-24 hours with 0.5 mg/ml X-Gal substrate (Fisher Scientific, Suwanee, GA) in X-Gal staining solution. Effectively transfected cells stained blue.

2.5. Experimental groups in the animal model

Three different sets of experiments were performed on 98 FTWs wounds. In the first set of experiments, 29 FTWs measuring 1.5 x 1.5 cm were created on the dorsum of two 4-month-old Yorkshire pigs. Wounds were transplanted with 5 x 10e5 of either allogenic (n=10) or autogenic (n=10) keratinocytes as a single cell suspension. As a control, a group of wounds were treated with saline only (n=9). Wound biopsies were performed on day 8.

In the second experiment, we investigated whether the rejection of allogenic cells would cause a breakdown of the wound and result in epithelial defects or clinical blister formation. 32 FTWs of 1.5 x 1.5 cm were created on the back of two 4-month-old Yorkshire pigs. Wounds were treated with either 5 x 10e5 AKCs (n=16) or saline (n=16). Wound biopsies were performed on post-operative days 8, 11, 14, 21, 28 and 35. Digital photography of individual wounds was used to assess clinical features such as colour, flexibility, hair growth, blister formation and desiccation.

The third set of experiments aimed at investigating the use of AKC as gene transfer vehicles for growth factors into the wound environment and their influence on wound healing. 36 FTWs of 1.5 x 1.5 cm size were created on the dorsum of two 4-month-old Yorkshire pigs.

Wounds were treated with 3 x 10e5 hEGF-expressing AKC (n=9), 3 x 10e5 untransfected AKCs (n=12) or saline as control (n=15). Wound biopsy specimens were taken at day 9 after the operation. To monitor viability of transplanted keratinocytes *in vitro*, the same amount of keratinocytes as transplanted into FTWs was seeded into 35-mm dishes. Cells were monitored for 3-5 days in vitro and medium from transfected cells was collected to determine levels of hEGF-expression after subcultivation.

2.6. Full-Thickness Wound Preparation in the animal model

Once a sufficient number of keratinocytes was cultured, the first operation (Day 0) was planned in the operation theatre under general anaesthesia as mentioned above. After cell count, using the trypan blue exclusion test, the cell suspension cultures were divided into individual 2cc syringes (one per wound) and transferred to the operation theatre.

Simultaneously, a second team anesthetised the pig. After anesthesia, waxing and skin preparation (cfr.supra), the pig's dorsum was prepped and draped. Square wounds measuring 1.5 x 1.5 cm were outlined with a surgical marker and template, and wound edges were retraced with a tattoo marker (Special Electric Tattoo Marker, Huck Spaulding Enterprises, Inc\., Voorheesville, NY), allowing planimetry of wound size and calculation of amount of wound contraction every third day.

FTWs were created with a # 11 blade at the inner margin of the tattoo mark to a depth of 1 cm, comprising a full-thickness skin layer down to the fascial plane. Following hemostasis with gentle bipolar coagulation, a thin layer of medical adhesive glue (Hollister, Libertyville, IL) was applied to the skin surrounding the wound and self-adhesive transparent vinyl chambers (TMED, Columbia, TN) were placed over each wound.

The cell suspensions were solved into serum- and antibiotic-free KBM and kept on ice in 2 CC syringes for transport to the OR theatre (see above). One syringe was used per wound. We injected the cell suspension cultures into each wound via the self-sealing injection pad incorporated in the chambers. Cells were allowed to settle for 2 hours, during which the pig was kept under anesthesia. At the end of the procedure, 1 ml of normal saline solution containing 100 U/ml penicillin and 100 μg/ml streptomycin was added to each wound. After recovering from the anesthesia, pigs were penned separately in custom-made, smooth-sided stainless steel cages. Pigs received 0.03 mg/kg BW buprenorphine IM every 12 hours as analgesia for one week following surgery.

2.7. Post-operative Follow-up

Every 20-24 hours post-surgery we collected wound fluids until the experiment was terminated. Pigs were premedicated in the sling and inhalation-anaesthesia was performed via the rubber self-sealing snout mask as mentioned above. During the intervention pigs received 1.5-2.0 % isoflurane and 4 L O_2. Pulse oximetry, heart rate and rectal temperature were also monitored throughout these short procedures. Where leaking, new chambers were applied and 1.5ml of normal saline solution containing 100 U/ml penicillin and 100 μg/ml streptomycin was added to each wound.

Wound fluid from each experimental group was pooled, transported on ice and stored at -80°C. Wounds were examined daily for signs of infection, such as redness and heat around the wound edges, putrid or cloudy wound fluid, elevated body temperature, or other illnesses. Every third day wound surface linings were traced for scanning and measurement by planimetry.

The last day of wound collection, pigs were euthanized after induction with isoflurane and oxygen, by IV injection of 20 mg/kg BW pentobarbital through a peripheral vein. Half cm wide full-thickness skin biopsies were harvested from each wound and placed in 4% formalin solution and sent to the Pathology Laboratory at the Brigham & Women's Hospital for routine histological processing and hematoxylin and eosin (H&E) staining.

2.8. Analysis of wound healing

Each H&E-stained biopsy slide was scanned (Epson Perfection 636 U with slide attachment, Epson America Inc., Long Beach, CA) into a computerized image (jpeg format) and analyzed with Paint Shop Pro (v. 7.0, Jasc Software, Eden Prairie, MN), allowing measurement of wound length, epithelial tongues and keratinocyte islands. The lateral margins of the wounds were histologically detectable by the tattoo mark. Starting at the lateral margin, length of epithelial tongues covering the wound surface was measured. The extent of reepithelialization was determined by dividing the sum of the length of epithelial tongues by the total length of the wound times 100 and expressed as a percentage.

To measure wound contraction, planimetric measures of each wound were scanned into 24-bit bitmap computer images (Epson,USA). Scion Image software (Scion Corporation, Frederick, MD) was used to measure the area of each wound tracing and a contraction percentage was calculated for each wound on each day, with day 0 as a reference.

2.9. Analysis of hEGF

We determined hEGF-expression by a hEGF-specific enzyme-linked immunosorbent assay (ELISA) in cell supernatant of keratinocyte cultures in vitro, as well as in the daily collected wound fluids in the animal model.

Anti-hEGF monoclonal Ab (R&D), anti-hEGF polyclonal Antibody (Santa-Cruz), and an HRP-goat-anti-rabbit polyclonal antibody were used as primary, secondary and tertiary antibodies, respectively. Results were evaluated by a V Max Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) at 450 nm with SoftMax Pro Software (v. 4.0). Results were averaged for each group and compared graphically.

2.10. Statistical Analysis

To determine the significance of hEGF expression, reepithelialization and wound contraction, variances within the data were analyzed with the F-test for variance. Based on these results the Student's t-test for equal or unequal variance was used to determine significance between the different treatment groups. All tests were two-sided and a P value of 0.05 or less was considered statistically significant.

3. Results

3.1. Influence of AKC on Wound healing of Full-Thickness Wounds

In the first experiment, biopsies were taken on postoperative day 8. We measured a mean reepithelialization percentage (MRE%) of 41.7% in wounds treated with AKCs (n=7), 42.2% in wounds treated with autogenic keratinocytes (n=7) and 41.1% in wounds treated with a mixture of AKC and autogenic cells (n=7). Saline treated controls (n=7) had a MRE % of 31% and dry treated wounds of 18.1% (Fig. 1).

Figure 1.

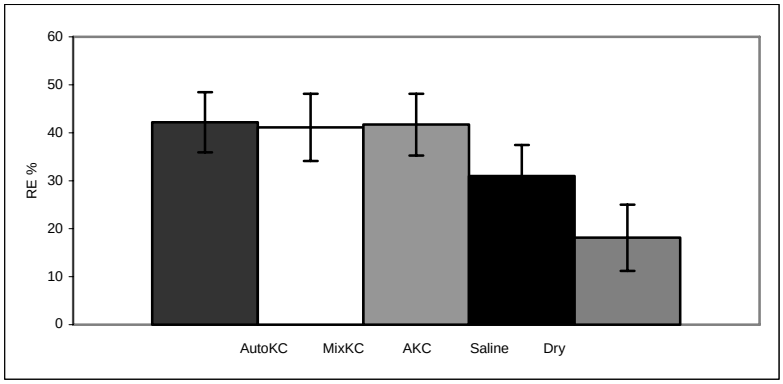


Fig. 1 Mean reepithelialization percentages of full-thickness skin wounds treated with suspensions of AKC, autogenic keratinocytes, mixed AKC/Autogenic cells with saline and dry treated wounds as controls. Biopsies were taken at day 8.X-axis: groups, Y-axis: reepithelialization percentage.
Significantly higher rates of reepithelialization were measured in wounds treated with cell suspensions than in saline treated wounds. No significant difference was noted in reepithelialization percentage between wounds treated with AKC, Autologous KC or mixed AKC/AutoKC.Legend: Auto KC= 5x10e5 autogenic keratinocyte cell suspensions, AKC= 5x10e5 allogenic keratinocyte cell suspensions, MixKC= mixed cell suspensions of 2.5x10e5 Auto KC and 2.5x10e5 AKC.

There was no statistical difference in MRE% between wounds treated with AKCs, autogenic or mixed AKC/autogenic keratinocytes. However, MRE% in the AKC, autogenic and mixed keratinocyte treated wounds was significantly higher when compared to saline control wounds ($p<0.001$), whereas saline treated wounds had a significantly faster MRE% than dry-treated wounds ($p<0.001$).

Histological examination of wound biopsies revealed an inflammatory infiltrate in the granulation tissue. The inflammatory response in wounds treated with AKC exhibited the same intensity in H&E-stained sections as in saline control wounds. Keratinocyte cell clusters were visible throughout the granulation tissue in all cell treated wounds.

Planimetry of wound surfaces showed no significantly different contraction rates between any of the cell versus saline treated wounds.

All wounds treated in a wet wound environment (all cell treated and saline treated groups) demonstrated a significantly lower contraction ratio than the dry treated wounds ($p<0.03$)

3.2. Long-term Assessment of Wounds Treated with AKC

Reepithelialization

In the second set of experiments, wound biopsies were taken at different time points up to post-operative day 35 to determine long-term effects of AKC on stability of FTWs.

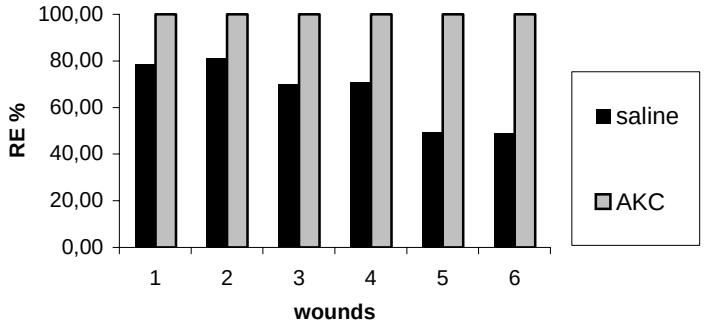
On post-operative day 8, when FTW at the midline of the dorsum were compared, wounds treated with AKC (n=3) reepithelialized 100% and saline control wounds (n=3) reepithelialized at a rate of 66.6% (Fig.2A). The difference between the groups was statistically significant ($p=0.002$). On days 11 (n=4), 14 (n=2), 21 (n=3), 28 (n=2), and 35 (n=2), wounds of both groups were 100% reepithelialized.

Histology demonstrated an increased presence of inflammatory cells throughout the 35 days of experiments. Multiple foci with granulomatous and mixed lymphocytic cells were observed. The degree of inflammation was scored on a 0 to 4 (max) scale by the histologist. Also, the thickness of the epidermal layer and size of rete ridges was evaluated. No significant differences were noted between the allogenic versus autogenic cell suspension treated wounds. The wounds treated with AKCs did not reveal clinical signs of strong inflammation or epithelial defects upon macroscopic and subsequent histological examination (Fig 2 B).

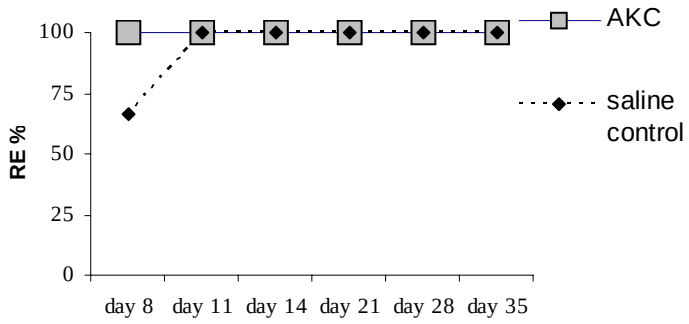
Wound Contraction

Wound contraction in wounds treated with AKCs was 44.1% on day 8 (n=16) and 61.5% on day 10 (n=13), as compared to 42.2% (n=16) and 54.9% (n=13) in saline control wounds (n=16), respectively. On day 21, wound contraction was 65.6% in AKC-treated wounds and decreased to 61.8% on day 28 and 58.7% (n=4) on day 35 (n=2). Saline control wounds contracted 57.7% on day 21 (n=7), 60.7% on day 28 (n=4), and 71.5% on day 35 (n=2). Differences in wound contraction between the groups were never statistically significant.

Figure 2.



A



B

Fig. 2: *A) Reepithelialization percentages were measured in wound biopsies taken from wounds that were treated with 5x 10e5 allogenic KC or saline in a mirror image pattern aside the midline of the dorsum for optimal comparison. All wounds treated with AKC had a 100 % epithelial cover at biopsy day 8. The mean healing rate for saline-treated wounds was 66%.*
B) The remaining wounds were biopsied at later time points to determine the long-term clinical and histological stability of the newly formed epidermis. No epithelial breakdown or blister formation was noted by day 35 in either group AKC= allogenic keratinocyte cell suspensions, RE % = percentage of reepithelialization

3.3. Influence of hEGF Transfected AKC on the Healing Time of Full-Thickness Wounds

Reepithelialization

The third set of experiments determined whether AKCs could function as gene transfer vehicles to deliver growth factors into the wound environment and further enhance wound healing. To emphasize the effect of hEGF-transfected keratinocytes on wound healing, we reduced the number of transplanted keratinocytes to 3 x 10e5 cells/wound. On post-operative day 8, wounds treated with AKC (n=12) had a reepithelialization percentage (RE %) of 45.38% and wounds treated with transiently transfected-hEGF expressing AKCs (n=9) had a RE % of 56.96%. We found a RE % for the Saline-treated control wounds (n=15) of 42.01% (Fig. 3).

Figure 3.

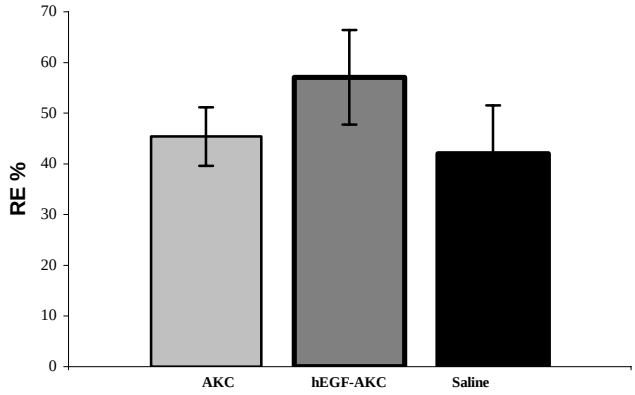


Fig 3 Full-thickness skin wounds measuring 1.5 x 1.5 cm were created on the back of two 4-month-old Yorkshire pigs prior to transplantation of 3x 10e5 hEGF transfected or 3x 10e5 untransfected AKC in single cell suspension or saline controls. Reepithelialization percentages were measured on post-operative day 8. Reepithelialization was significantly greater in wounds treated with hEGF-transfected AKCs, than in wounds treated with untransfected AKC or saline.
No significant difference in reepithelialization rates was found on day 8 when only 3x10e5 AKC were used versus saline.

The differences in RE% between the AKCs and hEGF-expressing AKCs (p=0.02), and between saline control and hEGF-expressing AKCs (p=0.009) were statistically significant (Fig.4-6). No difference in RE % could be detected between wounds treated with untransfected AKCs and saline-treated control wounds (p=0.42) when 3x10e5 cells were used per wound.

In Vitro expression of EGF.

To determine successful transfection in vitro, one dish of keratinocytes was transfected with LacZ-containing plasmid under conditions similar to hEGF transfection. 48 hours after transfection, cells were stained for LacZ expression, revealing a 15-25% transfection rate of keratinocytes.

To determine the ability of transfected keratinocytes to express hEGF, medium from hEGF-transfected and -untransfected keratinocytes was collected 24 hours post-transfection up to post-operative day 5. Levels of hEGF expression were 16782 pg/ml 24 hours after transfection and 9022 pg/ml 48 hours after transfection.

Figure 4.

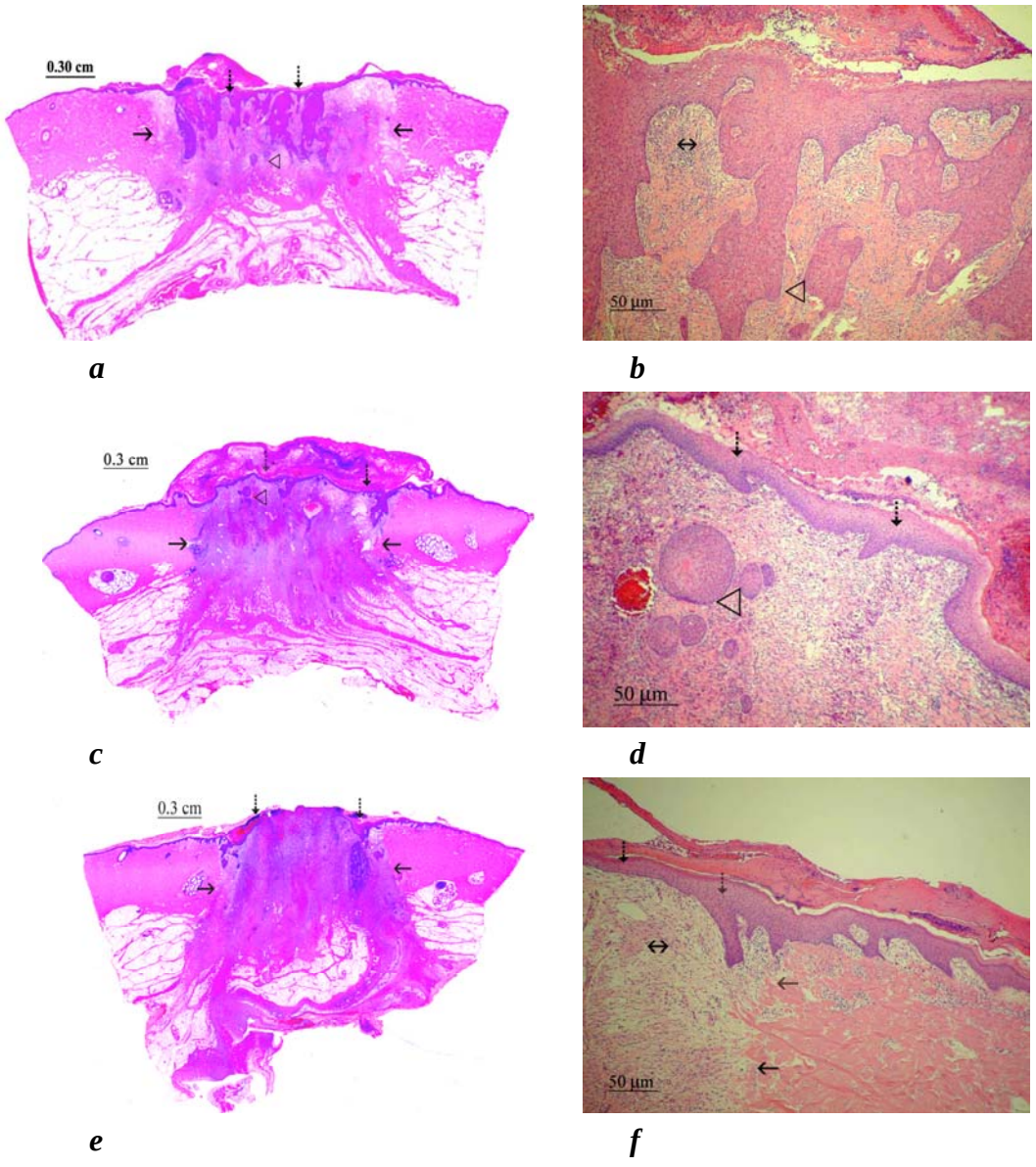


Fig. 4 a-f : H&E stained histological sections of FTW.

a-b/ FTW treated with 5x10e5 AKC EGF-expressing AKC at day 8 after wounding. The fully restored epithelial layer stains purple (↓) on top of the granulation tissue which is filled with epidermal cysts which will migrate further and incorporate in the epithelium (◁). Arrows indicate the transition zone between forming neodermis (wounded area) and intact non wounded dermis (←). Multiple granulomatous and mixed lymphocytic cells are observed in the inflammatory infiltrate in the neodermis (↔).

c-d/ FTW treated with 3x10e5 allogenic keratinotyes at day 8 after wounding. The fully restored epithelial layer stains purple (↓) on top of the granulation tissue and migrated underneath a thick scab attached on the wound. Only few epidermal cysts are visible in the granulation tissue (◁). Arrows indicate the transition zone between forming neodermis (wounded area) and intact non wounded dermis (↔).

e-f/ FTW treated with plain saline at day 8 after wounding.
The neo-epithelium expands as epithelial tongues (↓) over the granulation tissue. There are no epidermal cysts in the granulation tissue. Multiple granulomatous and mixed lymphocytic cells are observed in the inflammatory infiltrate in the neodermis (↔).Arrows indicate the transition zone between forming neodermis (wounded area) and intact non wounded dermis (←).

In Vivo Expression of hEGF

In the first experiment, hEGF levels in wounds transplanted with hEGF-transfected keratinocytes (Fig 5) revealed 920.8 pg/ml hEGF on day 1 and 624.5 pg/ml on day 2. Levels of hEGF decreased to 94.2 pg/ml on day 3, 68.9 pg/ml on day 4, and 36.9 pg/ml on day 5, and ranged between 12.7 and `6.5 pg/ml on days 6-9. In wounds treated with untransfected AKC or saline, hEGF was between 6.6 pg/ml and 0.98 pg/ml (Fig. 5). In the second experiment, hEGF expression was lower: 188 pg/ml on day 1, 88pg/ml on day 2, and 106 pg/ml by day 3. Expression decreased to 56 pg/ml by day 4 and 18 pg/ml by day 5. No expression was detectable after day 5. Control wounds did not reveal any expression of hEGF. Besides the lower peak expression, the ratio of hEGF expression in wounds treated with hEGF-transfected AKCs versus untransfected AKCs and saline controls was similar in both experiments.

Figure 5.

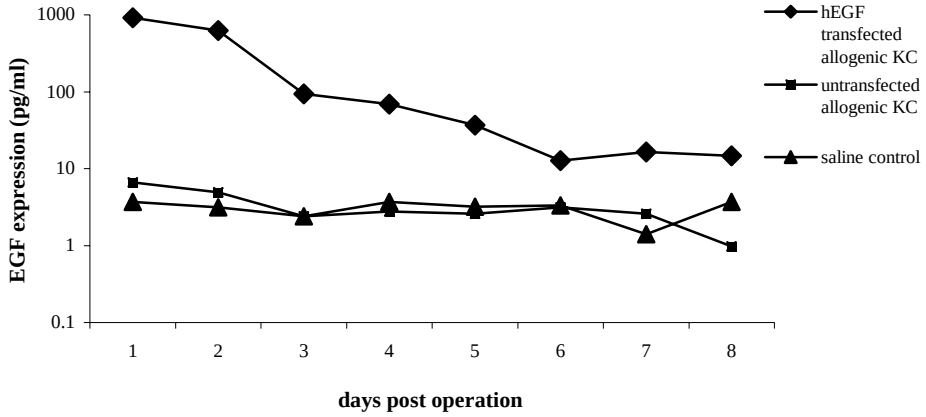


Fig 5. In vivo expression of human epidermal growth factor (EGF) in wound fluid retrieved daily from the wound chambers and determined by ELISA.

4. Discussion

It is generally accepted that allografts (homografts) are efficient biodressings that temporarily cover the floor of chronic wounds and acute burns and induce granulation and vascularization[18-21]. It has also been demonstrated that such allografts are rejected from wounds after 10-14 days. Several authors have reported that AKCs reject at a slower rate than allogenic skingrafts. Aubock et al.[22] investigated the properties of allogenic and autogenic cultured epidermis and concluded that Langerhans-cell free allogenic cultured epidermis does not survive permanently, but the rejection rate was delayed by 4-5 days, compared with allografted skin grafts.

It was postulated that absence of the MHC proteins could lead to a diminished host response against allografts[23,24]. Dierch et al.[25], however, showed that both MHC-I and MHC-II knockout allografts still underwent rejection, which was considered to be the result of the existence of 'Minor Antigens'.

Hunt et al.[26] reported that allogenic cultured keratinocytes grown from MHC-II knock-out mice created fewer immunogenic cells. They concluded that host priming, rather than Target Antigen Type, determines rejection rate in their mouse model. By using 'xenogeneic-syngeneic' mixed epithelial sheets, a histologically well organized epidermis could be grown, presenting basal and suprabasal cell layers and an active dermo-epidermal junction. The xenogeneic cells were 'selectively' eliminated without rejection of the entire 'mixed' implant, in contrast to purely allogenic cells, which had a 100% rejection rate. Immunofluorescence staining showed that syngeneic cells gradually replaced the AKC[27,28].

We were able to demonstrate that AKC covered the wound surface from within the wound and promoted reepithelialization compared with saline controls. After completion of wound coverage, neither an increased inflammatory reaction, nor epithelial thinning, nor wound breakdown could be detected in wounds over a period of 35 days post-transplantation. Based on these findings and the knowledge that AKC is gradually replaced by autogenic keratinocytes, it appears that transplantation of AKC not only provides accelerated wound coverage but also allows for ingrowth of autogenic keratinocytes. Therefore, transplantation of AKC in a single cell suspension can reduce the need for re-transplantation and therefore reduce the cost of wound treatment in the wound healing model used in this study. Moreover, to further eliminate the impact of rejection of allogenic cells in larger wound settings, a mixture of allogenic and autogenic keratinocytes can be used for wound treatment. This strategy reduces the amount of autogenic keratinocytes required for wound coverage while offering accelerated healing of large wounds.

Gene therapy justifiably gains interest as a treatment modality for wound repair and tissue regeneration. In vivo gene transfer of growth factor-encoding genes into the wound environment by 'gene gun' or microseeding may lead to high expression of growth factors in the wound[8,16] but does not add supplementary cell substrate to the defect. In large full-thickness skin wounds such as extensive third-degree burns or large chronic wounds, this lack of substrate might be critical to repair. Ex vivo gene transfer of transgene-expressing cells brings both cell substrate and growth-promoting genes into wounds, which might lead to more effective treatment than either therapy alone.

Stable gene expression remains a difficult issue in gene transfer protocols. Creating stable autogenic cell lines that have been selected and cultivated to express a particular gene represents an elaborate and time-consuming process[17].

In a clinical context, cell cultures need to be readily available for treatment, such as in an extensively burned patient, where rapid debridement of the burn scar must be immediately followed by coverage of the defect[18]. Stored allogenic cell cultures can be thawed, grown, transfected, and subsequently used for coverage after 7-10 days[19,20].

We found that AKCs transiently transfected with the hEGF gene expressed high levels of EGF in wound fluids over a period of 5 to 6 days. Wounds treated with these transfected cells showed a significantly greater reepithelialization rate than untransfected AKCs and saline-treated control wounds.

AKCs are effective carriers for gene transfer of growth factors to the wound environment. Given the fact that keratinocytes do not express EGF, the transient transfection of keratinocytes with this growth factor resulted in a prolonged expression and acceleration of wound healing in these experiments.

5. Conclusions

Our results demonstrate that the transplantation of EGF-expressing AKC as a single-cell suspension accelerates wound healing in the model employed in this study. Rejection takes place gradually and does not lead to epithelial thinning or defects. Furthermore, AKC, as single-cell suspensions, can serve as efficient gene transfer vehicles to the wound environment resulting in prolonged transient hEGF expression and further enhancement of wound healing.

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Corresponding Author:

Feng Yao, PhD, Laboratory of Tissue Repair and Gene Transfer
Division of Plastic Surgery, Brigham & Women's Hospital
75 Francis Street, Boston, MA 02115, USA
Phone: 617- 732-4836, Fax: 617-278-6918
Email: fyao@rics.bwh.harvard.edu

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You must feel chaos within
to give birth to a dancing star.
H.F. Nietzsche

Part 5 - Ex vivo gene transfer to full thickness skin wounds.

- *Induction of matrix formation and angiogenesis*
- *Regulation of gene expression*

"Regulable $VEGF_{165}$ overexpression by ex vivo expanded keratinocyte cultures, promotes matrix formation, angiogenesis and healing in porcine full thickness wounds."
Dickens S., Ing., Vermeulen P., M.D., Hendrickx B., M.D., Van den Berge S.,M.D.,
Vranckx J.J., M.D., FCCP
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Regulable $VEGF_{165}$ overexpression by ex vivo expanded keratinocyte cultures, promotes matrix formation, angiogenesis and healing in porcine full thickness wounds.

Dickens S., Ing., Vermeulen P., M.D., Hendrickx B., M.D., Van den Berge S.,M.D., Vranckx J.J., M.D., FCCP.

Laboratory of Plastic Surgery and Tissue Engineering Research
Dept. of Plastic & Reconstructive Surgery
KU Leuven University Hospital, KU. Leuven, B-3000 Belgium.

Abstract

The intricate wound repair process involves the interplay of numerous cells and proteins. Using a porcine full thickness wound (FTW) healing model, we hypothesized that the ex vivo gene transfer of VEGF-transfected basal keratinocyte cell suspensions (KCs) may generate cross talk, induce matrix formation, angiogenesis and accelerated healing. Moreover, to regulate $VEGF_{165}$ overexpression and its impact on healing, we introduced a tetracycline (TC)-inducible gene switch in the expression plasmid. Autologous basal KCs were cultivated from the porcine donor and transfected by cationic liposomes. A dose-response curve was established to determine optimal activation of the gene switch by TC. In vivo, FTW were treated with VEGF-transfected KCs versus controls. Wound fluids were collected daily and examined by ELISA. Biopsies were evaluated by H&E and immunostaining for fibronectin, CD144 and Lectin BS-1.In vitro, highest regulable $VEGF_{165}$-expression was obtained with 1µg/ml TC. In vivo, after induction of the gene switch by adding 1µg/ml TC to the FTW, we obtained upregulated $VEGF_{165}$ levels, enhanced fibronectin deposition and found more endothelial cell (EC) tubular formations and higher rates of reepithelialization than in controls. This ex vivo gene transfer model may serve as a platform for vascular induction in full thickness tissue repair.

Abbreviations

$VEGF_{165}$	Vascular Endothelial Growth Factor isoform 165	ECM	Extracellular matrix
FTW	Full Thickness skin Wounds	MMP	Matrix metalloproteinases
KCs	Keratinocytes	MT-1 MMP	Membrane type-1 MMP
FBs	Fibroblasts	TC	Tetracycline
ECs	Endothelial cells	FITC	Fluoresceinisothiocyanate

1. Introduction

The response of tissues to injury forms the foundation of all reconstructive procedures. In patients with chronic wounds and significant concomitant morbidity, it seems futile to count on intrinsic protein production by the debilitated mediating cells in the wound. In such microenvironment, extrinsic proteins could be added to induce, support and modulate the wound repair processes[1].

However, topical administration of recombinant growth factors as proteins has major shortcomings such as inefficient delivery to target cells, enzymatic inactivation by proteinases in the wound, low bioavailability and short shelf life[2]. Gene therapy offers an appealing strategy for direct delivery of growth factor genes into the cells and can offer targeted local and persistent delivery of *de novo* synthesized growth factors to the wound environment over many days[3,4]. Therefore, we developed a wound healing model in swine because of the homology with human wound healing and skin architecture. For all wounds, we use transparent flexible wound chambers to create an incubator-like wound milieu which improves healing[5,6].

Angiogenesis is the corner stone for wound repair and regeneration. Without proper vascularization, wounds fail to heal, tissues necrotize and tissue engineered constructs cannot thrive**.** Coupled with its ubiquitous expression in angiogenic tissues, it is clear that VEGF is the primary angiogenic factor[7]. $VEGF_{165}$ specifically binds to VEGF receptor-1 (Flt-1) which induces VEGF release and VEGF receptor-2 (Flk-1) for EC proliferation, migration and survival. This dual pathway explains $VEGF_{165}$ proangiogenic actions in tissues[8-10]. Activated migrating KCs express VEGF during wound healing[11]. Several clinical studies have reported the use of sheets of autologous KCs to resurface burn wounds and skin disease[12-13]. Single cell suspensions of basal KCs can also regenerate structurally intact epidermis in FTW[14]. KCs provide an epidermal cover that prevents fluid loss and delays bacterial invasion, but also mediates cell-cell interactions within the wound milieu. KCs interact with fibroblasts (FBs) through growth factors. FBs are activated to secrete extracellular matrix (ECM) components such as fibronectin, which contribute to the formation of a proangiogenic matrix and favour KC migration. Furthermore, secreted VEGF stimulates migration, proliferation, sprouting, and tube formation of ECs in this matrix[15-17]. We merged these elements using *ex vivo* transfected porcine basal KCs overexpressing $VEGF_{165}$ and examined its impact on angiogenesis, matrix formation and FTW healing. To regulate VEGF-expression by the KCs and analyse the precise influence of VEGF overexpression on FTW healing, we integrated a tetracycline (TC)-inducible gene expression switch in the plasmid.

2. Material and Methods

2.1. Cloning of $VEGF_{165}$

$VEGF_{165}$ was digested from pBluescript II KS(+) vector and pcDNA4/TO was digested using EcoRI and EcoRV (MBI Fermentas, Germany). Samples were analyzed on 1% agarose gel in TBE buffer (Invitrogen, Belgium) by electrophoresis at 100V for 90min, plasmid DNA stained with ethidiumbromide (1µg/ml; Invitrogen NV, Belgium) and visualized under UV light.

Appropriate bands were excised, cleaned with a QIAquick Gel Extraction Kit (Qiagen, The Netherlands) and ligated with T4-DNA ligase (MBI Fermentas, Germany) to form the regulatory pcDNA4/TO-VEGF plasmid.

2.2. Sequence analysis

Cycle sequencing was performed using the ready-reaction mix (ABI Prism Bigdye Terminator Cycle Sequencing kit; Applied Biosystems, USA) according to the manufacturer's instructions and using the CMV forward primer and BGH reverse primer. Analysis was performed on the ABI Prism 310 capillary. Assembly and editing was studied with GeneBase (Applied Maths, Belgium). The obtained full sequence was compared to all known sequences in the Genbank using Blast 2.0.

Restriction digest

Blank pcDNA4/TO and pcDNA4/TO-VEGF were digested with restriction enzymes. Simultaneously, two digests were performed: a single SalI and a double EcoRI-EcoRV digest. A total of 1µg plasmid DNA was used as template in a digest containing 2µl 10x buffer O and 0.5µl SalI or 2µl 10x buffer Tango, 0.5µl EcoRI and 0.5µl EcoRV diluted to 20µl with sterile H_20. Samples were incubated for 2h at 37°C and 20min at 65°C to inactivate the restriction enzymes. Samples were analyzed on a 1% agarose gel in TBE buffer by electrophoresis, stained with ethidiumbromide and visualized under UV light.

2.3. In vitro cell growth

Porcine KCs were isolated and pooled from a 6cm² skin graft, harvested from a 2 month old Yorkshire pig under general anaesthesia in the OR (cfr. Ch.2.8). Fresh skin grafts were washed and incubated for 3h with 2.4U/ml dispase II (Roche Diagnostics, Belgium). Dermis and epidermis were separated and basal KCs were harvested from the dermal fraction with 0.05% trypsin-EDTA. KCs were seeded in KC medium, which contains L-glutamine, 50µg/ml bovine pituitary extract, 5ng/ml human recombinant EGF (Invitrogen, Belgium) and supplemented with 2.5% calcium depleted fetal bovine serum (Chelex 100; Bio-Rad Laboratories, Belgium). Single cells were grown on collagen I coated dishes at 37°C and 5% CO2. 3rd and 4th passage cells were used.

2.4. Cloning of the Tetracycline inducible regulatory plasmid

The major component of the regulatory system is the inducible expression plasmid pcDNA4/TO from the T-Rex system (Invitrogen, Belgium). $VEGF_{165}$ expression from the inducible expression vector is controlled by the strong CMV promoter into which 2 copies of the TC operator 2 (TetO2) sequence have been inserted in tandem. Each TetO2 sequence serves as the binding site for 2 molecules of the TC repressor. The second major component is the pcDNA6/TR regulatory vector which expresses high levels of the TetR gene 18. In the absence of TC, the TC repressor forms a homodimer that binds with high affinity to each TetO2 sequence in the promoter of the inducible expression vector 19,20. Binding of the TC repressor homodimers to the TetO2 sequences and represses $VEGF_{165}$ transcription.

Upon addition, TC binds with high affinity to each TC repressor homodimer and causes a conformational change in the repressor that renders it unable to bind the TetO2. As a result, $VEGF_{165}$ transcription is induced. To integrate this regulatory system, we cotransfected the pcDNA6/TR and pcDNA4/TO-VEGF into our porcine KCs cell line.

2.5. Liposome-based transfection mechanism

When KCs reached 70% confluence, transfection experiments were performed with Lipofectin according to our standardized protocol. Briefly, plasmid DNA and Lipofectin were mixed in serum-free Waymouth medium, added to the KCs and incubated for 9h at 37°C. A 1:6 DNA ratio and a 1:4 DNA to Lipofectin ratio were applied. After transfection, medium was replaced by growth medium and left untouched for 24h prior to testing the transgene expression.

2.6. In vitro tetracycline dose-response analysis

Cotransfection was performed with 0.5µg pcDNA4/TO-VEGF, 3µg pcDNA6/TR and 14µg Lipofectin reagent. A dose-dependency curve for TC (0, 0.5, 1, 2 and 5µg/ml) was analyzed for induction of the transgene expression. Following a recovery period of 24h after transfection, all medium was removed and stored at -80°C (time point 0h). After 24h and 48h, cell supernatants were collected for subsequent ELISA analysis.

2.7. Animal preparation

All animals received human care in compliance with the principles of laboratory animal care (Royal Decree of 14 November 1993, Belgium) and the European Directive 86/609/EEC. Yorkshire pigs (female, 2 months old, ±30kg) were premedicated with 5ml IM zolazepam/tiletamine (Zoletil 100; Vibrac, France) and 2ml xylazine (Xyl-M 2%; VMD, Belgium). Pigs were placed in a custom made mobile sling. After induction and intubation with a 7.5 mm endotracheal tube, anaesthesia was maintained with 0.7-1.5% isoflurane (ISOBA, Abbott Laboratories, UK). Oxygen saturation and heart rate were continuously measured with pulse-oxymeter ear sensors. In a first and short intervention, a split thickness skin graft was harvested using an electrical dermatome set at 0.012 inch (Padgett Instruments Inc, USA) to obtain the KCs.

When sufficient 3rd passage cells were cultivated ex vivo, the operative day 0 was planned. The porcine back was stripped with depilatory cream and the skin was surgically prepped and draped. All 2.5x2.5cm wound sites were outlined and retraced using a black ink tattoo gun. FTW were created using a #11 bistouri scalpel blade to a uniform 1cm depth to the fascia. After haemostasis, all wounds were covered with an adhesive-backed sterile, transparent and flexible polyvinyl chamber with a self-closing injection pad. Experimental groups were assessed randomly. The injected cell or saline solutions were allowed to settle for 2h in the wound. After operation, the pigs were housed in individual cages and IM analgesia (Temgesic 0.3mg/ml buprenorfine-hydrochloride; Shering-Plough, Belgium) was administered twice a day.

Every 24h post surgery, pigs were sedated with isoflurane and oxygen via a snout mask. Pigs were examined for signs of wound infection or other illness. Wound fluids were collected, leaking wound chambers were replaced and skin was cleaned extensively. Maintenance was followed until day 10 post operative. Finally, pigs were euthanized via 10ml T61 intravenously (Intervet, The Netherlands).

2.8. In vivo experimental outline

32 FTW were created on the back of 2 pigs (n=32). Each wound was randomly assigned into a group (Table1). Group I received a 2ml NaCl 0.9% saline into the wound chamber (n=8).

Group II received a 2ml VEGF transfected KCs solution (5x105 cells/ml) (n=12) and group III received a 2ml VEGF transfected KCs solution (5x105 cells/ml) with 1µg/ml TC (n=12). Wound fluid was aspirated daily and stored at -80°C. Tattooed wound borders were copied every 4th day on transparent sheets and scanned into computer images for planimetry. At day 8 and 10 post surgery, 5mm wide biopsy strips were harvested comprising the entire wound width plus a margin of adjacent intact skin. The biopsy sites were closed with Ethilon 2-0 and covered with sterile gauze. Wounds were randomly assigned to the 1st or 2nd set of biopsies.

Table 1: Experimental group outline.

	Group I (Saline)	Group II (VEGF-TC)	Group III (VEGF+TC)
	Physiological NaCl 0.9%	$5x10^5$ VEGF-transfected keratinocytes/ml	$5x10^5$ VEGF-transfected keratinocytes/ml
Tetracycline	0µg/ml TC	0µg/ml TC	1µg/ml TC
Total volume	2ml	2ml	2ml

Wet wound healing is obtained by using adhesive wound chambers filled with 2ml of liquid solution. To control group only physiological saline solution is added (group I). To group II, VEGF-transfected KCs were added and dissolved in physiological saline solution (VEGF-TC). In Group III, VEGF-transfected KCs were added to a solution of 1µg/ml TC (VEGF+TC).

2.9. Enzyme-Linked Immuno Sorbent Assay

Cell supernatant and wound fluids were diluted with PBS. $VEGF_{165}$ was determined with an ELISA assay kit (R&D Systems, USA) according to the manufacturer's protocol. Absorbance was measured using a micro plate reader (Thermo Electron, USA) at 450 nm and λ corrected at 540nm.

2.10. Histological examination and immunohistochemical staining

Wound biopsies were immediately embedded in OCT and snap frozen in liquid nitrogen. Once frozen, 7µm sections were prepared for (immuno)histochemical staining, fixed in acetone for 10min and stored at -20°C.

Haematoxylin and eosin staining was performed and visualized by Axioplan 2 Imaging microscope and AxioVision Rel. 4.4. software (Carl Zeiss Vision, Belgium) to generate accurate length measurements. By measuring the length of newly formed epithelium tongues, the overall rate of reepithelialisation at the center of the FTW was determined.

For immunohistochemistry, slides were thawed, washed with PBS for 5min and incubated for 30min in PBS containing 1% Normal Goat Serum and 2% BSA. For FITC-conjugated iso-lectin Bandeiraea simplicifolia 1 (1:3 BS-1; Sigma Aldrich, Belgium) staining, slides were incubated for 45 min.

For anti-CD144 (VE-Cadherin) or anti-fibronectin staining, a 30min incubation was performed with anti-CD144 (1:100; Serotec, UK) or anti-fibronectin antibody (1:200; Sigma Aldrich, Belgium) followed by a 30min incubation with a secondary Texas Red conjugate (1:100; Sigma Aldrich, Belgium) or FITC conjugate (1:200; Sigma Aldrich, Belgium). Finally, slides were washed 3 times and nuclei were stained with Vectashield hardset mounting medium with DAPI (Labconsult, Belgium) and cover slipped. Analysis was performed by Axioplan 2 Imaging microscope and AxioVision Rel 4.4. Software using a Plan-Apochromat 10x lens. Neo-vascularisation stainings were quantified by counting clusters of stained endothelial cells. Fibronectin deposition in the ECM was analyzed by green fluorescence intensity.

2.11. Statistical analysis

Results were averaged for each group and statistical significance analysis was generated using non-parametrical Mann-Whitney test. Two-sided p-values of <0.05 were considered statistically significant.

3. Result

3.1. Confirmation of VEGF-cloning

Restriction digestion of EcoRI-EcoRV resulted in a 598bp fragment of $VEGF_{165}$ out of pBluescript II KS(+) vector and a 5066bp fragment for the induction vector pcDNA4/TO. After ligation of both fragments, we created a 5664bp fragment, named pcDNA4/TO-VEGF. The $VEGF_{165}$ protein is now under the control of a TetO2 region, regulable by TC and a CMV promoter. Sequence analysis shows a positive cloning for all crucial regions of pcDNA4/TO-VEGF (Fig.1).

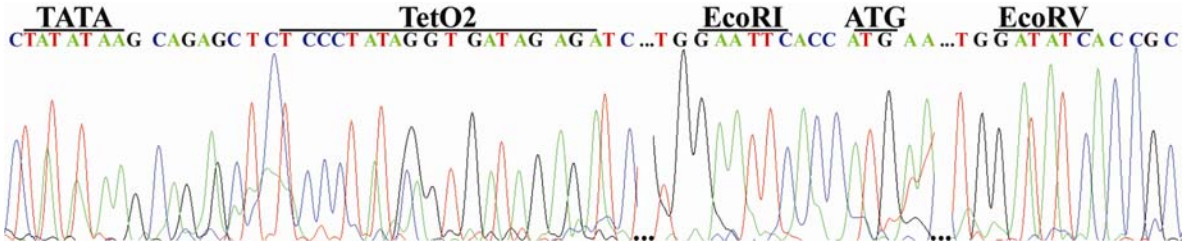


Fig. 1 Sequence analysis of pcDNA4/TO-VEGF.
Cycle sequencing was performed using CMV forward primer 5´-CGCAAATGGGCGGTAGGCGTG-3´ and BGH reverse primer 5´-TAGAAGGCACAGTCGAGG-3'. Visualization was obtained with Chromas software, recovering the following sequence for all crucial regions of pcDNA4/TO-VEGF:
CTATATAAGC...GCTCTCCCTATAGGTGATAGAGATCTC...GTGGAATTCACCATGA...CTGGATATCACC and sequence close-up visualisation of the TATA-box, TetO2 region (TC binding site), restriction enzyme EcoRI, ATG start region of transcription and restriction enzyme EcoRV.

The regulatory region TetO2 is well preserved just downstream of the TATA-box. $VEGF_{165}$ translation starts at the ATG codon downstream the EcoRI site. In addition, 2 restriction digests with SalI and EcoRI/EcoRV were performed to verify the correct composition of the pcDNA4/TO-VEGF plasmid (Fig. 2A,B). NCBI blast search with the obtained sequence shows 100% similarity with homo sapien $VEGF_{165}$.

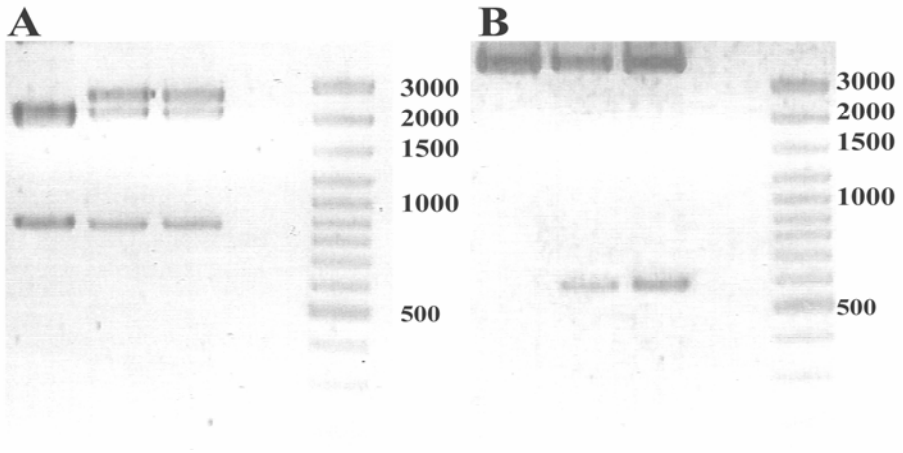
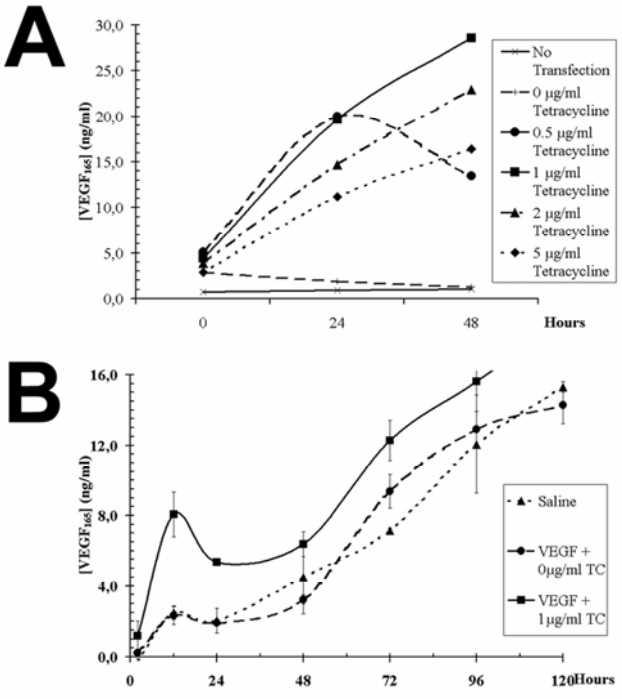


Fig. 2 Restriction digest of naked pcDNA4/TO and pcDNA4/TO-VEGF.
(A) Single restriction digest with SalI obtaining 3 fragments for naked plasmid (2185bp–2029bp–864bp) and pcDNA4/TO-VEGF (2627bp–2185bp–864bp).
(B) Double restriction digest with EcoRI–EcoRV obtaining 1 fragment for naked plasmid (5066bp) and 2 fragments pcDNA4/TO-VEGF (5066bp–598bp).

3.2. In vitro tetracycline dependency (Fig. 3A)

Non-transfected KCs demonstrate a basal VEGF expression below 1000pg/ml (—✕—). Transfected KCs not activated with TC follow an expression pattern similar to non-transfected KCs (—+—) and show similar basal VEGF expression at 48h. With TC concentrations of 0.5 and 1µg/ml, VEGF expression increases to 20000pg/ml VEGF after 24h. Nevertheless, decreased expression is seen in the 0.5µg/ml group after 48h (—●—), whereas VEGF concentration of the 1µg/ml group increased to 28600pg/ml VEGF at 48h (—■—). Highest TC concentrations (2 and 5µg/ml) result in a similar increase in VEGF concentration over time as compared to the 1µg/ml group, yet lower final concentrations of 22900pg/ml (20% decrease) and 16385pg/ml (43% decrease) were obtained, showing inhibition of expression by higher TC concentrations (—▲- & - ◆-).



(A) In vitro, transfected KC, without addition of TC, show a basal $VEGF_{165}$ expression, similar to the non-transfected control group. 1µg/ml TC shows a 25 fold up regulation after 48h compared to the control group, whereas other concentrations show a lower $VEGF_{165}$ expression.

(B) In vivo, the saline control and transfected KC group without TC activation (VEGF+0µg/ml TC), show similar increased $VEGF_{165}$ expression. Addition of 1µg/ml T (VEGF+1µg/ml TC) results in an upregulation of VEGF expression.

Fig. 3 In vitro and in vivo $VEGF_{165}$ expression measured by ELISA.

In vivo VEGF expression (Fig.3B)

Saline treatment of the FTW (-▲-) demonstrates an endogenous expression of $VGEF_{165}$ over the first 5 days. VEGF concentrations are 205pg/ml 2h after saline application and increase in an almost linear fashion to 15300pg/ml at 5 days. Wounds treated with transfected KCs, without activation with TC, show an increase from 416pg/ml after 2h to 14300pg/ml after 5 days, which is almost similar to the increase found in saline treated wounds (—●—), whereas wounds treated with VEGF transfected KCs, after addition of 1µg/ml TC, show a distinct higher $VEGF_{165}$ expression pattern over 5 days (—■—): after 12h, we measured a 3.5-fold increase compared to the saline group ($P<0.01$) and the non-activated VEGF group ($P<0.01$). This higher $VEGF_{165}$ expression follows a similar curve.

3.3. Reepithelialisation and wound contraction measurements (Table2)

We analyzed H&E stainings 8 days post surgery. On average, saline treated wounds had a reepithelialization percentage (RE%) of 24%, whereas wounds treated with VEGF but without TC had a RE% of 59% ($P<0.05$). VEGF-transfected KCs treated wounds with 1µg/ml TC had a RE% of 64% ($P<0.01$). 10 days post surgery, closure of saline treated wounds was 51% on average, 80% for VEGF-transfected KCs without TC ($P<0.05$) and 94% VEGF-transfected KCs with 1µg/ml TC ($P<0.01$). Moreover, the difference in RE% between VEGF-transfected KCs with or without TC was significant ($P<0.05$). There was no significant difference in wound contraction rates among the 3 different groups.

Table 2: Statistic analysis.

	Saline	VEGF-TC	VEGF+TC
Reepithelization			
Day 8 (%)	24 [16-42]	59 [17-71] *	64 [35-100] ** #
Day 10 (%)	51 [37-95]	80 [46-100] *	94 [58-100] ** #
Endothelial Cell			
Lectin (Count)	3 [0-8]	5 [1-32] **	10 [5-42] ** ##
CD144 (Count)	0 [0-4]	2 [0-8] **	4 [1-14] ** #
Fibronectin ($x10^3$)	0.91 [0.68-44.5]	33.3 [1.34-86.9] *	48.3 [4.0-185.3] **

* Significantly different to saline (P<0.05) ; ** Significantly different to saline (P<0.01)
# Significantly different to VEGF-TC (P<0.05) ; ## Significantly different to VEGF-TC (P<0.01)

Statistical analysis using non-parametrical Mann-Whitney test for the percentage of reepithelialization by haematoxylin and eosin staining, endothelial cell staining and fibronectin staining for the 3 experimental groups, namely saline, VEGF-transfected KC group, without addition of TC (VEGF-TC) and VEGF-transfected KC group, with addition of 1µg/ml TC (VEGF+TC). Data is represented as mean and minimal and maximum values. Two-sided p-values of <0.05 were considered statistically significant.

3.4. Immunohistochemical staining of neo-vascular structures and matrix deposition

To confirm the EC structures in FTW, phenotypical studies with lectin BS-1 and anti-CD144/VE-Cadherin were performed on sections harvested on postoperative day 10. We preferred CD144 to the commonly used marker CD31, since the latter might yield false positive results because of expression of T-cells21 and macrophages[22], some of the key modulators in early wound healing. Lectin stainings (Fig.4-Table2) show a difference between the saline group and the non-activated KCs group (P<0.001) suggesting that healing of FTW with KCs cell suspensions alone give rise to enhanced neo-vascularisation. Nevertheless, significantly more neo-vascular structures were present in VEGF-expressing KCs activated by TC as compared to saline (P<0.0001) and non-activated KCs (P<0.0001). Anti-CD144 staining (Fig.4-Table2) confirms previous Lectin staining. Wounds treated with VEGF-expressing KCs activated by TC show more ECs compared to the saline (P<0.0001) and non-activated KC groups (P<0.05).

Fibronectin deposition is visible in all treated wounds (Fig.5-Table2). KCs treated wounds show enhanced deposition in the matrix (P<0.05 & P<0.01) compared to saline control wounds. The addition of TC (resulting in VEGF overexpression) does not cause a significant difference.

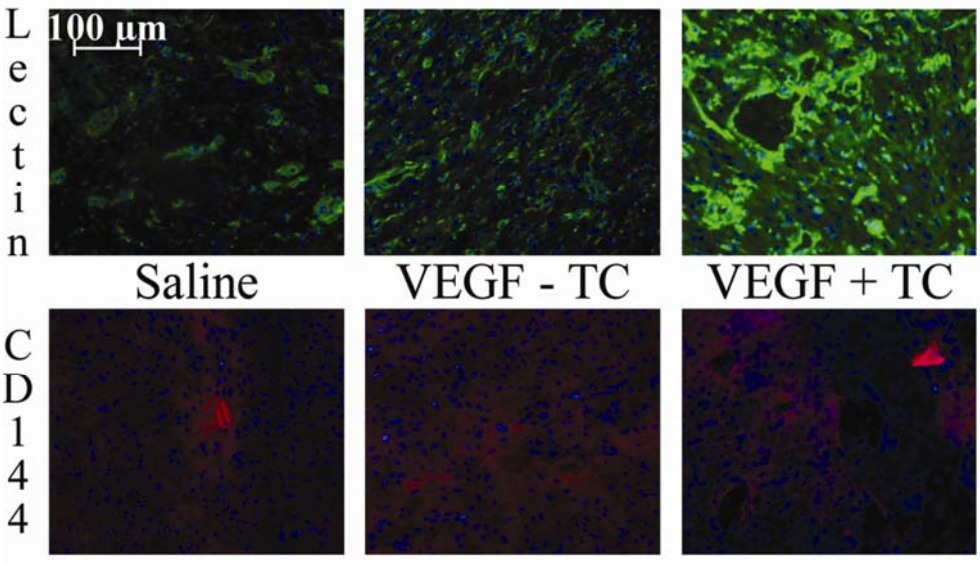


Fig. 4 Immunohistochemical staining for neo-vascularisation by lectin and CD144 (10x magnification). Immunohistochemistry of the 3 experimental groups, namely saline, VEGF-transfected KC group, without addition of TC (VEGF-TC) and VEGF-transfected KC group, with addition of 1µg/ml TC (VEGF+TC). Nuclei are stained with DAPI (blue) and lectin with FITC (green) or CD144 with Texas Red (red).

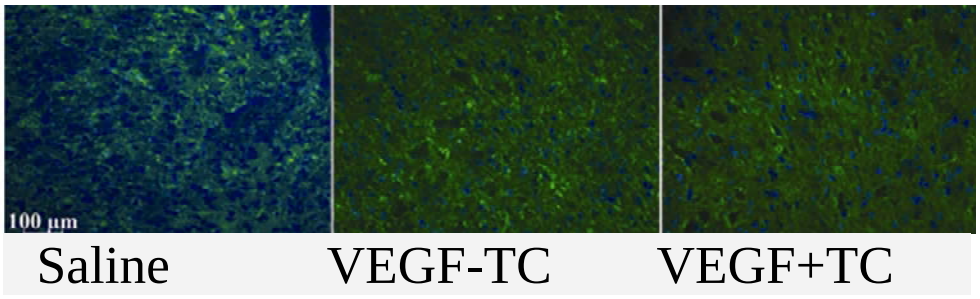


Fig. 5 Immunohistochemical anti-fibronectin staining (10x magnification) for extracellular matrix deposition. Immunohistochemistry of the 3 experimental groups, namely saline, VEGF-transfected KC group, without addition of TC (VEGF-TC) and VEGF-transfected KC group, with addition of 1µg/ml TC (VEGF+TC). Nuclei are stained with DAPI (blue) and fibronectin with FITC (green).

4. Discussion

The crosstalk among KCs, FBs, ECs, resident and infiltrating leukocytes and the ECM is complex and contributes to the maintenance of normal epidermal function and the epidermal response to injury[4,23,24]. In this ex vivo gene transfer approach, we used basal KC cell suspension cultures as temporary local production units for exogenous VEGF in the site of tissue repair and as an autologous bioscaffold with high clonogenic capacity. Since wound healing is a time-limited process, the temporary expression after cationic liposomal transfection is not disadvantageous in wound repair[24]. Furthermore, the success of gene therapy is not solely determined by its transfection efficiency.

In this study, liposomal mediated gene transfer shows sufficient angiogenic VEGF activity to achieve a significant biological outcome. In previous studies, we used an adenoviral linked Ad- $VEGF_{121}$ plasmid which was microseeded in vivo into FTW and we found impaired healing in those FTW treated with the highest VEGF concentrations.[6] Therefore, ex vivo gene transfer using transfected KCs as vehicles for VEGF expression represents a useful strategy that brings substrate and 'intelligence' into the wound repair site. VEGF is a key-factor in this strategy; it has the ability to stimulate all required processes for angiogenesis and vasculogenesis[25,26]. In this ex vivo study, we also found a relatively high basal VEGF-expression in saline treated wounds and KC-treated wound conditions without activating the genetic switch for VEGF production. Howdieshell et al also reported significant basal $VEGF_{165}$ production in control groups in a porcine wound model.[27] This basal VEGF secretion is part of the intrinsic inflammatory process following injury. In the early phase of tissue injury, alpha granules of platelets release growth factors such as VEGF, TGF-β_1 and PDGF that further attracts macrophages, FBs and ECs[1]. Furthermore, coagulation can reduce blood flow to tissues downstream resulting in hypoxia and VEGF up-regulation[27]. After 48h, macrophages, leucocytes and mast cells express a high level of mRNA of proangiogenic factors such as VEGF[28]. Brown et al showed that VEGF was up-regulated as early as 3 days after injury induction and this expression lasted for at least 7 days. Subsequent studies have shown that in FTW, VEGF mRNA expression declines around day 13 and after 3 weeks VEGF expression decreases to normal levels[7,11]. In this study, we found a consistent first peak and a dip in VEGF expression around post-operative day one before a larger second peak around day 5 in all FTW. This VEGF expression pattern coincides with previous results from treated and control FTW[6]. The consistent early peak expression may be explained by the reduction in blood flow caused by the obstructing clot and tissue edema. These events may induce hypoxia, which may temporarily reduce cellular VEGF production or hinder VEGF receptor binding and are followed by hypoxia-induced VEGF upregulation[27,29]. Using a rat model, Deodato et al describes increased vascular density and more rapid wound closure. However, a greater deposition of granulation tissue was observed with the use of a recombinant AAV vector encoding $hVEGF_{165}$[30]. VEGF inhibition via neutralizing monoclonal antibodies reduce granulation tissue formation indicating that VEGF is involved in stromal development through which capillaries grow and lead to wound closure and healing[31]. This may be explained by the increase in vascular permeability through VEGF. Extravasation of plasma proteins results in the formation of a highly pro-angiogenic ECM.

This pro-angiogenic matrix is rich in plasma derived fibrin and fibronectin, rather than interstitial products such as hyaluran and collagen.

Capillaries grow through this plasma-derived ECM for the first few days and then through fibroblast-derived granulation tissue for the next 2 weeks[32,33].

Fibronectin is an ECM glycoprotein, predominantly secreted by FBs and activated by KC-signalling to form and maintain the structural integrity of connective tissue[34,35]. KCs themselves produce integrins and fibronectin receptors to allow KC migration throughout the wound by cell-matrix interaction[36,37]. This profound fibronectin involvement demonstrates its commitment to the wound healing process and its use as an appropriate tool for the measurement of newly formed ECM16. In accordance, we found denser fibronectin deposition in wounds treated with KCs compared to saline controls.

In this study, VEGF expression of the KCs was under the control of a tetracycline-inducible switch. Yao et al improved the use of the TC-inducible regulatory system[49] and demonstrated that TC can function as a potent modulator to regulate gene expression in mammalian cells[20]. With correct positioning of $VEGF_{165}$ downstream of the TC-inducible operator and TATA box, we illustrated a dose-dependent regulation of $VEGF_{165}$ expression in vitro and in vivo. By administering KCs into FTW, we showed that $VEGF_{165}$ levels can be controlled by the addition of varying concentrations of TC directly into the wound. In this study, this mechanism allowed us to specifically analyse the impact of additional VEGF-overexpression in the setting of KC delivery to FTW. This system, which enables the downregulation of gene expression for safety concerns and the optimization of sequence and appropriate timing or expression of genes ad libitium, is essential when using more stable genes.

In conclusion:

In this porcine full thickness wet wound healing model, cell suspension cultures of VEGF-transfected basal KCs under regulation of a TC-inducible gene switch enhanced fibronectin deposition, EC tubuli formation and accelerated reepithelialization of FTW.

The effects may be explained by the complex crosstalk between KCs, FBs, the ECM, VEGF and ECs. We currently use this platform of modifiable ex vivo gene transfer to enhance angiogenesis in tissue engineered constructs.

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Correspondance:

Jan J.Vranckx, MD,FCCP.
Laboratory of Plastic Surgery & Tissue Engineering Research,
Dept.of Plastic & Reconstructive Surgery,
KUL Leuven University Hospitals,
49 Herestraat, B-3000, Leuven, Belgium,
32-16-348721 tel, 32-16-348723 fax,
jan.vranckx@uz.kuleuven.ac.be

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We must dare to think about
"unthinkable things" because when
things become "unthinkable" thinking stops
and action becomes mindless.
J. William Fulbright

Chapter IV: General Discussion

1. The wet wound healing animal model

All valid opportunities to obtain a smart full thickness construct start at the regular wound healing process with the cells involved during the inflammatory reaction, and the proteins expressed by featured cells for messaging, chemoattraction, proliferation, migration, and differentiation[7-9]. In our research experiments, we first focused on developing an animal model which could represent human wound repair in full thickness skin defects, mimicking tissue destruction of a defect down to the fascia level. Accordingly, we developed an appropriate standardized porcine model.

Unlike rodent skin, porcine epidermal, dermal, and junctional layers are remarkably similar to human skin in its biomechanical parameters, its immuno-histochemical and histological features, and also in its clinical behavior to inflammation and tissue repair[33,34]. Of all mammals, the skin of domestic pigs “sus scrofa” resembles human skin and wound repair best. We performed comparative immunohistochemical tests on full-thickness skin biopsies with involucrin, collagen VII, chondroitine sulfate, fibronectin, and vimentin antibodies. All layers correspond (Fig. 1). Obviously the predictive value of this model towards human FTW repair can only be determined after validation in ‘exploratory human outcome trials’. Nevertheless, we may presume that this porcine wound healing model predicts human tissue repair best of all available animal models.

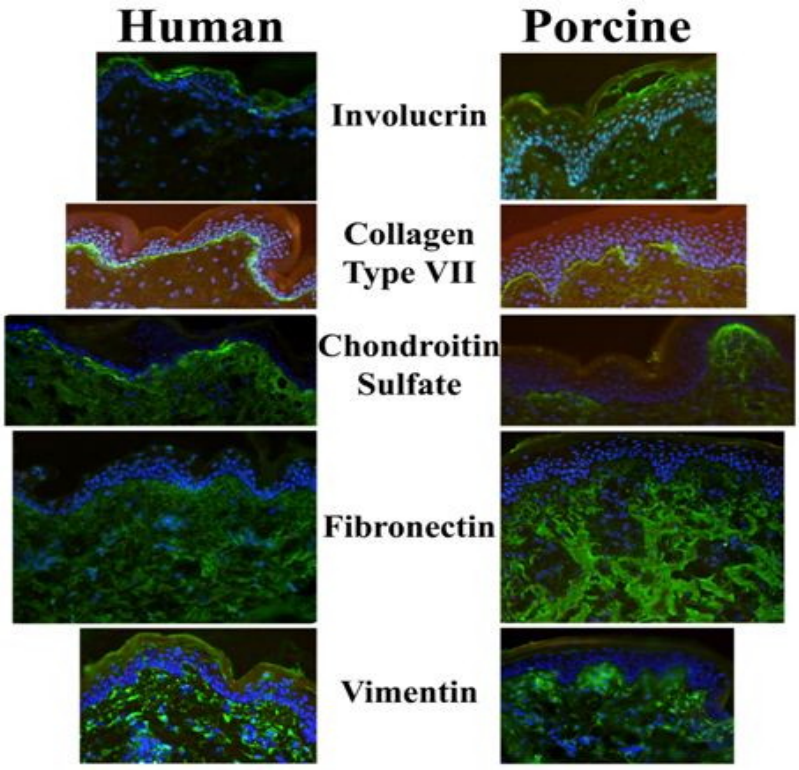


Fig. 1 Immunohistochemical staining of human and porcine full thickness skin.

We used young female Yorkshire pigs (3-4 months old) or hairless Yucatan Mini-Swine up to seven years of age. The latter represent an “aged” wound-healing model with intrinsic lower growth factor secretion and retarded healing.

We designed two custom-made mobile slings to transport the swine from their stainless steel cages to the operation theater (fabricated by Universal Metals, MA, USA).

These strong but light aluminium slings allow us to lift the pigs, after premedication, via a standardized and stress-less fashion in a ventral position using a custom made hammock. This sling also serves as a mobile operation table.

The ventral position facilitates intubation, induction, prepping, and draping. All interventions require inhalation anesthesia (Isoflurane/ N2O/O2) after premedication[35,36].

The standardized wound repair model consists of creating full thickness skin wounds (FTW) measuring 2.5 x 2.5 x 1 cm on the swine’s dorsum. These FTWs are all covered with a custom made highly flexible polyvinyl wound chamber that creates a wet wound healing environment as “mini-incubator.” This milieu allows for juxtacrine and paracrine influences by secreted growth factors and cytokines on neighboring cells in the FTW.

A dry wound is a dead wound. This paradigm sounds logical now, but was revolutionary in wound healing treatment two decades ago. Even nowadays, we often are confronted in clinics with chronic wounds treated elsewhere in a dry healing environment. We reported the healing of 25 chronic non-healing ulcers in patients with significant generalized morbidity such as diabetes, cardiovascular disease, corticosteroid treatment, and extensive burns. These persistent ulcers were ultimately healed by using a wet wound healing treatment after appropriate debridement with high local and nonsystemic antibiotic concentration[37,38].

We translated this clinical treatment technique to a standardized experimental swine model. When creating a wet wound healing environment, we found significantly faster healing rates as compared to dry treated wounds. Therefore, we used this wet wound treatment for all further experiments and for all "negative control wounds" which would be treated with plain saline injection into the wound chamber, creating a wet wound healing microenvironment. As such, all significant differences found in FTW repair by cell based therapies or by in vivo and ex vivo gene transfer treatments, were compared to ad-hoc clinical state-of-the-art wound treatment.

For purposes of future proof-of-principle research in mice and rat models, we also designed custom-made titanium mouse and rat chambers, which were fabricated at the atelier of the Aeronautics Department at Massachussets Institute of Technology in Boston. These titanium chambers were further modified for use in a pig model, and baptized "The Bo-chamber"[39].

2. Growth factors and Gene therapy for wound repair

2.1. Growth factors in tissue repair

The complex tissue repair process involves the cross-talk of a plethora of cells, proteins and humeral factors[40-42]. Cells orchestrating the several phases of wound repair secrete growth factors which in turn coordinate the further phases of tissue repair and stimulate cellular migration, proliferation, differentiation, matrix formation and angiogenesis.

However, in patients with chronic wounds and significant co-morbidity, it seems futile to count on intrinsic protein production by the debilitated mediating cells in the wound.

Since growth factors seem elementary in tissue repair, application of growth factors to such wounds seems then a logical strategy; extrinsic growth factor proteins could be added to induce, support, and modulate the wound repair processes[35,43]. We demonstrated that, with aging, the endogenous growth factor profiles such as VEGF and EGF are diminished. Since tissue repair also retardates in aged patients, we hypothesized that growth factor administration would enhance tissue repair.

The most obvious approach for applying growth factors to wounds is as proteins expressed in solutions, ointments, or dressings.

However, topical administration of recombinant growth factors as proteins have major shortcomings such as short shelf life, low bioavailibility, enzymatic inactivaton by proteinases in the wound, and inefficient delivery to target cells[21-23].

Gene therapy offers an appealing strategy for direct delivery of growth factor genes into the cells in the wound or the tissue-engineered construct. Cells are reshaped into mini-production units of the selected growth factor(s) with the intent of altering protein synthesis in the cellular apparatus in order to modify the healing response[16,47].

Skin is a fascinating target for gene therapy not only because of its accessibility and abundant vascularity, but also for its capacity for regeneration.

Gene therapy can offer targeted local and temporary persistent delivery of de novo synthesized growth factor to the wound environment . There are viral ‘transduction’ and non-viral ‘transfection’ methods to introduce effector genes into host cells. Viral ‘transduction’ is based on the natural ability of viruses to infect cells. Attenuated recombinant viral vectors differ in terms of integration in host cells, packing abilities, and their capacity to ‘transduce’ dividing and/or non-deviding cells. Besides small packing ability for effector genes, potential insertional mutagenesis and strong inflammatory host immune responses are a risk of viral gene transfer. Non-viral delivery systems involve physical or chemical transfer of genetic material and are dependent on cellular transport mechanisms for uptake and expression in the host cell[23,47].

Two major strategies are available for gene transfer to tissues: direct in vivo gene delivery into the wound environment[48-50] and ex vivo gene transfer by transplantation of transgene cell cultures into wounds[36,51]. In vivo gene transfer techniques bring growth factor DNA plasmids into FTW in the pig by direct injection, particle bombardment with the gene-gun, or by Microseeding. Using the Microseeding technique on skin wounds, Eriksson et al. demonstrated a threefold higher yield of hEGF than in wounds treated by particle bombardment with the 'gene gun,' and seven-fold higher yield than gene transfer by single injection[26]. We used this in vivo Microseeding procedure to transfer Ad-VEGF to FTW. High VEGF titers were measured in wound fluids following a dose-response curve.

Non-viral strategies attain much lower transfection efficiency, but there is no risk of virus-related morbidity. Also, non-viral transfection is currently unable to induce permanent genetic modification. Nevertheless, non-viral gene therapy holds great promise for selectively elevating or down-regulating expression of a particular growth factor in the wound microenvironment in order to promote tissue repair and regeneration, particularly if one considers that healing of a wound is a local event and requires high levels of transgene expression only for a limited period of time[23].

Evenmore, we demonstrated retarded healing of porcine FTW after in vivo gene transfer with highest Ad-VEGF concentrations by microseeding[35], as well as after ex vivo gene transfer of a stable PDGF-BB-expressing cell line of autogenic keratinocytes[52,53]. In context of wound repair, it seems that a 'minimal efficient concentration' of growth factors may be advisable compared to the highest concentrations obtained with viral transduction strategies or with stable cell lines. Therefore, we further focused on non-viral transfection strategies in our porcine wet wound healing model because of lower anticipated morbidity. Moreover, while in vivo gene transfer strategies may be appealing for superficial chronic wounds, extensive and deep wounds, such as third degree burns, may be too large to only rely on induction of repair processes by growth factors (or proteinase blockers). Adding cell substrate to the wound repair site may be obligatory.

Since the full thickness skin is destroyed in third degree burns, there is no vascular network available for potential circulating progenitor stem cells to aid in rebuilding a vascular network and in inducing repair. This may be the reason why we do not see any attempt for endogenous repair and regeneration from a stem cell population in extensive burns, despite the emergency of the situation.

Ex vivo gene transfer approaches are more likely to induce repair in such deep and extensive wounds, since growth factors are secreted by cultivated cell populations that form the substrate for repair. Ex vivo gene transfer may also be used to deliver 'intelligence' to three-dimensional tissue engineered matrices, by adding autogenic or allogenic cells that secrete targeted growth factors.

The major challenges facing wound repair are identifying an appropriate gene (or a cluster of genes) that is (are) effective in tissue repair and then making certain that the therapeutic gene is expressed reliably at beneficial levels[55].

2.2. In vivo gene transfer of Ad-VEGF to full thickness wounds.

Previous research indicated that endogenous VEGF and PDGF-BB concentration peaked later and at a four-fold lower level in aged pigs as compared to younger age groups. Rivard et al. stated that the angiogenesis responsible for collateral development in limb ischaemia is impaired with aging. Responsible mechanisms include age-related endothelial dysfunction and reduced VEGF expression[43-46]. We found a complete re-epithelialization around day 12 in 18 m/o Yucatans, while mean re-epithelialization was around 50% in 6 y/o Yucatans at day 21. These data suggest a relation between VEGF expression, aging, and wound healing. All five Yucatan minipigs we used were retired breeders. Taking into account the age of fertility (at 6 months) and the maximum age (10-12 y/o) as compared to humans, a 6-7 year old Yucatan pig represents a mean age category of 80 y/o in humans.

We demonstrated that microseeding the Ad-VEGF cDNA in the FTW resulted in a high concentration of VEGF protein in the wound fluid ,and that this VEGF expression follows a dose-response pattern[35] . However, despite these high yields, we could not demonstrate accelerated wound repair on histological sections taken on day 16 and 21 after creating the full thickness standard wounds. Paradoxally, the highest seeding concentrations of VEGF resulted in impaired healing. These results raise several questions.

We used an adenoviral linked $VEGF_{121}$ construct for this study. It has been documented that the use of viral vectors leads to higher tranduction efficiency than direct plasmid injection. Deodato et al. demonstrated a significant shortening in healing time of excisional wounds in a rat model using recombinant AAV-vector encoding human $VEGF_{165}$[54], and Ailawadi et al. reported enhanced vascularity and bursting strength of healing abdominal fascia by adenovirus vector-mediated VEGF transfer in a mouse-model[55]. However, all of the abovementioned studies have been performed on rat and mice models. Healing in these mammals results mostly from contraction by a strongly developed panniculus carnosus (‘musculus cutaneus maximus’) and only to a smaller extent by granulation and re-epithelialization.

This healing process is far from comparable to healing in human or porcine skin wounds. $VEGF_{121}$ is present predominantly in a soluble form, which may be appropriate for the microseeding technique to FTW in a wet wound healing model.

However, unlike $VEGF_{165}$, $VEGF_{121}$ lacks exon 7 of the VEGF gene, which confers a heparin binding capability. This property may generate diversity in growth factor signaling.

The heparin binding ability of $VEGF_{165}$ may enable the restoration of damaged $VEGF_{165}$ function in processes such as inflammation or wound healing, unlike $VEGF_{121}$[56].

$VEGF_{165}$ specifically binds to VEGF receptor-1 (Flt-1) which induces VEGF release and VEGF receptor-2 (Flk-1) for endothelial cell proliferation, migration, and survival. This dual pathway explains $VEGF_{165}$ proangiogenic actions in tissues[107].

The success of gene therapy is not solely a function of transfection efficiency or protracted gene expression. Despite relatively low transfection efficiency associated with the use of injection of naked DNA, studies in severely ischaemic limbs with arterial gene transfer of VEGF demonstrated that the angiogenic activity of VEGF is sufficiently potent to achieve a meaningful biological outcome[57] .In contrast, in our in vivo gene transfer study, elevated expression in wound fluids even led to diminished wound repair. This finding was confirmed in later ex vivo gene transfer studies using stable keratinocyte cell lines as carriers for PDGF-BB[53]. Elevated expression by viral gene transfer or stable cell lines appears not to be a prerequisite for wound repair protocols. Also, the microseeding procedure might play a role in delayed healing: with a rate of 4000 RPM, the needles of the Microseeding device create 20000 puncture holes in the wound bed and borders. The resulting biomechanical injury and prolonged inflammatory response may delay wound repair.

A whole spectrum of growth factors and cytokines is released in a sequential fashion in various concentrations and with highly interactive profiles in order to coordinate each of the phases of the regular wound healing processes: coagulation, debridement, extracellular matrix formation, neovascularization, and tissue remodellation. VEGF is probably the most important proangiogenic growth factor, but not the only one. Various reports indicate that TGF-b isoforms and PDGF family members play a significant role in neovascularization (REF). It is plausible that in this porcine FTW model, neovascularization takes place regardless of relevant $VEGF_{121}$ concentrations. Other factors may play a larger part in the proangiogenic process due to many interactions and synergisms between growth factors. We found a similar outcome for in vivo gene transfer studies using PDGF-BB as an effector gene (REF).

We have demonstrated that in old Yucatan pigs, endogenous VEGF concentration is strongly diminished but it is not reduced to zero. A veritable pathogenic porcine wound healing model should be developed that is recognized for reduced growth factor levels, such as in diabetes. However, a standardized porcine model with steady-state biochemically induced diabetes is much harder to establish than in lower mammals. When the pancreas is destructed by streptozotocin, the pigs require ‘intensive care’ and constant monitoring. Also, pigs require high and therefore expensive doses. We used Aloxan to induce diabetes based on available local protocols for diabetes induction in rats. However, it appeared to be very difficult to titrate the exact required individual doses needed to develop a steady-state hyperglycemia in adult pigs. Similar doses of Aloxan per weight led to hyperglycemia and multiple organ-failure over a few days in some Yorkshire pigs, but in others resulted in a minimal rise in glycemia and only for short period without clinical signs of distress (non-published results). Therefore, we currently focus on streptozotocin-induced diabetes[58] to create a pathogenic model to assess the impact of selected growth factors (and growth factor blockers).

Finally, in large and deep wounds such as in extensive burns, it is unlikely that growth factors would induce sufficient tissue repair; cells should be added to deliver ‘tissue substrate’ to bridge the FTW. ‘Intelligence’ could then be added to these cells to influence and modify repair and regeneration by ex vivo gene transfer protocols.

Given the outcome of these in vivo gene transfer experiments, we therefore focused on ex vivo gene transfer strategies using cell suspensions of highly proliferative basal cell keratinocytes as vehicles for gene transfer.

3. Establishing a defined porcine basal keratinocyte cell suspension culture system for ex vivo gene transfer under serum-limited conditions.

Ex vivo gene transfer represents a promising strategy to treat full thickness skin defects by adding cell substrate and 'intelligence' using cells as gene transfer vehicles. Before translating such strategies into a clinical setting, standardization of the optimal culture and transfection protocol is mandatory. In this study, we first analyzed the impact of different growth media on the cultivation parameters of porcine basal cell keratinocyte suspension cultures (BCKs) under serum-reduced conditions. The best medium was further applied in ex vivo gene transfer studies to investigate transfection efficiency of six distinct transfection reagents.

3.1. Cell growth of BCKs under serum-reduced conditions

BCKs have high clonal capacity and remain strongly attached to collagen I coated cell ware. Nakamura et al. described in 18% of these BCKs CD 90 expression, a probable marker of keratinocyte stem cells[59]. This BCK cell population therefore represents a promising target cell population for gene transfer and cell engineering for wound repair.

Porcine keratinocytes (KC) are acknowledged to be even more fragile than human KC cultures, and their overall take is disappointing. Therefore higher seeding-densities are required and high concentrations of bovine serum (FBS) are used as a rich source of mitogens for KC proliferation[60,61]. However, reduction or even elimination of serum in cell-media is obligatory in translational research: FBS has a large physiological variability and contains a wide range of minor components that may have a considerable effect on cell growth. Serum also has a short shelf life and poor consistency and may be contaminated with viruses that might interfere with cell growth. On the other hand, serum-proteins also have protective and detoxifying actions and the search for serum-free functional media may require different formulations to replace the growth enhancing capacities of serum[62,63]. Rosdy et al. demonstrated that the cultivation of cells and the development of a reconstructed epidermis could be produced without serum and dermal factors[64]. We tested growth parameters in porcine cell suspension cultures of cryopreserved young and primary cultured adult BCKs under reduced serum conditions. Human BCK thrive well in serum-free SFM, but porcine BCKs thrived best in a 2.5% FBS culture condition at primary culture. After 3rd passage, porcine BCKs also proliferated fairly in serum-free culture conditions. The migration rate was highest in SFM 2.5 % followed by our previous standard KGM 8%. The larger degree of differentiation in KGM II and Optimem may be the reason for diminished migration rates and lower viability scores after passaging. The regular cuboid shaped morphology of KGM 8% was mimicked by a slightly flatter morphology of SFM 2.5 % cultivated BCKs. In Optimem and KGM II, cells displayed irregular sizes and stages of differentiation.

For cultivation of stratified keratinocyte layers, these latter media will perform well, but not for specific cultures of highly proliferative basal cell keratinocytes as single cell suspensions.

Dispase and trypsin both are used to separate the epidermis from the dermis. Dispase II is a thermolysin-like neutral protease. Green et al. reported that the detachment of KC sheets by dispase II had virtually no effect on the viability of cells[65]. However, given sufficient time and concentration, even neutral proteases as Dispase II will damage cell surface proteins[66]. Trypsin is an aggressive serine protease .A trypsin/EDTA solution disrupts the intercellular junctions by calcium chelation, and it provides proteolytic degradation of the intercellular matrix[67]. The metabolic activity is strongly influenced by temperature. Cell distress by trypsinizing can be avoided by using low trypsin concentrations of 0.05-0.0625% or 'cold trypsinization' at room temperature or 4°C and also by adding EDTA. Several reports indicate that short incubation with trypsin/EDTA leads to higher post-seeding proliferation than when using trypsin without EDTA, thermolysin, or dispase[68,69]. When using SFM 2.5% as basal enriched serum–reduced medium for porcine BCKs, trypsin/EDTA 0.0625% was more efficient, consistent, and safe than dispase II in passaging steps.

Moreover, short incubation of BCKs by proteases such as trypsin may induce an episode of proliferation. Meyer-hoffert et al. demonstrated increased KC proliferation by activation of the protease-activated receptor PAR-1, which is expressed by KCs[70]. Trypsin activates PAR-1. Therefore we only use dispase to separate the epidermis from the dermis at primary culture. All other cell separation steps for BCKs are performed with trypsin/EDTA 0.0625%.

Cultures of irradiated human or mouse cells have been used since the 70’s to promote proliferation, particularly with low-density inocula[71]. High energy irradiation can completely suppress cell division long before general metabolism is affected. Since an irradiated "feeder" cell population continues to metabolize actively, the non-multiplying cells provide diffusible and short-lived growth plus conditioning factors to the medium. Human KCs can be cultured and serially passaged without a fibroblast feeder layer using a defined low calcium environment and altering seeding density, pH, and incubation conditions[72,73]. We used collagen I or fibronectin coated dishes to grow porcine BCKs in a standardized fashion. The primary function of fibronectin is cell adhesion to the ECM through an interaction of the cell binding domain with fibronectin-specific cell surface receptors[74,75]. Other domains interact with collagen, heparin, and cell surface glycosaminoglycans[76]. We found no significant difference in cell migration and colony forming units of cell cultures seeded on fibronectin coated dishes versus the collagen coated dishes. Collagen or fibronectin seem not to be a requirement for epidermal growth and maintenance, but they may select for attachment of basal cells and discourage attachment of cells committed to differentiation[77].

High calcium and serum concentrations induce differentiation, which results in extensive desmosomal cell attachment and stratification, and it is associated with the expression of a variety of differentiation marker proteins[78,79]. Ca2+ seemed to be most important in determining whether a colony would form, rather than how rapidly a colony would expand.

However, we found that with Ca2+ concentrations below 0.075 mM, cultures did not survive passaging well, but at levels around 0.4 mM, cells grew rapidly but stratified and formed an upper layer of squames as they reached confluence.

Hennings et al. have shown that lowered medium calcium concentration can significantly prolong the culture lifespan of epidermal cells by shifting the pattern of growth and differentiation in favor of continued proliferation[80]. There is an inverse relationship between growth response (extent of clonal growth) and the differentiation response (extent of differentiation)[81]. To optimize the role of BCKs as vehicles for gene transfer, we focused on serum-reduced growth conditions that favor migration and proliferation and impair differentiation.

3.2. Non –viral transfection protocols for basal cell keratinocyte cultures

Gene therapy to selectively elevate or down-regulate expression of a particular growth factor in the wound milieu is promising in promoting tissue repair, especially for wound healing, which is a local event and requires only temporary high levels of transgene expression[23]. Previously, we documented our choice for non-viral gene transfer in our ex vivo gene transfer protocols. Skin is a useful target for gene therapy not only because of its accessibility and abundant vascularity but also because of its capacity for regeneration[8,41,47].

In BCKs under serum reduced conditions, Lipofectamine 2000 and Fugene 6 lead to inferior transfection efficiency (TEE) when compared with Lipofectin. Lipofectin is a synthetic cationic lipid polymer which is able to fuse with membranes of mammalian cells. In previous experiments we found that cytotoxicity is high in cells derived from aged pigs. After five hours of incubation, these cells are markedly stressed with high losses at subsequent passaging. In young cells however, no morphologic changes were seen, even after overnight incubation. Fugene 6 was found to have minor cytotoxicity.

To improve TEE, we increased the time needed to form the complex up to two hours; following the recommendation of the manufacturer and because of low cytotoxicity, we increased the amount of both Fugene 6 reagent and DNA (4 ug). Neither of these steps led to higher TEE in adherent BCKs. Kiefer et al. found minimal TEE for Fugene 6 in serum reduced media, but high efficiency in serum-containing medium[82]. However, our aim was to focus on high tranfection efficiency in serum-reduced conditions.

In a subsequent set of experiments, we compared Lipofectin with newer transfection reagents PIE, Effectene, and Fugene HD for ex vivo gene transfer of BCKs with LacZ and GFP as reporter genes. PEI is a transfection reagent directed at epithelial and endothelial cells with high efficiency and low cytotoxicity[83] . In our porcine BCKs, PEI-based transfection resulted in higher efficiency than Lipofectin. Cross-linking of small PEIs with judiciously designed amide- and ester-bearing linkers may boost their gene delivery efficiency both in vitro and in vivo[83] and might be promising for future applications, especially when combining BCKs and endothelial progenitor cells to improve angiogenesis. Effectene is another promising transfection reagent. Only a small amount of DNA is required, cytotoxicity is low, and cells do not need to be highly confluent.

Zellmer et al. found a 20-fold higher transfection efficiency of Effectene compared to Lipofectin and Lipofectamine in human keratinocytes[84]. Using our BCKs, TEE was higher than with the previous Lipofectin based standard protocol, but consistently lower than with Fugene HD. Fugene HD is a next generation non-liposomal multicomponent reagent suitable for transfection of mamalian cells for protein expression and may also be used with or without serum. Its complexes interact with nucleic acids and promote efficient transfer across cellular membranes with minimal cytotoxicity and physiological and morphological cell changes. In these BCKs, Fugene HD resulted in highest TEE using different plasmid concentrations and in different ratios of DNA.

Fugene HD transfected BCKs kept their viability and proliferation capacity after all passages as measured via GFP expression. By further optimizing the transfection methods in regard to the time of transfection, the ratio of cells versus the lipid: DNA complexes and the ratio of lipid versus DNA, TEE may be further enhanced.

Currently, we also investigate Nucleofection (Amaxa,Koln,Germany), an electroporation-based transfection method that enables the DNA to enter directly into the nucleus. Distler et al. investigated six programs which differ in strength of the electric field and length of electric pulses[85]. They reported an optimal TEE of 56% in primary human keratinocytes and the number of non-viable cells was low (14-16%). An advantage is that results can be analysed within seven hours.

A disadvantage is the need for the Nucleofector device itself. Nucleofection may offer novel opportunities for various research applications for therapeutic procedures in which gene transfer is required.

4. Ex-vivo gene transfer of growth factors to full thickness wounds using keratinocyte cell suspension cultures as vehicles

Gene therapy justifiably is gaining interest as a treatment modality for wound repair and tissue regeneration. In vivo gene transfer of growth factor-encoding genes into the wound environment by ‘gene gun’ or microseeding may lead to high expression of growth factors in the wound but does not add supplementary cell substrate to the defect (see 2.2). In large full-thickness skin wounds such as extensive third-degree burns or large chronic wounds, this lack of substrate might be critical to repair. Ex vivo gene transfer of transgene-expressing cells brings both cell substrate and growth-promoting genes into wounds, which might lead to more effective treatment than either therapy alone[86].

In a clinical context, cell cultures need to be readily available for treatment, such as in an extensively burned patient, where rapid debridement of the burn scar must be immediately followed by coverage of the defects and sparse donor sites for split thickness skin grafts must heal rapidly so they can be be reused. Autogenic cell cultures will become fully integrated in the defect while allogenic cells will gradually be replaced by endogenous cells and serve as a temporary biomatrix and supplier of growth factors to the wound.

The advantage of allogenic cells is that they can be cultivated, expanded, cryopreserved, stored and thawed again when required[87,88]. Therefore allogenic cells may be very useful as carriers of growth factor expressing plasmids to FTW.

Previous experiments demonstrated that fibroblast cell cultures served as efficient carriers for growth factors into FTW, but did not consistently lead to accelerated repair (non published data). In this study, we used cell suspensions of autogenic and allogenic porcine basal cell keratinocytes (and not differentiated allogenic keratinocyte sheets) as vehicles for human Epidermal Growth Factor (hEGF) after cationic liposomal mediated ex vivo gene transfer. EGF is produced by platelets, monocytes, and macrophages and is present in high quantities in the early phase of wound healing[23,89,90]. EGF likely increases wound healing by stimulating the proliferation and migration of epithelial cells and mesenchymal cells. We demonstrated that allogenic keratinocytes serve as efficient gene delivery vehicles for hEGF and also further accelerate wound healing similar to autogenic BCKs. Moreover, when overexpressing hEGF, the allogenic BCKs further improved repair.

It is generally accepted that allografts (homografts) are efficient biodressings that temporarily cover the floor of chronic wounds and acute burns and induce granulation and vascularization[91]. It also has been demonstrated that such allografts are rejected from wounds after 10-14 days. Several authors have reported that allogenic keratinocytes reject at a slower rate than allogenic skingrafts. Aubock et al.[92] investigated the properties of allogenic and autogenic cultured epidermis and concluded that Langerhans-cell free allogenic cultured epidermis does not survive permanently, but the rejection rate was delayed by 4-5 days when compared with allografted skin grafts.

Using single cell allogenic keratinocyte suspension cultures, rejection may be further delayed. It was postulated that the absence of MHC proteins could lead to a diminished host response against allografts[93,94]. Dierch et al.[95], however, showed that both MHC-I and MHC-II knockout allografts still underwent rejection, which was considered to be the result of the existence of ‘minor antigens’.

Hunt et al.[96] reported that allogenic cultured keratinocytes grown from MHC-II knockout mice created fewer immunogenic cells. By using ‘xenogeneic-syngeneic’ mixed epithelial sheets, a histologically well organized epidermis could be grown, presenting basal and suprabasal cell layers and an active dermo-epidermal junction. The xenogeneic cells were ‘selectively’ eliminated without rejection of the whole ‘mixed’ implant, in contrast to purely allogenic cells which had a 100 % rejection rate. Immunofluorescence staining showed that syngeneic cells gradually replaced the allogenic keratinocytes[97,98].

We were able to demonstrate that allogenic BCKs covered the wound surface from within the wound and promoted re-epihelialization as compared with saline controls. After completion of wound coverage, neither an increased inflammatory reaction, nor epithelial thinning, nor wound breakdown could be detected in wounds over a period of 35 days post-transplantation.

Based on these findings and the knowledge that allogenic BCKs are gradually replaced by autogenic BCKs, it appears that transplantation of allogenic BCKs not only provides accelerated wound coverage but also allows for ingrowth of autogenic keratinocytes. Therefore, transplantation of allogenic BCKs in a single cell suspension can reduce the need for re-transplantation and therefore reduce the cost of wound treatment in the wound healing model used in this study. Moreover, to further eliminate the impact of rejection of allogenic cells in larger wound settings, a mixture of allogenic and autogenic keratinocytes can be used for wound treatment. This strategy reduces the amount of autogenic keratinocytes required for wound coverage while offering accelerated healing of large wounds.

5. Skin substitutes and smart temporary 3-D scaffolds

Although artificial scaffolds may improve the morphologic characteristics of the wound bed, dermal regeneration remains limited histologically. This is probably secondary to constraints on fibroblast repopulation and on remodeling of the scaffold, as well as inflammatory responses secondary to the polymer framework. Unlike the extracellular matrix which represents a millenary natural evolution, artificial biomaterials do not have such a complex structure and chemical composition[14,28,99]. In consequence, the trade off to large-scale production and standardization of these 'man-made devices' is the very low bio-information content and the scarce quantity of signals they transmit to cells.

Allogenic or xenogenic materials also carry a risk of viral transmission and could be potentially immunogenic. Moreover, currently used biodegradable scaffolds in cardiovascular tissue engineering showed toxic degradation products and elicited significant inflammatory reactions[12,13]. The shorter this biodegradation, the higher the load of toxic side products that is introduced into the microenvironment.

Furthermore, actual dermal templates integrate on the wound surface by vascular diffusion and therefore have very limited volume. In full thickness restoration of skin after deep burns, the application of dermal templates, such as Integra, lead to enhanced elasticity but still limited tissue volume, as previously shown[5,10,11]. Current skin equivalents therefore do not represent our final needs for burn patients and further intensive research and development is required to obtain authentic full thickness skin equivalents. However, such a full thickness skin equivalent should be autogenic and vascularised; hence cell engineering, gene transfer technologies and biomaterial sciences may be required.

The concept of combining synthetic carriers with the cell-recognition sites from naturally derived biomaterials is very attractive. Such hybrid materials could carry desirable properties from both origins[15,17,99]. However, cells adhere and interact with their extracellular environment via integrins, and their ability to activate associated downstream signaling pathways depends on the character of adhesion complexes formed between cells and the extracellular matrix, which in turn determines which interactions take place in cytoskeletal organization, growth factor responses, and cell cycle patterns. Therefore, the development of materials that can specifically and molecularly interact with cells in the defect has become an emerging field in research.

6. Matrix deposition and induction of angiogenesis by ex vivo gene transfer

In clinical tissue reconstruction, large defects need to be reconstructed with well vascularized tissues by transplantation and microanastomosis to a local blood vessel network[1-3]; the absence of a vascular network capable of distributing oxygen and nutrients within the matrix is the key limiting factor in the overall success of custom-made tissues produced by tissue engineering. Artificial skin constructs undergo imbibition, fibroblast migration, neovascularization, and vessel maturation while the dermal layer resorbs[7] .This process takes weeks to months, and during this period the construct is fragile and prone to infection. So far, tissue engineering protocols have been focused on limited thickness to aid these constructs in surviving the initial episode of hypoxia by diffusion of nutrients until neovascularization takes over[11]. The ultimate difficulty in tissue engineering is to incorporate an inherent vascular network, such as a capillary bed, that is ready to be connected to the host vascular system. A viable capillary network within the tissue construct represents the missing link between the host and the engineered implant[12-14]. The formation of such a vascular network depends on guidance molecules within the matrix[101-103]. The adult vascular network remodels itself by arteriogenesis, with the enlargement of existing collaterals induced by shear forces as well as by the formation of completely new vessels from existing vessels (arterialisation). Post-natal vasculogenesis occurs via migrating dedifferentiated EPCs and monocytes which form tubules. Remodelling is mediated by monocytes and endothelial progenitor cells[104-106]. The extracellular matrix functions as a reservoir of growth factors to induce incoming blood vessels (angioinduction) as well as a scaffold for EPCs to participate in arteriogenesis.

We demonstrated, in our standardized porcine full thickness wet wound healing model, that cell suspension cultures of VEGF-overexpressing BCKs under regulation of a TC-inducible gene switch had the effect of enhancing fibronectin deposition, endothelial cell tubuli formation, and accelerated reepithelialization of FTW. The effects may be explained by the complex crosstalk between KCs, FBs, the ECM, VEGF, and ECs[101-103]. VEGF is a key factor in this interplay; VEGF has the ability to stimulate all required processes for angiogenesis and vasculogenesis[107] and also increases vascular permeability. Extravasation of plasma proteins results in the formation of a highly pro-angiogenic ECM. This pro-angiogenic matrix is rich in plasma-derived fibrin and fibronectin, rather than interstitial products such as hyaluran and collagen. Capillaries grow through this plasma-derived ECM for the first few days and then through fibroblast-derived granulation tissue for the next two weeks[108-109].

Fibronectin is an ECM glycoprotein, predominantly secreted by FBs and activated by KC-signalling to form and maintain the structural integrity of connective tissue[101].

KCs themselves produce integrins and fibronectin receptors to allow KC migration throughout the wound by cell-matrix interaction[102]. This profound fibronectin involvement demonstrates its importance in the wound healing process and its possible use as an appropriate tool for the measurement of newly formed ECM . In accordance, we found denser fibronectin deposition in wounds treated with KCs as compared to saline controls. Mitsi et al[102] suggested that VEGF interacts with fibronectin through conformational changes in heparin binding sites.

This suggests a significant contribution of the ECM to modulating angiogenesis. Moreover, in research practice, fibronectin is the preferred coating for in vitro endothelial progenitor stem cell growth, indicating a direct interaction between fibronectin deposition, EC adherence, and vasculogenesis[110,111]. In all KC-treated wounds, we measured elevated fibronectin deposition and more EC structures relative to saline controls. Moreover, TC-triggered-KCs demonstrated an increase in $VEGF_{165}$ concentration in the wound microenvironment and a further elevation in EC counts.

These data suggest that high VEGF expression further induces the recruitment and proliferation of ECs in the fibronectin-rich matrix of FTW. Cai et al demonstrated that the EC response to VEGF is dependent on the local concentration of VEGF, VEGFR-2 phosphorylation, and intracellular regulators such as the protein tyrosine phosphates and eNOS[112]. Upon VEGF binding, VEGFR-2 activation initiates a cascade of intracellular downstream signalling events leading to increased proliferation, migration, and EC survival[113]. Also, the availability of ECs may have a beneficial effect on revascularisation and angiogenesis in hypoxic tissue such as FTW[114]. Metalloproteinases (MMP) and their tissue-derived inhibitors (TIMPs) determine the architecture of the ECM by digesting basement membrane and ECM proteins. Specifically, membrane type 1 MMP was recently demonstrated to be involved in the polarization of ECs to form tubules during capillary sprouting[115]. These MT1-MMPs are expressed by ECs, FBs, and basal KCs[116,117]. We found stronger EC staining and tubular formation in wounds treated with VEGF-expressing KCs (Fig.2). This finding may be explained by MT1-MMP secretion by the ECs and VEGF-transfected basal KCs within the fibronectin-rich proangiogenic matrix. Since MMPs have been recognized as vital mediators of angiogenesis, they may represent a potential target for our future tissue repair therapies.

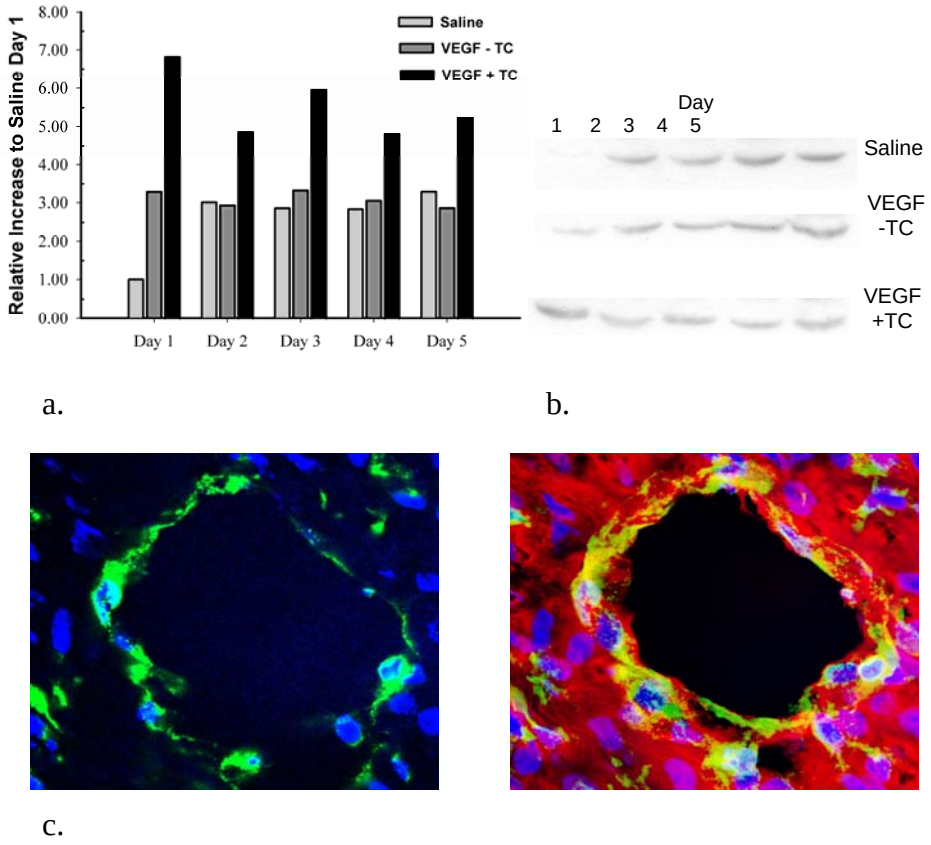


Fig.2. a/ MT1-MMP expression in FTW treated with KC overexpressing VEGF by activation of the gene switch (VEGF+TC), is significantly higher than controls during 5 consecutive days. b/ Western Blots indicate the higher MT1-MMP expression in the VEGF+TC group. c/ Confocal microscopy confirms the 3-D tubular structure and lumen; blue: cell nuclei (Dapi), Green: EC (Lectin), red: fibronectin (Safranin O).

Since the appropriate cocktail of cells and growth factors may strongly induce angiogenesis and tissue growth, we must be able to regulate timing and gene overexpression levels to avoid progression of healing towards hypertrophy, sclerosis, or even cancerogenesis[118,119]. In this study, VEGF expression of the KCs was under the control of a tetracycline-inducible switch.

7. Gene regulation for gene transfer protocols in tissue engineering

Successful implementation of gene therapy relies not only on delivering a therapeutic gene into target cells efficiently, but ultimately on developing a genetic device in which expression of a therapeutic gene could be regulated in a predictable and effective way.
New strategies involve the inclusion of promotor regions that are only active in specific tissues or at specific stages of cell differentiation or that can be regulated by exogenously supplied drugs. To date there only are four such systems[23].

The common feature lies in their employment of a minimal mammalian cell promotor, which by itself exhibits little basal activity, combined with a transactivator whose activity is regulated by a pharmacological molecule, such as tetracycline in the tetracycline inducible system[120].

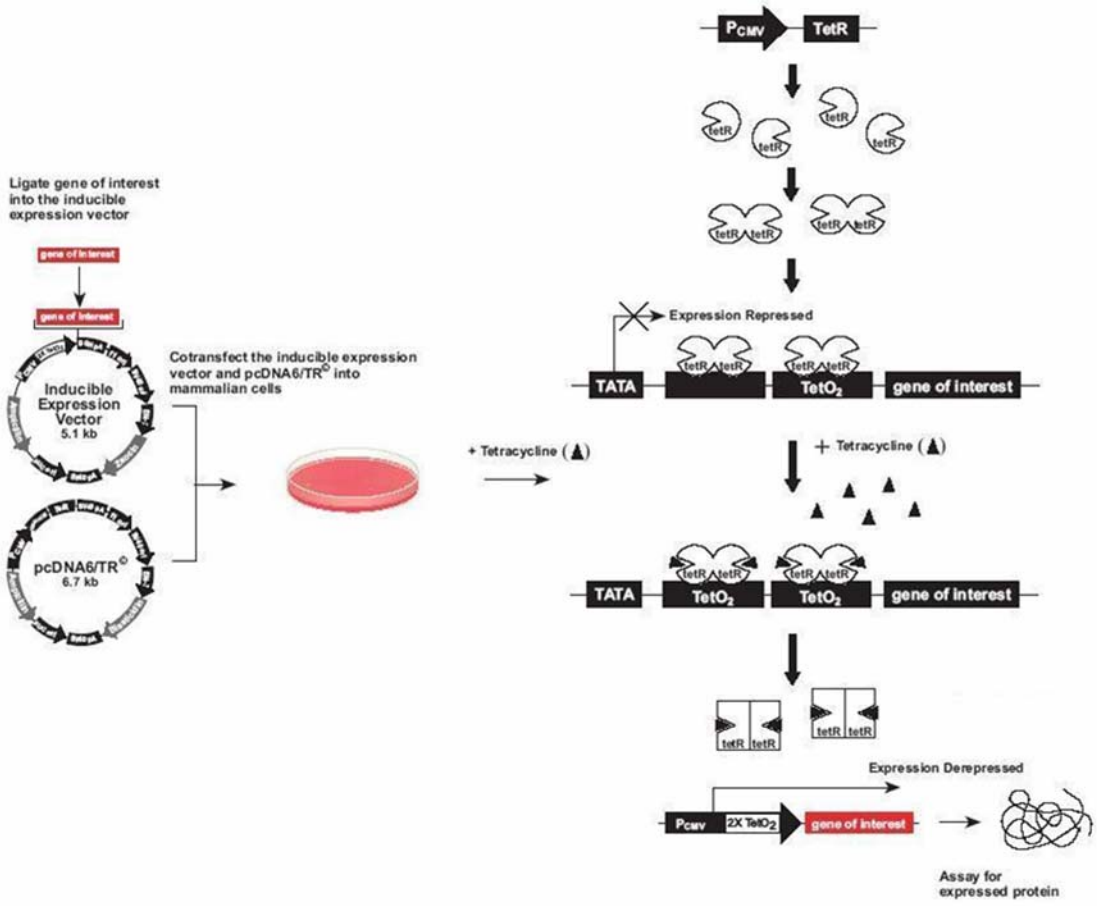


Fig. 3. The Tetracycline regulated mammalian expression vector uses a repressor mechanism to block transcription from the promoter in the absence of tetracycline. The system consists of two components: The pcDNA™6/TR regulatory vector provides high-level expression of the tetracycline repressor (TR) protein and the expression vector which contains the gene of interest (GOI). Inserted between the TATA box of the CMV promoter and the transcriptional start site of your GOI are two tetracycline operator sequences ($TetO_2$). When mammalian cells are transfected with these two components, expression of TR from the regulatory vector binds to the $TetO_2$ sequence and represses transcription of the GOI. Tetracycline added to the wound chambers binds with high affinity to and alters the confirmation of TR which is then released from the $TetO_2$ sites, allowing transcription of the gene of interest (Yao et al. Human Gene Therapy, 1999).

Yao et al improved the use of the TC-inducible regulatory system[31,32] and demonstrated that TC can function as a potent modulator to regulate gene expression in mammalian cells (Fig. 3).

The major immediate-early enhancer promotor can be regulated by tetR over three orders of magnitude in response to tetracycline when the reporter is cotransfected with the tetR-expressing plasmid in transient expression assays.

We integrated a tetracycline-inducible regulating gene switch in an EGF and VEGF expression plasmid to allow in vivo gene regulation in the full thickness skin wounds. With correct positioning of $VEGF_{165}$ downstream of the TC-inducible operator and TATA box, we illustrated a dose-dependent regulation of $VEGF_{165}$ expression in vitro and in vivo.

By administering BCKs into FTW, we showed that $VEGF_{165}$ levels can be controlled by the addition of varying concentrations of TC directly into the wound. In this study, this mechanism allowed us to specifically analyse the impact of additional VEGF-overexpression in the setting of KC delivery to FTW. This gene switch enables the downregulation of gene expression for safety concerns; the optimization of sequence and appropriate timing or expression of genes ad libitum is essential when using more stable genes.

8. Actual clinical applications of growth factors and tissue engineering concepts

8.1. Platelet Rich Plasma Gel.

Studies indicate that platelets may provide a critical link between coagulation and inflammatory cascades via regulated production of growth factors and cytokines:
PDGF-AA and BB, IGF-1 and 2,TGF- β1, and IL-1β signal the local mesenchymal and epithelial cells to migrate, divide, and increase collagen and matrix synthesis required for bridging and closing the wound defect[121,122]. Autologous platelet-rich plasma gel (APG), obtained by sequestering and concentrating platelets by gradient density centrifugation from autologous whole blood, may be used clinically to deliver these growth factors in high concentration to the wound microenvironment.

In an experimental setup using our standardized wet wound healing porcine model, we recently analysed the capacity of PRP-gel to serve as a temporary autogenic biomatrix for ex vivo gene transfer protocols[123].

$VEGF_{165}$ overexpressing BCKs were seeded in fresh porcine platelet rich plasma activated by autogenic thrombine (derived from the platelet poor plasma fraction). High concentrations of PDGF-BB, IGF-1, and VEGF were found in fluids from wounds treated with PRP.

Therefore, the platelet gel can also be used as a natural autogenic growth factor delivery system to wounds[124]. In a retrospective study in ten burn patients with 3rd degree burns, we found that skin graft donor sites healed faster than nontreated areas. When PRP gel was sprayed over split thickness skin grafts, we found a higher graft take, diminished need for dressing changes, and less pain at donor sites. Additionally, we actually conduct a prospective clinical study using autogenic PRP gel in donor and recipient sites of 3rd degree burn wounds. A Color Laser Doppler method is used to determine exact depth of wounds for optimal symmetrical analysis. The impact of PRP gel is determined by analyzing healing parameters, graft take, dressing changes, pain assessment scales and long-term scar formation.

8.2. Vacuum assisted closure

Also known as negative pressure therapy, vacuum assisted closure has gained its rightful place in the armamentarium of physicians dealing with extensive wounds. Vacuum assisted closure uses foam dressings with fixed pore size and steady continuous or controlled intermittent suction, and has settled in the niche between wound debridement and reconstruction in a straightforward and efficient fashion.

The VAC system allows for intensive wound exsudate management, creating a moist environment based on circulation of wound fluids[125]. The aspiration of these wound exsudates reduces the amounts of bacterial clusters and proteinases initially present in high concentrations in chronic wounds.

Once the wound enters an anabolic and thus healing stage, growth factors are no longer broken down by the now reduced levels of proteinases, and their function is facilitated by the wet wound healing environment created in the foam dressing.

The negative pressure creates a gradient that biomechanically favors the outgrowth of granulation tissue by stimulation of proliferation and migration of cells[126].

This stimulus presumably is given by the constant alterations on the cellular cytoskeleton due to the vacuum: integrin bridges of the cytoskeleton are disrupted by subathmospheric pressure, triggering the release of intracellular secondary messengers which in turn upregulate cell proliferation[127]. It was demonstrated that intermittent suction promotes this granulation tissue outgrowth even more intensively (Fig. 4).

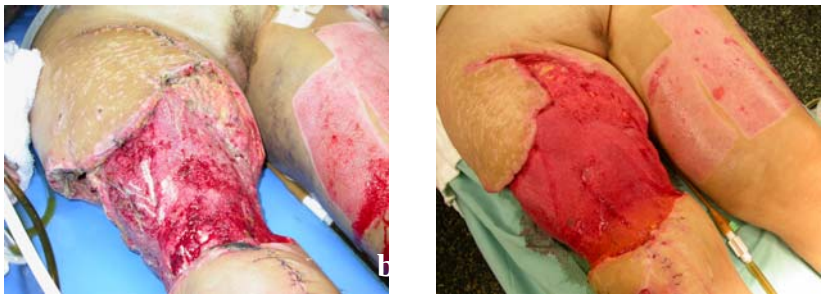


Fig.4. Severe Crush-avulsion trauma of the tigh. Status after serial debridements (a) Status after multiple VAC applications under intermittend pressure cycles (b). The wound is now prepared for skin grafting.

Intermittent vacuum therapy also enhances perfusion to the wound borders each time the aspiration cycle starts. Some reports described an increased local bloodflow that lasts 5-7 minutes. This enhanced blood flow delivers increased oxygen and leukocytes[125,127].

By gradual aspiration of interstitial oedema caused by inflammation, the distance between healing cells and capillaries diminishes and tissue oxygenation improves.

However, vacuum-assisted closure in all its efficiency does not replace appropriate debridement nor does it offer an instant treatment for highly contaminated wounds.

To avoid the risk of promoting a deeper or systemic infection, the wound should always be completely debrided before applying VAC.

All of these features might explain why chronic contaminated wounds, after an intial debridement of debris, can be successfully treated with vacuum assisted closure with foam replacements every 3-4 days, whereas coverage with meshed allografted skin at that stage often cannot withstand the unfavourable healing environment[125].

Through biopsies and protein assays on wound fluids, we currently try to determine alternating growth factor and cytokine profiles of wounds before, during and after VAC treatment. These data could provide us with important knowledge of key factors in the repair and regeneration process.

8.3. Prefabrication

In prelamination and prefabrication procedures, the natural anatomic boundaries of tissues are altered in order to procure more optimised donor tissues for reconstruction.

Prelamination of a flap at a distant site can provide the missing layers for reconstruction of extensive and complex defects by suturing tissue layers on top of a well-defined axial vascular territory that serves as a composite vascular carrier.

In prefabrication procedures, a vascular source is transposed into a non-axial area to provide an alternative blood supply through neovascularization of a tissue[128].

The purpose is to create a well-defined axial vascular territory in tissue areas that are not outlined by a specific angiosome/venosome structure.

Several strategies for prefabrication exist. Tanaka et al. and Tark et al. analysed the potential for angiogenesis by "tissue engineering" skin flaps using various types of vascular carriers[129,130]. Both the arteriovenous shunt loop and the arteriovenous bundle groups showed marked angiogenesis in the adventitia of the femoral artery and vein and in the surrounding tissue. This observation is noteworthy since angiogenesis was previously reported to occur only in arterioles and venules[131].

Additionally, the wall of the femoral vein appeared to have many holes, representing luminal sprouting. When promoting factors such as VEGF dominate over inhibitory factors, pericytes become separated from endothelial cells, which are rendered reactive to the promoters. These stimulated endothelial cells produce various proteases that digest the vascular basement membrane and adjacent extracellular matrix, and initiate endothelial sprouting in the direction of the stimuli[132]. Endothelial cells that have migrated through the basement membrane into the matrix further migrate and proliferate, and cell-cell adhesion by vascular endothelial-cadherin leads to the formation of a tubular structure. Other growth factors such as basic FGF and TGF-beta play a role in supporting this formation within the framework of the extracellular matrix[133,134]. Inflammation, hemorrhage, and coagulation associated with surgical trauma help to promote this angiogenesis[135,136].

Clinically we use prefabrication and vascular delay procedures for complex reconstructions. For instance, we wrap and vascularize a 4 cm trachea segment with a forearm fascia flap.

After 3 months –but already feasible after 2-3 weeks- this vascularized 'axial' trachea segment is opened U-shaped, and transferred to reconstruct a hemilaryngeal defect after unilateral laryngeal cancer resection[137]. In nose reconstruction, a newly reconstructed nose cartilage framework can be covered with a pedicled forehead skin flap that is sutured over this nose cartilage but left attached and rooted to its vascular supply at the eyebrow vessels.

After 2-3 weeks of vascular delay during which small bloodvessels form in the reconstructed nosetip, the vascular supply can be cut, and the skin flap trimmed and modified for optimal lining[138].

In tissue expansion techniques, the relative hypoxia created by the expansion process, induces enhanced VEGF expression in combination with mechanical strain and stress induced tissue regeneration. This concept we often use after deep burns of the scalp to generate more hair-bearing skin[139].

The concept of three-dimensional vascularised tissue engineering with incorporated blood vessel networks stands far from a clinical context ad hoc.

Future approaches might consist of prefabrication of blood vessel networks by combining gene therapy of growth factors and Endothelial Progenitor Stem Cells seeded on porous biomaterials[140-143], or authentic bio-engineering strategies to fabricate vascular channels by silicon micromachining or micro and nanotechnology for replacement microvessels[144,145]. Awaiting such innovations, prefabrication procedures might gradually be merged with tissue engineering protocols and gene therapy to create vascularized tissue equivalents

9. Conclusions

So far, the construction of an autologous or even allogenic bio-inducible three-dimensional living matrix has not been achieved yet. The required matrix needs to mimic the regular wound healing processes, the mediators and cells involved, and the complex interactions with the wound microenvironment. Ex vivo gene transfer and engineering technologies may succeed in seeding biocompatible matrices with autologous stem cells that overexpress selected genes.

This gene expression leads to the production of proteins elementary to the repair processes. Introduction of such proteins in the matrix and in the healing environment may strongly influence the micro-niche around the wound, the paracrine influences to local and distant cells, and may determine the final fate of stem cells and progenitor cells migrated to the wound microenvironment. The impact of such proteins on wound regeneration, healing, and tissue engineering might be extensive; precise gene regulation in vivo therefore is paramount to safety and control.

To treat extensive burns, congenital skin deformities, or deep skin defects after trauma and skin tumor excisions, an autologous full thickness ‘smart’ skin equivalent would deliver the required autologous treatment for tissue reconstruction, improving functional and esthetic outcome, social reintegration, pain relief, and quality of life. Ad hoc novel strategies in wound repair are based on changing the wound microenvironment, eliciting repair and regeneration processes, vascularization, and cell growth. This actual multidisciplinary approach brings together the molecular biology of cells and genes, the material sciences of matrices and scaffolds, and the clinical expertise of tissue and wound repair, and might well lead to the cultivation and production of a veritable authentic and autologous full thickness skin equivalent.

The point of living and of being an optimist
is to be foolish enough to believe the best is yet to come.
Peter Ustinov

Chapter V: Current concepts & research perspectives

We used the platform described in this thesis project to found the 'Lopster' Laboratory of Plastic Surgery and Tissue Engineering Research at the settings of the KUL Leuven University Hospitals.

With the 'smart autogenic 3-D vascularized tissue engineering' concept in mind, we further investigate the development of vascular networks within a three-dimensional biomatrix that we built up with autogenic elements.

Recently we cultivated an autogenic matrix laminated with fibroblast sheets and covered with VEGF-expressing basal cell keratinocytes. We currently analyze cell behavior and lamination properties. We also continue our projects using autogenic platelet rich plasma gel as a resorbable biomatrix in which we induce angiogenesis by the ex vivo gene transfer approach.

In recent experiments, laminated cultured fibroblast sheets were used embedded in PRP in a synergistic fashion (our 'calzone- principle').

Angiogenesis is induced by $VEGF_{165}$-expressing cell cultures and endothelial progenitor stem cells (EPCs)[146].After having identified human and porcine EPCs by PCR using multiple primers, immunohistochemistry, and uptake assays, we actually perform in vitro and in vivo angiogenesis models to analyze the development of endothelial networks in various 3-D substrates, among which the above mentioned laminated fibroblast sheets. Currently these promising strategies are translated into in vivo studies in our standardized porcine model. These experiments are performed with enthusiastic support and cooperation of Dr. A. Luttun and his team at the Centre of Molecular and Vascular Biology at the KUL Leuven.

In another project we use lipoaspirate-derived mesenchymal stem cell fractions to cultivate chondrocytes,osteocytes and adipocytes[147]. The adipocytes are cultured to obtain adipose tissue which is required to built up the subcutis in full thickness skin, or as substrate for breast reconstruction, in the treatment of scars or even in aesthetic augmentation surgery. The chondrocytes will serve in tissue engineering protocols to cultivate and generate a cartilage framework that may be used for nose and ear reconstructions. Major difficulty is to regulate the differentiation of stem-or progenitor cells in in vivo experiments and once in loco, to trace the origin and destination of these progenitor cells.

For 3-dimensional constructs we need a 3-dimensional shaped biomatrix as a template. We work with rapid prototyping technologies, such as 'stereolithography' and 'fused deposition modeling' in a study design developed with collaborating laboratories of biomaterial sciences abroad. From data obtained by CT imaging, three dimensional shaped images are generated and translated into a bioplotted template[148]. This custom-made porous template is further treated with ex vivo gene transfer protocols, using autogenic cells that gradually invade the template while secreting pro-angiogenic growth factors which induce and coordinate matrix deposition and angiogenesis (Fig 1).

Major difficulty is the synergism between the structure and compliance of the resorbable material, its toxicity at resorbtion and the impact on the progenitor cells applied to the matrix. Our ultimate aim is to generate custom-made vascularised three-dimensional tissue constructs built up by autogenic donor cells for use in the broad domain of plastic and reconstructive surgery.

To achieve this goal, a multidisciplinary strategy is elementary. While rapid prototyping translates clinical scan data into 3-D scaffolds, biomaterial sciences will further integrate engineering principles in the development of rigid or flexible, permanent or resorbable scaffolds in which applied (stem)cells can migrate, proliferate and reorganize into tissues (Fig 2). These cells may be harvested in an autogenic fashion and after separation and purification cultivated. Gene transfer may introduce further ‘intelligence’ by transfecting (stem)cell cultures with protein expressing DNA plasmids to promote eg. cell migration and proliferation, matrix formation, angiogenesis and remodeling.

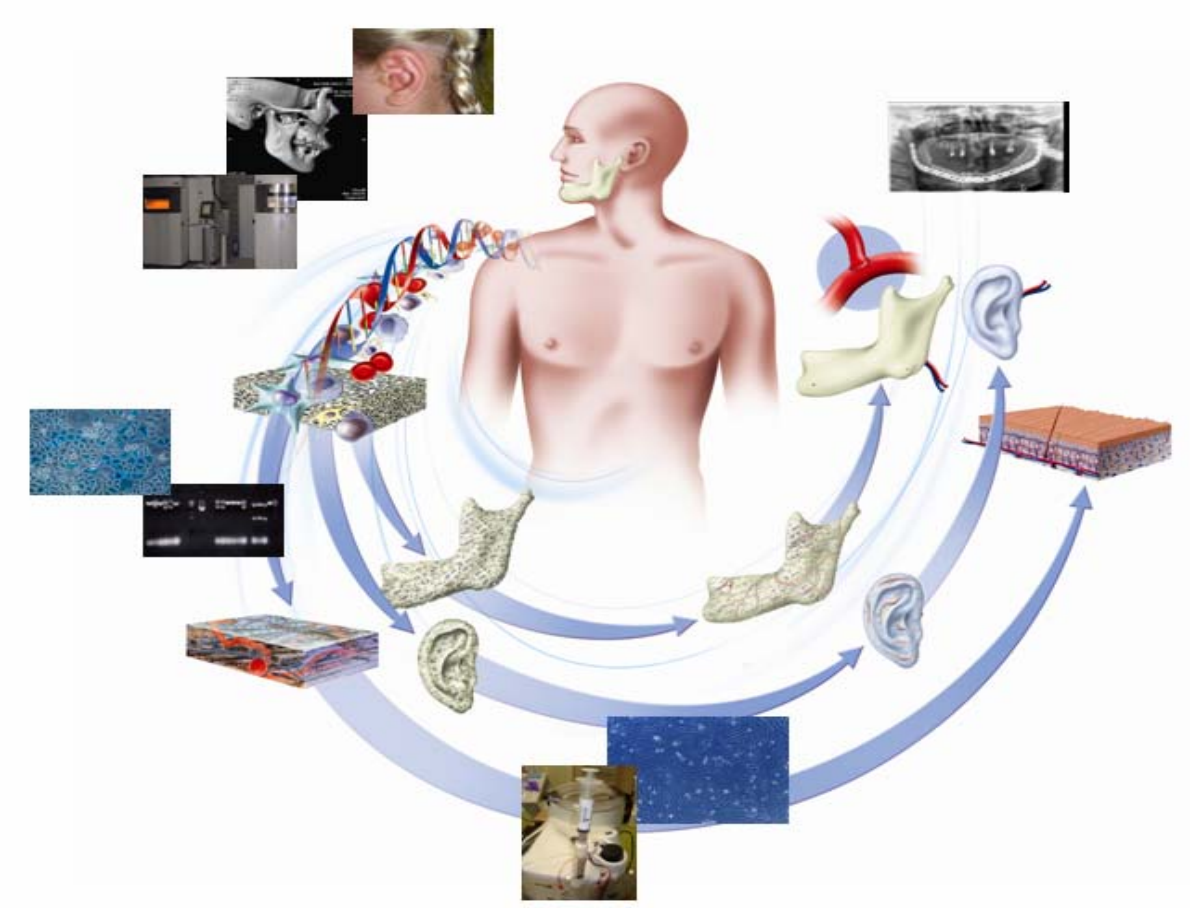


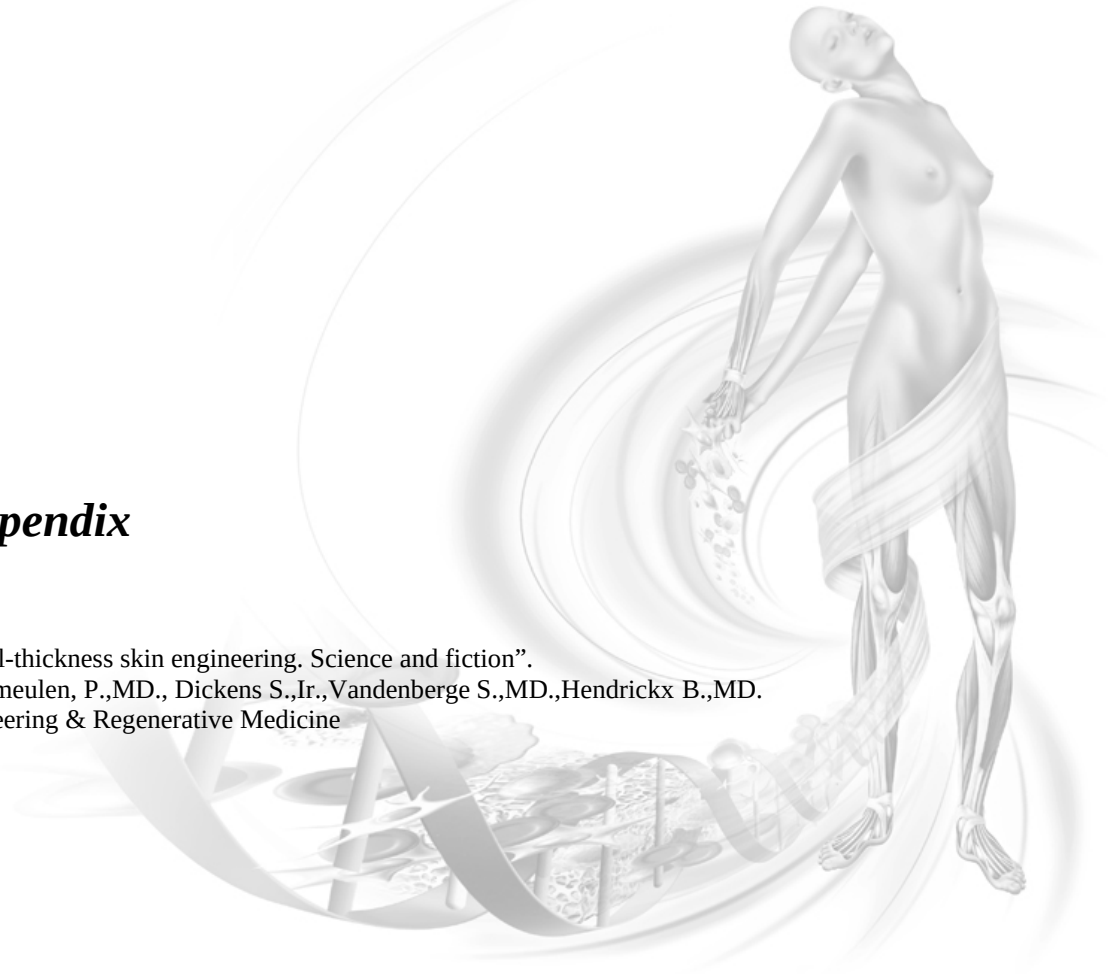
Fig. 2. The smart autologous tissue engineering concept:
CT-scans or real-time pictures are used as digital model for rapid prototyping into a custom-shaped 3-dimensional scaffold in bioresorbable material. This porous scaffold is seeded with ex vivo transfected autologous cell cultures that overexpress selected growth factors, cell adhesion molecules or cytokines, such as VEGF. These proteins create a micro-niche that stimulates cell migration, proliferation and extra cellular matrix formation. The induction of intrinsic vascular networks occurs in this matrix by seeding endothelial progenitor cell cultures and by the VEGF overexpression by cells. Finally, the 3-D custom-made vascularized construct needs to be integrated in the in vivo defect by ligating its intrinsic blood vessels to in situ bloodvessels near the defect by (supra)microsurgery.
(picture courtesy of JJ.Vranckx).

State-of-the-art clinical approaches to tissue repair and regeneration, tissue transplantation and prefabrication may serve as the macroscopic platform that ultimately 'transplants' these vascularized tissue engineered autogenic constructs into the defect in vivo.

The Greek word "*plasticos*" signifies 'to mold / to shape' and these verbs may be translated into '*engineering*' using current terminology. Therefore, besides our intense clinical involvement in the "molding and shaping" of tissues, the *plastic* surgeon also is etymologically related to the intriguing and promising multidisciplinary field of Tissue Engineering and Regenerative Medicine.

These strategies will certainly be part of our future clinical armamentarium.

I've missed more than 9000 shots in my career.
I've lost almost 300 games.
26 times I've been trusted to take
the game winning shot and missed.
I've failed over and over again in my life.
And that is why I succeed.
I can accept failure, but I can't accept not trying.
Michael Jordan



Chapter VI: Appendix

"The quest for autogenic Full-thickness skin engineering. Science and fiction".
Vranckx JJ.,MD,FCCP, Vermeulen, P.,MD., Dickens S.,Ir.,Vandenberge S.,MD.,Hendrickx B.,MD.
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The quest for autogenic Full-thickness skin engineering. Science and fiction.

Vranckx JJ.,MD,FCCP, Vermeulen, P.,MD., Dickens S.,Ir.,
Vandenberge S.,MD.,Hendrickx B.,MD.

Dept. Plastic & Reconstructive Surgery & Burn Centre,
KULeuven University Hospitals, Leuven
Laboratory of Plastic Surgery & Tissue Engineering Research, KULeuven, Belgium

Abstract

The absence of a reliable and durable autogenic full thickness skin (FTS) equivalent that mimics human FTS with its elasticity, flexibility, sensibility and resistance to biochemical and biomechanical aggressors significantly hinders recovery of FTS wounds, especially in deep and extensive burns. Such a construct would represent an enormous improvement in restoration of physical function and aesthetic outcome. An autogenic adherent FTS equivalent will further improve pain relief and discomfort caused by environmental insults such as surface desiccation or exudate buildup, which retards healing.
The reason why no such autogenic FTS equivalent could be developed is directly related to the complexity of human FTS. The skin constructs currently available are constructed using xenogenic or allogenic cells and matrix components, which will be resorbed/rejected after weeks. Unlike the extracellular matrix, which represents a millenary natural evolution, actual artificial biomaterials do not have such a complex structure and chemical composition.

In consequence, the pay off for large-scale productivity and standardization of these artificial devices is the very low bio-information content they transmit to cells.
The development of materials, which specifically and molecularly interact with cells in the defect, has become an emerging field in research. Tissue engineering aims at the reconstruction of tissue loss by developing biological substitutes using the principles of cell cultivation and material engineering. Gene therapeutic strategies may deliver ‘intelligence’ to these biologic tissue substitutes.

Actual clinical tissue repair modalities aim to change the wound milieu actively by altering the microenvironment (eg. vacuum assisted closure), adding missing proteins (eg. recombinantgrowth factor therapy and plasma gels) or even by changing anatomical boundaries through surgical prefabrication and prelamination procedures.

In this overview, we focus on the required elements for autologous vascularised full thickness skin engineering. What is science and what is fiction so far and which actual clinical strategies anticipate these requirements.

Keywords
wound repair, tissue engineering, stem cells, gene transfer, growth factors

1. Introduction

Skin has two highly specialized layers with an effective mechanism of integration. The thin epidermis consists of a basal layer of clonogenic keratinocytes (KC) that is anchored through the basal lamina components with the papillary dermis. In the dermal layer, fibroblasts (FB) produce matrix molecules such as fibronectin, elastin and collagens which shape the extra cellular matrix (ECM). Endothelial cells (EC) line an intrinsic network of fine blood vessels. A deeper subcutaneous layer of fibrofatty structures delivers elasticity to the full thickness (FT) skin[1].
The actual skin constructs available for wound repair consist of the dermal or epidermal layer or are built up by xenogenic or allogenic cells and matrix components, which will be resorbed or rejected after weeks similar to allogenic skingrafts. These allogenic components exploit a strategic temporary function by preparing the wound bed and producing growth factors once in contact with ECM components[2].

Besides being immunogenic, allogenic or xenogenic materials also carry a risk of viral transmission[3]. Moreover, currently used biodegradable scaffolds in cardiovascular tissue engineering showed toxic degradation products and elicited significant inflammatory reactions[4]. A shorter biodegradation results in a higher load of toxic side products. Finally, artificial biomaterials do not represent FT skin with its complex structure and chemical composition, and therefore they contain and transmit only limited bio-information to the wound microenvironment[5].

Furthermore, cells adhere and interact with their extracellular environment via integrins. Their ability to activate associated downstream signaling pathways depends on the character of adhesion complexes formed between cells and the ECM, which in turn determine which interactions take place in cytoskeletal organization, growth factor responses and cell cycle patterns[6]. When using a FT tissue equivalent, this cross-talk and interplay between cells, matrix molecules and growth factors should be restored for optimal wound repair.Therefore, the optimal tissue equivalent should be autogenic to optimally integrate without rejection, three-dimensional to bridge deep defects, porous to allow cell migration, bio-inductive for cells to proliferate and topically produce extra cellular matrix components, bio-inductive and supportive for angio and arteriogenesis and chemotactic for cells that infiltrate from the wound surroundings and orchestrate, stimulate and regulate these processes. Proliferative cells with stem cell properties could be part of the construct to obtain an optimal degree of integration and proliferation with the intrinsic capacity to respond to a changing microenvironment.
Such constructs should also be applicable to defects induced by an unfavorable healing environment such as in diabetes or cardiovascular disease and under treatment with corticosteroids or cytostatica[7].

These constructs could be spiked with recombinant DNA plasmids or populated with transgene cells expressing wound-healing proteins that are deficient in the particular morbidity state or conversely, with proteinase inhibitors that neutralize the impairing effects of proteinases present abundantly in chronic defects[8].

Tissue engineering of skin equivalents heralds promising clinical treatment options in the restoration of skin continuity after burns, tumor removal and in congenital skin disease[9].

Tissue engineering may produce the elementary bioscaffolds required to restore large defects while in vivo or ex vivo gene therapy may deliver supplementary 'intelligence' to the wound healing niche by production of coordinating proteins[10].

2. Growth factors as directors of the regular wound healing process (Table 1)

The response of tissues to injury forms the foundation of all reconstructive procedures. The intricate wound healing and tissue repair process involves the complex interplay of numerous cells, proteins and hormones[11]. Routinely, the wound healing process is divided into three overlapping stages: the inflammatory, proliferative and remodeling phase. The inflammatory process starts with tissue injury. Platelets play a pivotal role in the modulation of the early repair responses; they release growth factors, especially the Platelet Derived Growth Factor (PDGF), Insulin-like growth factor (IGF-1), and Transforming Growth factor beta (TGF-β) isoforms. In this early inflammatory phase, the intrinsic coagulation cascade is activated. Tissue damage results in the release of thromboplastin that activates the extrinsic coagulation pathway. The ultimate result of both pathways is the formation of cross-linked fibrin. This assembled matrix is the structural temporary platform that allows monocytes, FB and EC to adhere and migrate into the wound defect[12,13]. In the later inflammatory phase, more cells are recruited to the site of injury by substances secreted by platelets and damaged cells and by substances generated in the coagulation cascade. These substances attract macrophages into the wound and they, in turn, also release PDGF, IGF-1,TGF-β, epidermal growth factors (EGF) and vascular endothelial growth factor (VEGF). Fibrin and hypoxia in turn trigger the release of more growth factors, cytokines and lactate, which will continue the process and stimulate fibroplasia, collagen deposition and angiogenesis[11-13].

An intense crosstalk exists between FB and KC with the purpose of closing the open wound as rapid as possible, either by wound contraction or reepithelialization, or preferably, both in balance[14]. FBs secrete growth factors, EGF and KGF (Keratinocyte Growth Factor), which influence KC migration and proliferation. FBs produce ECM molecules such as fibronectin and elastin.

Growth factor	Isoforms Related factors Influencing factors	Cell source	Biological activity
PDGF	AA,BB,AB 60 % homology	PLT, EC,FB,MP, NP,SMC	Mitogen for EC,FB. Stimulates ECM formation
IGF	IGF-1 (somatodinC) IGF-2 (sometidin) Both have separate receptors; Bound to carrier protein, 50 % homology with pro-insulin	FB,MP,PLT,NP, liver, lung, heart	KC, FB chondrocyte and osteoblast proliferation, adipogenesis, collagen synthesis, angiogenesis, EC activation
EGF	30% homology with TGF-α:	KC,MP,FB,kidney, salivary and lacrimal glands	KC proliferation and migration & collagenase activity
FGF	aFGF (FGF-1) has 50 % homology with bFGF (FGF-2).FGF-7 = KGF FGF 10 = KGF-2	EC,FB,MP,SMCs,CH	FGF-1 and 2 : migration and proliferation of KC,FB FGF-2: EC growth and migration,angiogenesis
KGF	KGF-1 has 50% homology with KGF-2. KGF-2 = FGF-10 (for 96%)	FB, EC	Mediator in dermo-epidermal function, KC proliferation and migration, increases mechanical wound collagen, stabilizes epidermal turnover and barrier function
VEGF	VEGF A: 121,165,189, VEGF, B and C, PIGF H2O2 and TNF-α induce KC to increase mRNA for VEGF	KC,FB,MP	Angiogenesis, EC mitosis, increases bloodvessel permeability
TGF-ß	TGF-$ß_1$, $ß_2$, $ß_3$	PLT,FB,KC,EC, bone,hepatocytes	$ß_1$ and $ß_2$:deposition ECM collagen, induces sclerosis and epidermal migration $ß_1$ inhibits KC basal cell proliferation $ß_3$ scarring antagonist TGF-ß modulates EGF,FGF2,MMP's, TIMPS
CTGF	IL-1 inhibits CTGF production in FB, CTGF activity largely controlled by TGF-ß	FB	FB proliferation,collagen synthesis
GM-CSF	G-CSF M-CSF GM- CSF	EC, KC,FB,LC	Stimulates proliferation and differentiation of hematopoietic progenitor cells, chemotaxis and mitogen for WBC,FB and EC in vitro
cARP			Angiogenesis, increased perfusion and granulation tissue
HGF	MSP	FB,SMC	Stimulates KC migration ("scatter factor") and proliferation, chemoattractant for MP
TNF-α		LC,MP,MC,KC	Keeps KC in activated state, activates immune responses by inducing production of signaling molecules

Growth factor	Isoforms Related factors Influencing factors	Cell source	Biological activity
IL-1	α en β forms present unprocessed in cytoplama of KC	EC,KC,MP,LC	Initiator of KC activation, migration, proliferation Autocrine KC, paracrine on EC's, chemotaxis LC, FB
IL-3		KC,MP,MAC,EC,FB	Stimulation, growth and differentiation of HSC
IL-6		KC,MP,MAC,EC,FB	Stimulation of HSC, acute phase response, T-cell activation
IL-8	Expression induced by IL-1	EC,FB,LC,MC	NP recruitment, Proliferation KC in vitro
NOS	cNOS iNOS	KC (cNOS) FB (cNOS & iNOS)	KC proliferation, KC differentiation, angiogenesis, communication between KC and FB role in psoriasis (iNOS) and hypertrophic scars (cNOS).
MMP-9	MMPs = family of 28 Zn dependent endopeptidases antagonized by TIMPs MMP-2 & 9= gelatinases" MMP-1,8,13= "interstitial collagenase"	Expressed in advancing epithelium	Prognostic marker of wound repair, elevated in acute wounds; restructuring of ECM
MMP-1		Expressed by cells at wound edges	Activation of KC migration in early healing Also associated with impaired reepithelialization
MMP-2	Activated by MMP-14 MMP14 = MT1-MMP		Migration of KC Role in KC apoptosis along with MMP-14 MMP14: stimulates EC tubuli formation
TIMPS	TIMP-1, TIMP-2, TIMP-3, TIMP-4		1 and 3: expressed distally in proliferating KC 2: expressed at the epidermal tip

Table 1: growth factors and cytokines involved in wound healing

PDGF= platelet derived growth factor, IGF = insulin-like growth factor, EGF= epidermal growth factor, FGF= fibroblast growth factor, KGF= keratinocyte growth factor, VEGF= vasculoendothelial growth factor, H202= hydrogen peroxide, CH= chondrocytes, TGF= transforming growth factor, CTGF= connective tissue growth factor, GM-CSF= granulocyte macrophage colony-stimulating factor, cARP= cardiac ankyrin repeat protein,HGF= hepatocyte growth factor, MSP= macrophage stimulating protein, TNF= tumor necrosis factor, IL= interleukin, PLT= platelets, EC= endothelial cells, FB= fibroblasts, MP= macrophages, SMC= smooth muscle cells, NP= neutrophils, ECM= extra cellular matrix, KC= keratinocytes, HSC= hematopoietic stem cells, SC= Schwann cells, LC= lymphocytes, MC=monocyte,MAC= mastcells, NP= neutrophils, NOS= nitric oxide synthase, cNOS= constitutively expressed NOS, iNOS= inducible NOS, MMP= Matrix metalloproteinase, TIMP= tissue inhibitor of MMP'.

Fibronectin is predominantly secreted by FBs and activated by KC-signalling to form and maintain the structural integrity of connective tissue[15]. KC themselves produce integrins and fibronectin receptors to allow their migration throughout the wound by cell-matrix interaction[11,15,16].

Angiogenesis is regulated by FB and EC under the stimulation of VEGF, PDGF, bFGF and TGF. Cai et al demonstrated that the EC response to VEGF is dependent on the local concentration of VEGF, VEGFR-2 phosphorylation and intracellular regulators such as the protein tyrosine phosphates and eNOS[41]. Upon VEGF binding, VEGFR-2 activation initiates a cascade of intracellular downstream signalling events leading to increased proliferation, migration and EC survival[17,18].

Recently, several other mediators such as GM-CSF (granulocyte-macrophage colony stimulating factor), cARP, Matrix metalloproteinases (MMPs) and eNOS have been reported to play a regulatory role in angiogenesis and the required extra cellular matrix formation and degradation[19-21]. Fibronectin also seems to interact directly with VEGF through conformational changes in heparin binding sites[22]. This suggests a significant contribution of the ECM to modulate angiogenesis. Furthermore, MMP and their tissue-derived inhibitors (TIMPs) determine ECM architecture by digesting basement membrane and ECM proteins. As such, they have been recognized as vital mediators of angiogenesis. Specifically, membrane type-1 MMP is known to be involved in the polarization of EC to form tubules during capillary sprouting. These MT1-MMPs are expressed by EC, FB and basal KC[23,24].

Finally, tissue maturation ends the healing process, which leads to remodeling of granulation tissues. The TGF-β isoforms play an important role in these events[34]. Besides onset of growth factor expression and peak and steady state concentrations, duration, sequence and timing of expression are essential to tissue repair.

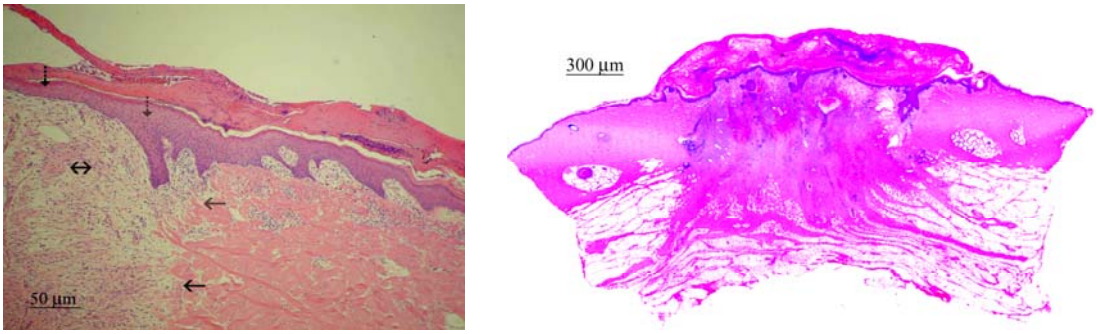
3. Growth factors in impaired wound healing

The incidence of chronic non-healing wounds is rising, due to a growing geriatric population with associated morbidity such as diabetes mellitus and cardiovascular disease. Furthermore, aging has been demonstrated to significantly diminish growth factor levels in the wound[25,26]. In chronic wounds, the disrupted balance between growth promoting proteins and protein-dissolving proteinases might lead to delayed healing or non-healing. Moreover, almost paradoxically, several reports documented impaired wound healing with VEGF and PDGF overexpression by in vivo and ex vivo gene transfer into full thickness skin wounds[27,28]. In skin tumors, the uninhibited production of growth factor induced angiogenesis might lead to uncontrollable cellular proliferation[29]. Therefore, the diificulty in growth factor research is to determine the application of the correct growth factor(s) at the right time, in the right sequence, for the correct period using a minimal efficient concentration. It will require many more research lives to explore this evolutionary process of wound repair in detail.

Elaborate protein analyses with innovative protein chip technologies might determine patterns of up-and down regulation of growth factors, their receptors, signaling pathways and their neutralizing proteinases. Once such proteins are identified, their corresponding DNA plasmids can be integrated into tissue engineered constructs or directly administered into the wound by gene transfer or slow-release vehicles[30-34].

4. Skin substitutes and temporary 3-D scaffolds for coverage of the full thickness defect

Skin ("cutis, integument") is the largest and most complex organ in the human body and the most affected in injuries. Skin provides a barrier against chemical, mechanical and infectious aggressors, provides thermal regulation and prevents dehydration.



a b

Fig. 1
a/ Healing wound at the transition zone between wounded (L) and intact (R)dermis (←). Epidermis stained purple (↓). Mark the inflammatory infiltrate in the granulation tissue (↔)
b/ Full thickness skin wound biopsy with epidermis (purple), dermis and subcutis. Mark the large blood vessels at both sides in the intact dermis and the central inflammatory infiltrate in the granulation tissue. In both sections the epithelial layers have migrated underneath the fibrin crust on top of the wound.

Skin consists of three well-balanced and interconnected layers (Fig. 1). The stratified epithelium (epidermis) is characterized by a continuous proliferation and maturation/differentiation process. The dermis represents a collagenous mesh work interwoven with varying contents of elastin fibers, proteoglycans, fibronectin and other components. In this network, fibroblasts are surrounded by blood vessels, lymphatic vessels, nerves, sebaceous and sweat glands, hairfollicles and nerve corpuscules. The basement membrane (BM) shapes and links the interface between the dermis and epidermis and provides mechanical stabilization to the skin via its hemidesmosomal structure. Anchoring fibrils pass from the BM into the dermal tissue[12,34]. Laminin-5, laminin-1,type IV collagen and the interaction with integrins alpha 3,5 and 6 are critical in KC adherence to the BM[12,33].

Superficial wounds can be covered with skin grafts (Fig. 2). The donor sites heal primarily within two weeks if all superficial dermal elements remain intact at the donor site. When meshed, these expanded skin grafts may cover large defects. In extensively burned patients, their might be a shortage of skin graft donor sites. Other techniques such as using larger meshed grafts or post-stamp grafts were developed to make optimal use of available donor tissue. Cell cultures may also be used to cover superficial defects.

Rheinwald and Green discovered that human KC could be cultivated and differentiated on plastic dishes on a carpet of irradiated murine 3T3 FB based on the finding that dissociated mature cells can reorganize themselves into native histologic structures when placed in appropriate tissue culture conditions[34]. This breakthrough led to the application of cultured KC on burns in 1981 and later proved to be life saving in extensive burn wounds with limited donor site for skin grafts[35].

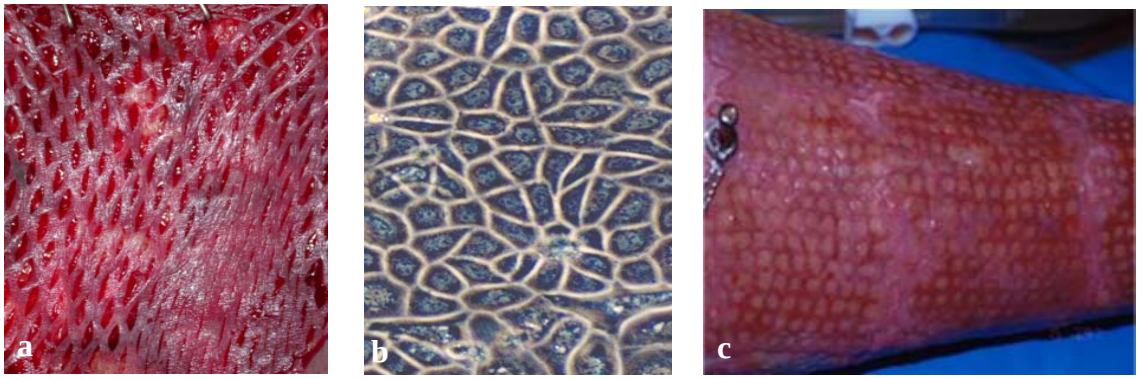


Fig. 2 (a) Close up of meshed split thickness skin graft. (b).Confluent basal cell keratinocytes cell layer. (c) In vivo expanding post stamp grafts (Meek-wall) on burn.

However, graft take remained below 70% in more than 50% of cases. Even after healing, the reconstituted epidermis remained excessively fragile mainly due to loss of the retepeg pattern of the epidermis.

In skin wounds deeper than the BM, reconstitution requires a restoration of all basement membrane components, thickness, texture and elasticity, while providing biomechanical resistance to aggressors[36,37]. Dermal tissue contributes to strength, durability, elasticity and aesthetic outcome in normal skin. When dermis is replaced by granulation tissue in deep wounds, scar tissue forms and myofibroblasts cause contractions. Also the BM forms slowly, which leads to blistering and disfigurement.

Several parameters can be taken into account to categorize "skin substitutes" or "skin equivalents;" the fabricated matrices may be *synthetic, semi-synthetic or biological* in origin. In biological matrices, a *xenogenic* animal source (bovine, equine) might have been used to distract collagens or matrix glycans (GAGs) or a human allogenic neonatal foreskin might have been harvested to cultivate fibroblasts and/or keratinocytes. As such, these skin substitutes might be cell-containing or cell free[38].

These scaffolds could be applied for short periods to stimulate autogenic healing and serve as biological dressings. These scaffolds may also be categorized by the complexity of their bio-content (table 2). Cell free biodegradable scaffolds may stimulate colonization by autogenic cells in the wound environment. Cell containing skin substitutes may provide immediate functional skin replacement[39-41].

So far, Integra represents the most investigated and most widely applied dermal scaffold. Preliminary clinical results on the use of 'Integra artificial skin' as a 'permanent wound cover' were published in 1981[42].

Both short and long term subjective evaluations by patients and surgeons demonstrated better or equivalent performance of the artificial dermis but poor resistance to infection. Especially in deep burns of the face, treatment with Integra artificial dermis and subsequent skin grafting led to good results with significantly better skin elasticity and pliability and restoration of facial mime[38-42].

However, cell free allogenic absorbable dermal scaffolds are prone to infection and are very expensive. In some countries, reimbursement for their use in deep burns of the face and over exposed joint area's after burns are in place[43].

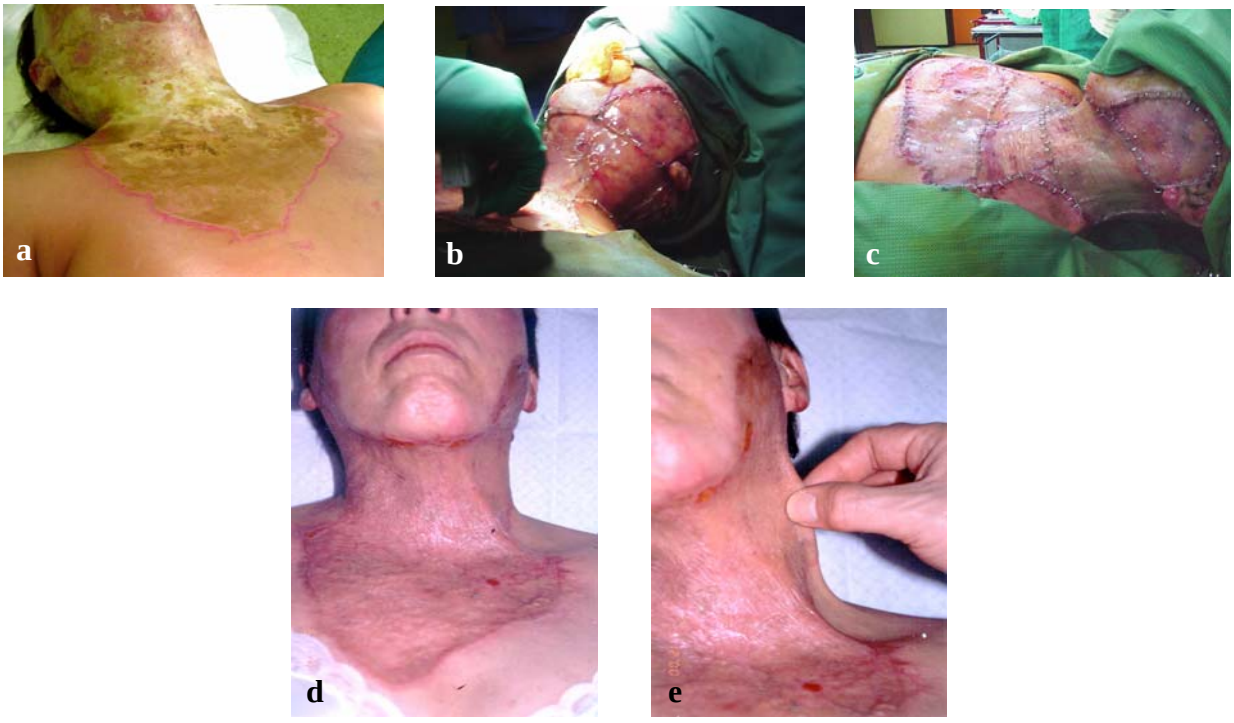


Fig. 3 a/ Deep burn of the neck. b/ After debridement application of Integra sheeth. c/ After peeling the silicone cover of Integra, the dermal matrix is covered with thin non-meshed split thickness skin grafts. d/ 3 months after final treatment. Full take of the grafts. e/ elasticity was partly restored with the Integra/skingraft treatment. Texture and color are good but not authentic to surrounding skin. (Pictures courtesy of Dr.P.Massage).

Recently, more compound scaffolds where presented, featuring dermal matrix elements in combination with allogenic foreskin keratinocytes and/or fibroblasts. Thus far, however, none of the cell-containing 'artificial skin equivalents' have clearly proven to add a significant clinical advantage to healing parameters despite their more corresponding structure and bio-content[44].

Name	*Supplier*	*Composition*	*Source*	*FDA approved*
Class I				
Epicel	Genzyme	CK	autogenic	CK graft
Vivoderm	ER Squib	CK on HA	Autogenic Synthetic	
Class II				
Alloderm	Life cell corp	Acellular dermis with basement membrane	allogenic	Full thickness burns
Dermagraft TC	ATS	FB in PGA mesh (biobrane) Silastic membrane	Allogenic neonatal Semi-synthetic synthetic	Diabetic foot ulcers
Integra	Life Sciences	Collagen-1+ GAG (CS) Silicone layer	Bovine synthetic	Deep Partial thickness and full thickness thermal injury to skin
Transcyte	ATS	Dermal tissue Silicone layer	Allogenic Synthetic	2nd & 3rd degree burns
Skin Temp Medifil	BioCore MT	Reconstituted collagen	Bovine	
Laser skin	FIDIS AB	HA membrane	semisynthetic	As KC carrier
Class III				
Apligraf	Organogenesis Novartis	KC + FB in collagen-1	Allogenic neonatal Bovine	Diabetic venous Foot ulcers
Composite cultured skin	Ortec Int	KC + FB in Bilayer collagen-1	Allogenic neonatal Bovine	Recess.Dystrophic Epid.Bull

Table 2 Artificial derm constructs and skin equivalents.

KC= cultured keratinocytes, FB= cultured fibroblasts, PGA= polyglycolic acid,GAG= glucosaminoglycans, CS= chondroitin–6-sulfate, HA= hyaluronic acid

The rationale of using an absorbable artificial dermis on deep wounds is the temporary scaffold it provides for cells that should be able to migrate into the construct from the wound environment[43,45]. Therefore, pore size should be around 100-250 μm. The collagen GAG dermal architecture of most dermal scaffolds approaches that of normal dermis and completely biodegrades after 30 days. Faster biodegradation might lead to toxic byproducts that impair healing. MHC class-1 and class-II antigens are found on all cellular components of the epidermis. During the processing of acellular dermis, these Major Histo-compatibility Complex molecules are fully eliminated but reappear following grafting indicating that cells can invade the scaffold and pore-size is sufficient[46]. Finally, major concern resides in those dermal components that are fashioned from bovine collagen, mainly derived from bones and hides.

There are risks of disease causing contaminants linked to prions that may infect and destroy nervous tissue. There also are concerns regarding hypersensitivity reactions to collagen-based products (Table 3). The future in ready-to-wear custom-made skin requires enormous advances in the fields of extra cellular matrix (ECM) biology[33,36,37]. The fabrication of the ideal construct requires that we further unravel the complex concerted process of ECM assembly and fully understand the macro and micro architecture of human dermis.

Integrating tissue-engineered scaffolds utilized in aberrant healing conditions when vascularization parameters are bad, anabolic tendencies are diminished due to cytostatica or corticosteroids or when chronic inflammation exists will be more complex. Adding biomolecular cues of wound healing and tissue regeneration into the 3-D scaffold might anticipate these adverse healing parameters creating a 'smart' tissue scaffold in which tissues migrate, integrate and proliferate[47].

5. Substrate for tissue engineering: stem cells

Stem cells have the unique ability to self renew, proliferate indefinitely and create offspring that differentiate into specialized tissues. These functional properties of stem cells have attracted significant interest from both basic and clinical science researchers. Stem cells represent unlimited therapeutic potential in tissue regeneration and reconstruction. Initially, researchers focused on embryonic and fetal tissues that in development clearly demonstrate their regenerative potential.

Embryonic stem cells, derived from the inner cell mass of a blastocyste, can differentiate into tissues of all three germ cell layers[48]. ES cells can be cultured and indefinitely maintained in vitro. These cells represent a potentially unlimited source for stem cell therapy. In animal research protocols, distinct differentiated tissues could be successfully generated from ES cell lines[49].

Human embryonic stem cell acquisition evokes an ethical dilemma that troubles many critics since it requires an excess human embryo or fetus. Moreover, the use of human fetal tissue, even if acceptable by society, involves heterologous transplantation, which would lead to tissue rejection and significant inflammation when applied in vivo. Finally, undifferentiated ES cells resulted in 20% in teratoma formation in several organ systems, which limit its actual use in human cell therapy[50].

Recent experiments have challenged the long-standing idea that adult stem cells or organ- specific stem cells are lineage restricted and that, once more differentiated, cells loose plasticity or ability to de-differentiate[51-55].

So far, adult stem cells have been isolated from several tissue sources, including bone marrow, skeletal muscle, central nervous system, retina, lipoaspirate from adipose tissue and skin. All of these stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin. Recent experiments, however, demonstrated that at least a fraction of stem cells in these populations can generate cells of a different embryonic lineage in vivo[56]. These findings indicate that adult tissues contain 'tissue-biased' stem cells that are much more' plastic' than previously appreciated.

Other experiments even suggested that adult stem cells from various organs can contribute to the regeneration of dissimilar organs with stem cells crossing germ layers. This led to the suggestion of a "stem cell highway," in which stem cells can transit through the circulation, with access to all organs of the body[57,58]. Homing signals (the 'niche' or microenvironment) might influence stem cell migration to specific sites.

The function of stem cells and their potential for "trans-differentiation" appears to depend largely on the local microenvironment and the "adequate lesion," which promotes the translocation of a sufficient number of stem and progenitor cells at the site of tissue damage[59]. Circulating multipotent stem cells could be latent and inactive, perhaps hardly detectable as 'sleepers' until an 'adequate lesion' occurs.

Stem cells and differentiation into a targeted population

The option of inducing an 'adequate lesion' by altering the microenvironment with external factors heralds further strategies in tissue repair, where differentiating cells into a targeted population is the focus of intensive research.

One of the important extracellular signals that controls stem cell fate is the secretion of growth and differentiation factors. TGF-β family members have remarkable instructive effects in ES cells[52]. GM-CSF and IL-3 and IL-6 as well as molecules from the Wnt family play a significant supportive role in diverse cell differentiation[55]. In addition to secreted factors, integral membrane proteins as well as integrins and the extracelluar matrix also contribute to the microenvironment of stem cells in determining their fate[60].

Recent findings imply that there is no intrinsic difference between tissue-specific adult stem cells and ES cells if a novel environment is provided[59,61].

Therefore, discoveries of new growth factors or new functions of existing factors could promote the progress of studies in stem cell expansion.

Skin represents an enormous and easy accessible source for uni-and pluripotent stem cells. Cell cultivation techniques on skin stem cells have been intensively studied and might provide a platform for designing innovative culture methods for ex vivo expansion of stem cells[60]. Umbilical cord blood (UCB) may be a valid alternative source to bone marrow or peripheral blood for HSC[62]. The characteristics and potential uses of UCB stem cells are currently under extensive examination. Rigorous standards need to be delineated to demonstrate stem cell fate and plasticity by a set of strict criteria[60].

Stem cell engineering for epidermal tissue engineering and regeneration

The epidermis is constantly renewed and maintains a fine equilibrium between proliferation and differentiation. Epidermal stem cells are the most accessible of all adult stem cells. The epidermis contains epithelial cells that are slow cycling (label-retaining cells 'LRCs') and cells that are clonogenic with extensive growth potential and which allow serial cultivation for clinical treatment, such as in burns[63,64].

At the upper bulge region, aAdult hair follicles contain 'pluripotent' stem cells that can reconstitute a wounded epidermis. Since the pool of stem cells in self-renewing tissues such as skin and blood must be maintained throughout life, the proliferation and differentiation balance must be rigorously controlled to avoid depletion or amplification of the stem cell pool[65,66].
As in ES, several cell surface markers are identified for adult HSC, MSCs and epidermal stem cells. These cells express high levels of beta-1 integrins and alpha-6 integrins and low levels of the CD71 transferring receptor[58,63-65]. Transient amplifying cells express high levels of alpha-6 integrins and CD71. However, they do not specifically express hematopoietic stem cell markers such as CD34 or Sca-1[67,68]. In a recent study, CD34 was expressed on stem cells isolated from the bulge area of mice hair folliclesm, but cells that were CD34- also demonstrated stem cell properties[56].
Further experiments must refine these epidermal and dermal stem cell marker analyses to obtain a purified stem cell population for cell-based therapies such as FT skin engineering.
After identification, selection and seeding, stem cell lines need to be further cultivated and prepared for transplantation into an in vivo wound environment. The challenge of maintaining the stem cell's undifferentiated multilineage potency during its long-term expansion is great[55,56]. Ideally, an *ex vivo* stroma- and serum-free system supported with known cytokines and growth factors needs to be developed for clinical applications.
To avoid the impact on cell growth and differentiation of undetermined proteins present in sera used to enrich cell cultivation media, serum-free cultivation techniques with pre-determined additives are preferred. Maintenance of the stem cell compartment ultimately depends on cell autonomous regulators modulated by external signals. Integrins hold cells in the right place in a tissue. Loss or alteration of integrin expression ensures departure from the stem cell niche through differentiation or apoptosis. ECM proteins can modulate expression and activation of integrins. These intrinsic and extrinsic signals such as growth factors and cytokines, regulate stem cell fate[57,61]. Certain aspects of the stem cell microenvironment or niche are conserved between tissues. These proteins can be exploited in the application of stem cells to tissue engineering and tissue repair[69].

6. Wound repair by cell engineering and gene transfer of growth factors

In patients with large chronic wounds and significant collateral morbidity, it seems futile to count on intrinsic protein production by the debilitated mediating cells in the wound. In this microenvironment, extrinsic proteins could be added to induce, support and modulate the wound repair processes[26,30]. Topical administration of recombinant growth factors such as proteins have major shortcomings such as short shelf life, low bioavailibility, enzymatic inactivaton by proteinases in the wound and inefficient delivery to target cells[34]. Gene therapy offers an appealing strategy for direct delivery of growth factor genes into the cells with the intent of altering protein synthesis in the cellular apparatus to modify the healing response. Gene therapy can offer targeted local and persistent delivery of de novo synthesized growth factor to the wound environment over many days[32,34].
This conformational change liberates the transcription binding complex and allows translation of the downstream inserted protein sequence[81,82].

7. Tissue engineering and vascularization: the missing link.

In reconstructive surgery and clinical tissue repair, the vascular perfusion of tissues in the defect determines the selection of the applied treatment[83]. Also, in tissue engineering protocols, the absence of a vascular network capable of distributing oxygen and nutrients within the matrix is the key-limiting factor. Thin artificial skin constructs undergo inhibition, FB migration, neovascularization and vessel maturation, while the dermal layer is resorbed. This process takes weeks to months. During this period, the construct is fragile and prone to infection[37,40,41]. So far, tissue engineering protocols have focused on thin constructs able to survive the initial episode of hypoxia by diffusion of nutrients until neovascularization occurs.

A viable capillary network in the tissue construct represents the essential missing link between the host and engineered implant[84]. The differentiation of precursor angioblasts into endothelial cells and the de novo formation of a vascular network is called 'vasculogenesis'.

'Angiogenesis' is the coordinated migration and proliferation of endothelial progenitor cells (ECs) and pericytes from the existing vascular bed and their subsequent maturation and stabilization by enveloping smooth muscle cells. These vessels are stimulated by hypoxia to proliferate[80,85]. The formation of such a network depends on guidance molecules within the ECM. The adult vascular network remodels itself by 'arteriogenesis' with the enlargement of existing collaterals induced by shear forces and the formation of completely new vessels from existing vessels[86,87]. Post-natal vasculogenesis occurs from migrating de-differentiated endothelial progenitor stem cells (EPC) and monocytes, which form tubules. Remodeling is mediated by monocytes and EPC[88]. The process of inducing incoming microvessel formation depends on growth factors used and the method of delivery.

Many growth factors and proteins have been reported to play an essential role in vasculogenesis[1,13,80]. The increase in vascular permeability through VEGF results in extravasation of plasma proteins and the formation of a highly pro-angiogenic ECM. VEGF modifies the ECM to allow migration of EC and FB[80,89].

Mitsi *et al*[17]suggested that VEGF also interacts with fibronectin through conformational changes in heparin binding sites. This suggests a significant contribution of the ECM to modulate angiogenesis. Further, upon VEGF binding, VEGFR-2 activation initiates a cascade of intracellular downstream signalling events leading to increased proliferation, migration and EC survival .TGFβ and bFGF mediates angiogenic sprouting and the formation of a guiding scaffold through ECM deposition[90,91]. Maturation of vessels occurs through PDGF pathways and Angiopoietin-1 and –2[28,92].

The primary stimulus and factor causative for capillary sprouting is hypoxia[27,29,80]. Other factors play a role such as Early Growth Response Factors Egr-1 and 3, Monocyte chemoattracted protein-1 (MCP-1) and matrix metalloproteinases[93,94].

Membrane type-1 MMP has been intensively investigated for its role on the polarization of ECs to form tubules during capillary sprouting[23,24]. These MT1-MMPs are expressed by ECs, FBs and basal KCs.

Micro-printing of biological molecules directly onto scaffolds, guided by a computerized pattern, spawns a new generation of strategies in smart tissue engineering[95]. In culture, ECs bind to an alkane-thiolate patterned gold or silver blueprint upon which the EC coalesce due to tissue fluidity. This process is repeated in layers and forms a 3-D structure. Subsequently, these constructs are cultivated in a bioreactor.

Moldovan et al. demonstrated that ECs and EPCs in bloodstream are able to drill holes through matrices[97]. Since biomolecules have nano-dimensions, nanotechnology could deliver an 'angiogenesis assist device' – 'angiochip'- on which EC clusters may be spatially distributed and interconnected by grooves that allow for proliferation and angioconduction and induction[96,97].

8. Growth factor therapy & Tissue engineering avant-la-lettre in clinical wound repair

Progressively, innovative technologies and techniques are introduced into clinics, based on growth factor release and tissue engineering principles. Their purpose is to stimulate repair and regeneration to modify the wound bed and its vascularization or modify the anatomical features of tissues by introducing new layers of vascular networks. Such novel treatment options gradually pave the road towards 'smart' autologous three-dimensional vascularised tissue engineering.

8.1. Wet wound healing

Reports on treatment of wounds in a liquid environment date back to the nineteenth century[98]. Burn patients were treated in bathtubs with water for periods of weeks to months. It reduced pain and increased survival. Only since the early '90s, the concept of treating wounds in a moist or wet wound healing environment became golden standard treatment and confirmed the adage of 'a dry wound being a dead wound'. A wet wound environment favors cross talk of cells through autocrine and paracrine secreted growth factors[99,100]. Pharmaceutical industry followed this change in philosophy by producing a plethora of dressings developed to manage the wound exudates and create a moist or wet healing environment.

8.2. Vacuum assisted closure

Vacuum assisted closure (VAC^{tm}, KCI, San Antonio, Tx, USA) has gained its rightful place in the armamentarium of physicians dealing with extensive wounds. Debrided wounds are covered with custom sized porous foam dressings, sealed with airtight transparent adhesive foils and set under continuous or intermittent vacuum suction[101].The VAC is used to bridge debridement and reconstruction (Fig. 4). This negative pressure therapy diminishes wound oedema by aspiration of wound exudates and reduces the amounts of bacterial clusters and proteinases present in high concentrations in chronic wounds. Progressively, growth factors are no longer broken down by the now reduced levels of proteinases while reduced oedema enhances vascularization.

The porous foam dressings create a moist or wet wound healing environment. Moreover, the subatmospheric pressure creates a gradient that disrupts integrin bridges of the cytoskeleton.

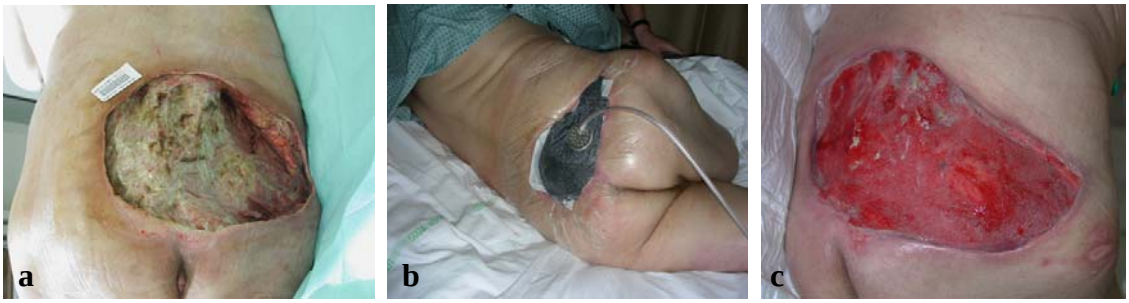


Fig. 4 (a) Extensive sacral + gluteal pressure sore after first debridement in a paralytic diabetic patient with severe vasculopathy. (b) Negative pressure therapy using 3 large foams and intermittent suction in cycles of 5 min.
(c) After 6 weeks well vascularised granulation tissue formed which oblitered the wound clefts.Wound now suitable for skin grafting. (Pictures courtesy of Dr.Vranckx JJ)

This triggers the release of intracellular secondary messengers which in turn upregulates cell proliferation, and thus the outgrowth of vascularised granulation tissue[102]. Clinical studies demonstrated that intermittent suction (eg. cycles of 5 minutes) promotes this granulation tissue outgrowth in a more intense fashion.

Morikwas et al. measured higher blood flow, which lasted 5-7 minutes when the aspiration cycle began[102]. This enhanced blood flow delivers increased oxygen and leukocyte concentrations. Managing interstitial oedema caused by inflammation also diminishes the distance between healing cells and capillaries.

However, the vacuseal assisted closure in all its efficiency does not replace appropriate debridement. Once meticulously debrided, inert wounds clearly recapture their propensity to granulate and heal.VAC decreases bacterial count in infected wounds.

However, to avoid the risk of promoting a deeper or systemic infection, the wound should always be completely debrided before applying VAC.

Moreover, extensive deep wounds may heal after long term vacuum assisted treatment, but without the flexibility and elasticity of full thickness tissues. Therefore, for the reconstructive surgeon, vacuum assisted closure perfectly prepares an extensive and deep wound for final surgical reconstructive treatment with pedicled or free flap tissue transfer. (Fig 5)

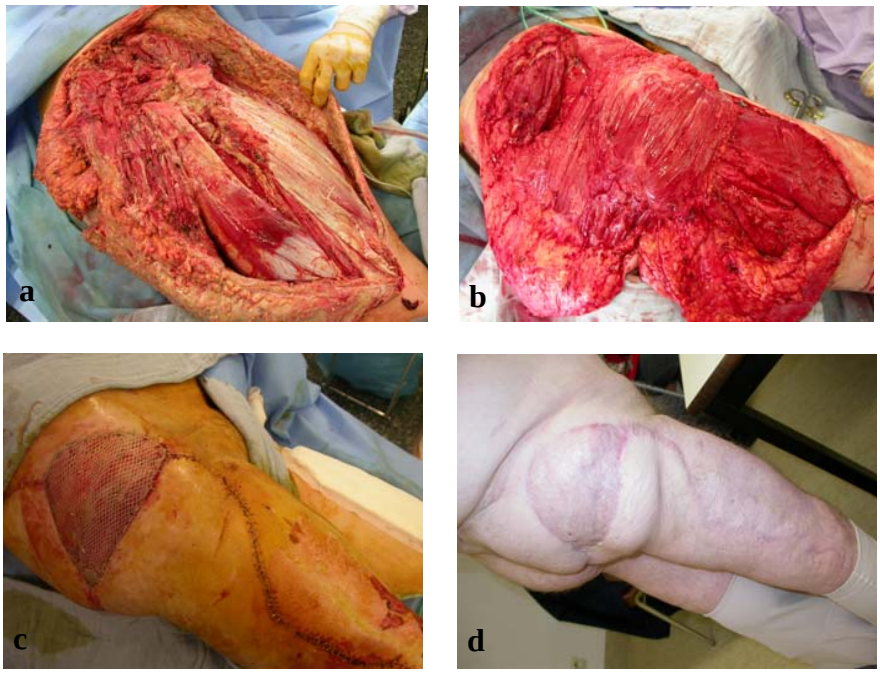


Fig. 5 (a) Necrotising fasciitis of the left gluteus and thigh with extension into the left hip joint. Status after multiple debridements. (b). After 5 weeks negative pressure therapy under intermittent pressure. (c)Gluteus maximus muscle transposition to the hip covered with skin graft. Local fasciocutaneous transposition for secondary closure.(d) Status after 6 months. The patient is fully mobile. (Pictures courtesy of Dr.Vranckx JJ)

8.3. Platelet rich plasma gel

Studies indicate that platelets may provide a critical link between coagulation and inflammatory cascades via regulated production of growth factors and cytokines:
PDGF-AA and BB, IGF-1 and 2 ,TGF- β1 and IL-1β signal the local mesenchymal and epithelial cells to migrate, divide, and increase collagen and matrix synthesis required for bridging and closing the wound defect.
Autogenic Platelet-rich plasma gel (APG), obtained by sequestering and concentrating platelets by gradient density centrifugation from autogenic whole blood, may be used clinically to deliver these growth factors in high concentration to the wound microenvironment[103,104]. Autogenic activated platelet rich plasma may also be used as a fibrin sealant in hemostasis and as tissue glue for efficient attachment of skin grafts (Fig. 6). The gel activated with autogenic thrombin may be used as an autogenic biomatrix to promote granulation formation for full thickness repair[105,106]. We use the autogenic platelet gel as an autogenic bioscaffold that supports split thickness skin grafts, cultured KC grafts and post-stamp grafts and accelerates migration and proliferation of those skin cells into confluent islands.

Fig. 6 Extensive IIIrd degree burn on the thorax and lef upper arm.Treated with split thickness skin grafts after tangential excision of the burns. (a). From autologous venous whole blood, platelet rich plasma (PRP) gel is prepared. From the platelet poor plasma fraction autologous thrombine is derived. (b) The PRP is mixed with the thrombin and sprayed over skin graft donor sides and over the skin grafted wounds. After 30 seconds a lightly adhesive gel forms.
(c) 3 days after opening the dressing, a full take of the skin grafts is seen on crests and creases.
(Pictures courtesy of Dr. Vranckx JJ.)

8.4. Prefabrication and vascular induction

In prefabrication procedures, the natural anatomic boundaries of tissues are altered to procure more optimised donor tissues for reconstruction. Prelamination of a flap at a distant site can provide the missing layers for reconstruction of extensive and complex defects by suturing tissue layers on top of a well-defined axial vascular territory that serves as a vascular carrier. In prefabrication procedures, a vascular source is transposed into a non-axial area to provide an alternative blood supply through neovascularization of tissues. Several strategies of prefabrication exist[107]. Both the arteriovenous shunt loop and the arteriovenous bundle groups showed marked angiogenesis in the adventitia of the femoral artery and vein and the surrounding tissue, noteworthy since angiogenesis was reported to occur only in arterioles and venules. The wall of the femoral vein appeared to have many holes, which represents luminal sprouting. When angiogenic factors such as VEGF dominate inhibitory factors, pericytes become separated from EC, which are rendered reactive to the promoters[108]. Inflammation, hemorrhage, and coagulation associated with surgical trauma help to promote this angiogenesis[109].

We use standardized prefabrication procedures in laryngotracheal reconstruction. For treatment of tracheal restenosis, we prefabricate buccal mucosa or ear cartilage patches on a highly vascularised radial forearm fascia flap[110] (Fig. 7).

After two weeks of vascular induction, this laminated prefabricated forearm flap is transplanted to the neck where it releases the stricture and covers the mucosal defect in the trachea with a well vascularised mucosal framework.

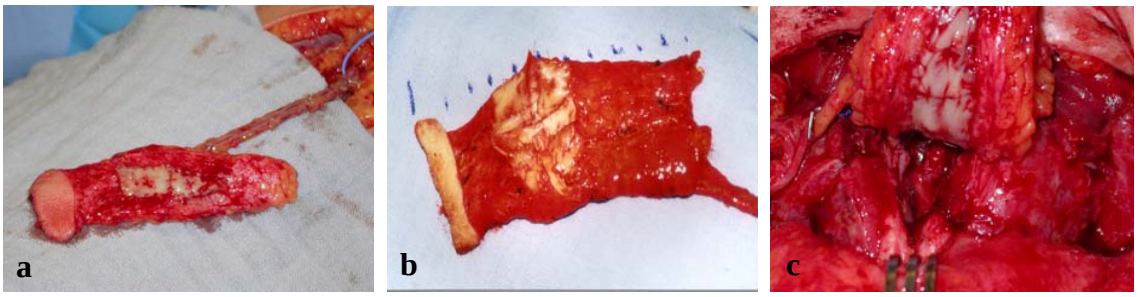


Fig. 7 (a) Free forearm fascia flap laminated with ucal mucosa and its radial vascular pedicle still attached on the donor site. (b) Free forearm fascia flap with ear cartilage framework and bucal mucosa after two weeks of flap prefabrication.(c). The mucosa patch on the fascia flap before inset in a splindle shaped open trachea defect. Note the vascular pedicle at the left side. (Pictures courtesy of Dr. Vranckx JJ., Dr. Delaere P.)

We use a more extensive prefabrication procedure to prefabricate a circular trachea segment with a similar radial forearm free flap anastomosed to the neck vessels[109]. After 4 months, this vascularised trachea patch is used to reconstruct a hemicricohemilaryngeal defect after cancer resection (Fig. 8).

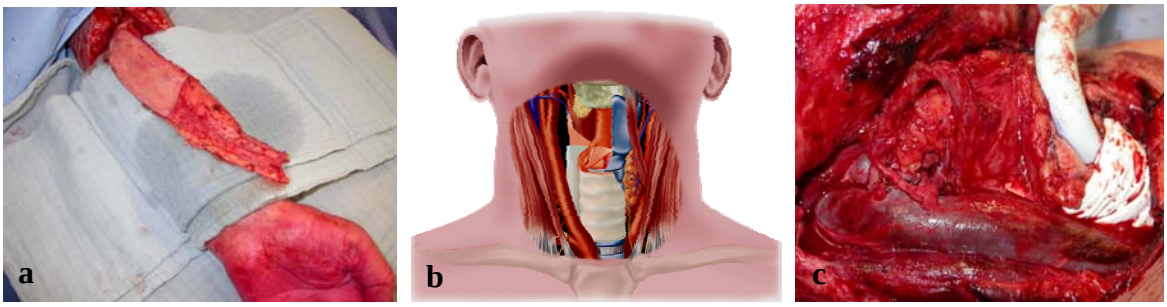


Fig. 8 (a) Prelevation of a compound radial forearm free flap from the non-dominant forearm.(b)This flap is used to prefabricate a 4 cm trachea segment and to restore the laryngeal defect after hemilaryngectomy.(c) The free flap in position. The fascia-wrapped trachea covered with a (white) GoreTex ® sheet. Note the microvascular anastomosis of the vein end-to-side on the internal jugular vein. The artery end-to-side on the superior thyroid artery.
(Pictures courtesy of Dr. Vranckx JJ. and Dr. Delaere P.)

The purpose is to restore patency of laryngeal structures, avoid a permanent tracheostomy and preserve speech in patients with unilateral glottic cancer. Similar vascular induction is seen using tissue expanders for reconstructive purposes such as in scalp expansion treatment for extensive post-burn alopecia[111]. The relative hypoxia caused by the expansion process is the trigger for angiogenesis.

In contrast, vascular delay procedures are also used to promote vascular ingrowth from the recipient tissues into a pedicled flap such as in “jump flaps” for nose reconstruction.

After three weeks, the pedicled tissues are integrated into the recipient vascular system and the original proximal vascular source can be severed.

The concept of three-dimensional vascularised tissue engineering with incorporated blood vessel networks stands far from a clinical context *ad hoc*. Awaiting such innovations, prefabrication procedures might gradually be merged with tissue engineering protocols and gene therapy to create vascularized tissue equivalents.

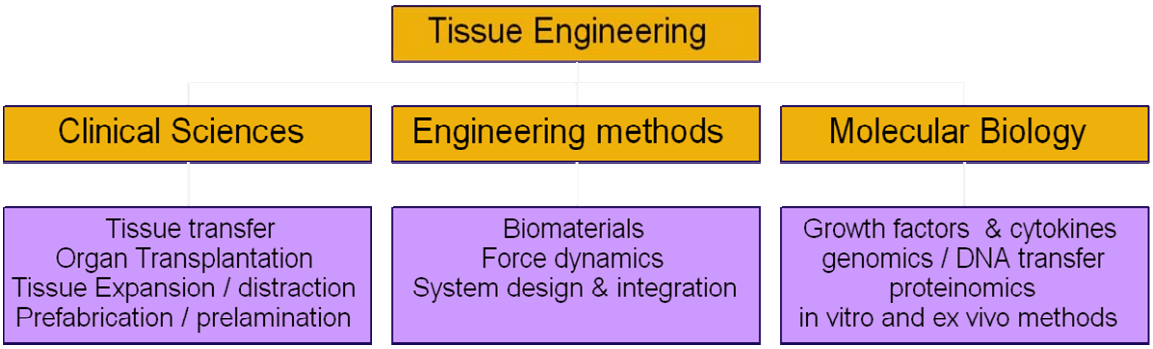


Fig. 9 Tissue engineering: from blackboard to bench to bed

9. Conclusions

The construction of an autogenic or allogenic bio-inducible three-dimensional living matrix has not been achieved. The required matrix needs to mimic or anticipate the intricate cross talk and interplay of cells and mediators involved in full thickness skin repair and regeneration. Gene transfer and tissue engineering technologies may succeed in seeding biocompatible matrices with autogenic stem cells that express selected genes, which lead to the production of proteins essential to the repair processes. The introduction of such proteins to the matrix and the healing environment may strongly influence the micro-niche around the wound, the paracrine influences to local and distant cells and may determine the final fate of stem cells and progenitor cells migrated to the wound milieu. The impact of such proteins to wound regeneration, healing and tissue engineering might be extensive; therefore, meticulous gene regulation in vivo is paramount to safety and control of expression. To treat extensive burns, congenital skin deformities or deep skin defects after trauma and tumor excision, an autogenic full thickness 'smart' skin equivalent should bring the required autogenic treatment for tissue reconstruction, improving functional and aesthetic outcome, social reintegration, pain relief and quality of life. Ad hoc novel strategies in wound repair are based on changing the wound microenvironment, and promoting repair and regeneration processes, vascularization and cell growth.

The actual multidisciplinary approach brings together cell and molecular biology, molecular genetics, material sciences of matrices and scaffolds and the clinical expertise of tissues and wound repair. This integration might lead to the cultivation and production of a veritable authentic and autogenic full thickness tissue equivalent.

Corresponding author:

Jan Jeroen Vranckx, MD,FCCP.
Dept. Plastic & Reconstructive Surgery
KULeuven University Hospitals
Leuven, Belgium.
32-16-348721 tel 32-16-348723 fax
Jan.vranckx@uz.kuleuven.ac.be

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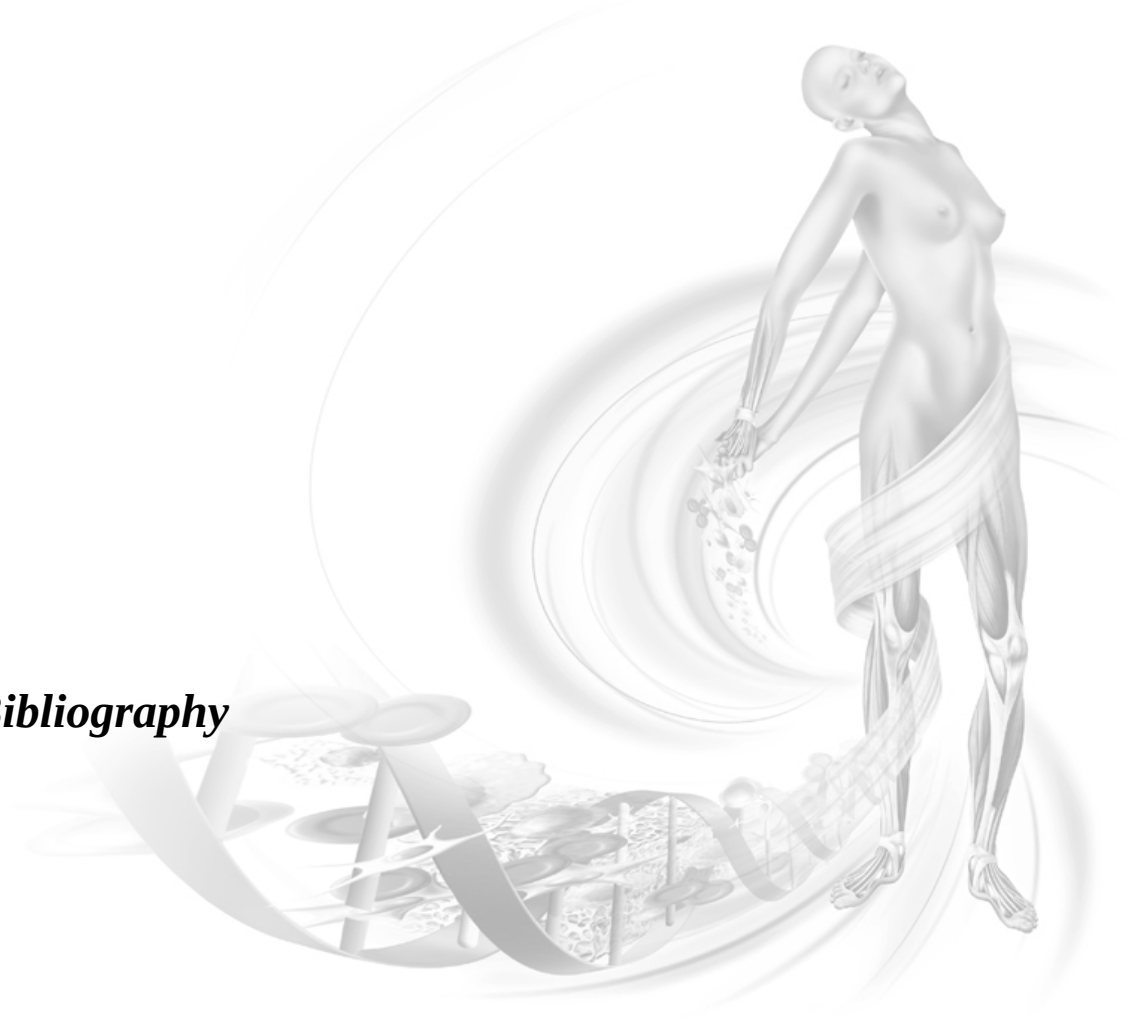
If a man takes no thought about what is distant
He will find sorrow at hand.
The Confucius Analects



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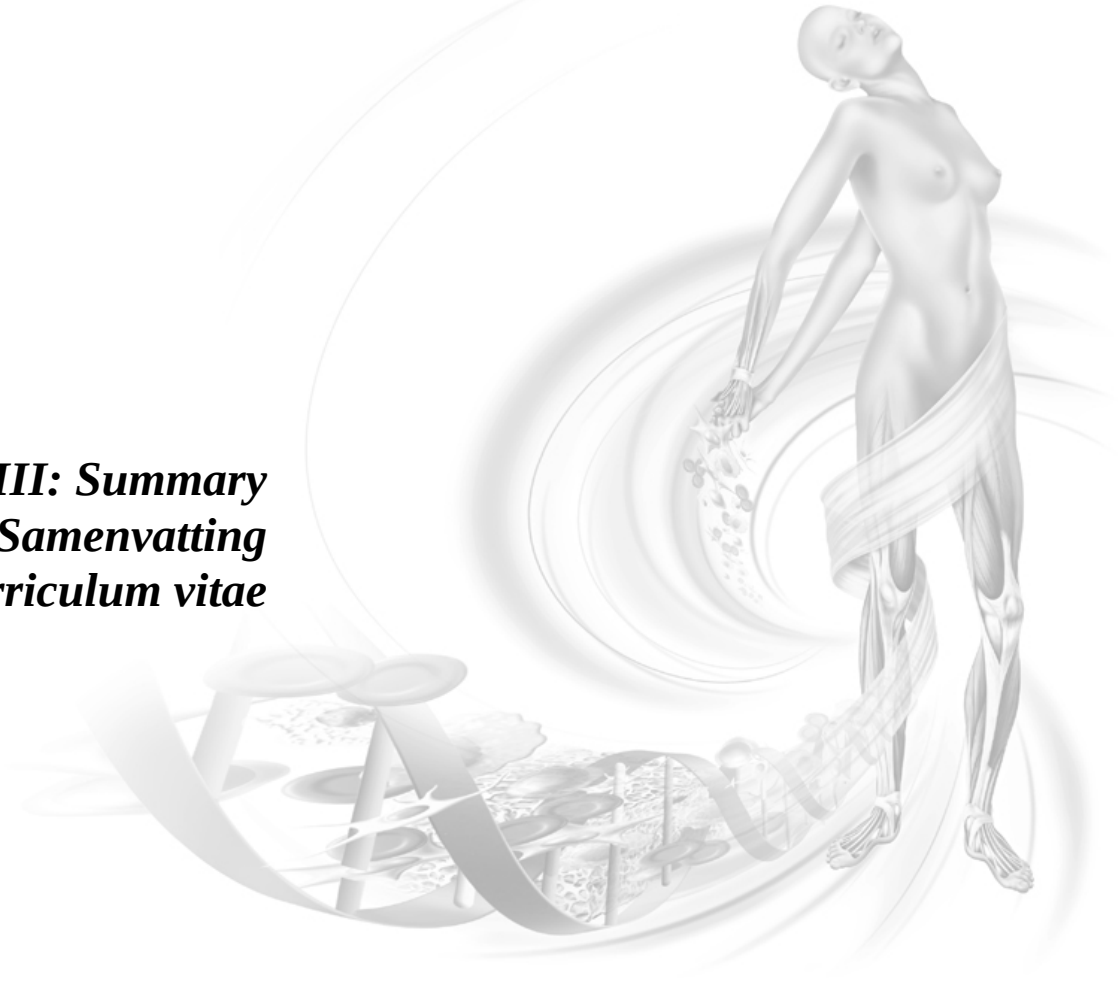
It is possible to fly without motors,
But not without knowledge and skill.
Wilbur Wright (1876-1912)



Chapter VIII: Summary
Samenvatting
Curriculum vitae

1. Summary

Tissue engineering was heralded as a promising novel strategy for the treatment of defects caused by tumor resection, trauma and burns. Because of the complex molecular biology of the wound repair processes and the demanding clinical requirements in tissue regeneration, there hasn't been much progress made ever since. In this project we hypothesized that cell cultures and gene transfer of growth factors would stimulate tissue repair and that his approach would form the backbone for the cultivation of a 3-D biomatrix for tissue engineering purposes. Cell cultures deliver the required tissue substrate for reconstruction whilst gene transfer of growth factors supplies 'intelligence' to the healing microenvironment.

Growth factors delivered as proteins to the wound are rapidly neutralized by enzymes. Gene therapy offers the template rather than the protein to cells in the wound. This transforms the cells into mini-production units of the selected growth factors.

We used custom made wound chambers on top of full thickness skin wounds (FTW) to create a wet wound healing environment which promotes wound repair. We established a porcine animal model because of the intrinsic similarities between porcine and human skin structure and wound repair.

In a first project we 'microseeded' high concentrations of the Vasculoendothelial growth factor VEGF directly into the FTW in order to promote angiogenesis and wound repair. We used an attenuated Adenovirus as carrier for the VEGF plasmids. Highest plasmid concentrations led to highest protein expression in the wound but at the cost of retarded healing. A minimal but efficient VEGF concentration rather seemed mandatory in wound repair protocols.

Subsequently we cultivated cell suspension cultures of basal cell keratinocytes (BCKs) from skin grafts and transfected these with Epidermal Growth Factor. EGF induces reepithelialization in wounds. The BCKs stimulated repair and served well as carriers for the EGF growth factor plasmids. By overexpressing EGF healing could even be accelerated.

A similar strategy but with overexpression of VEGF by the BCKs led to improved matrix formation, endothelial cell clustering and reepithelialization. In the presence of VEGF we also found an increased tubular formation of the endothelial cells indicating the formation of small blood vessels.We also integrated a tetracycline-inducible gene switch into the VEGF-expressing plasmid. When adding the antibiotic tetracycline into the wound, the switch is 'activated' and VEGF overexpression starts. Such a gene switch allows us to regulate timing and sequence of growth factor expression.

These data currently guide us towards the required platform for the cultivation of custom-made vascularized autologous tissue equivalents.

2. Samenvatting

Tissue Engineering werd verwelkomd als een veelbelovende strategie voor de behandeling van weefseldefecten na ondermeer tumor resectie, trauma en brandwonden. Door de complexe moleculaire biologie van wondheling enerzijds, en de klinische vereisten bij weefsel regeneratie anderzijds is tot op heden weinig vooruitgang geboekt. In dit project stelden we de hypothese dat *cel culturen* en *gen transfer* van groeifactoren wondheling stellig zouden bevorderen en dat deze methoden tevens de ruggegraat zouden vormen voor de verdere cultivatie van een 3-D biomatrix voor tissue engineering waarbij de celculturen het weefselsubtraat leveren voor reconstructie van het defect en de groeifactoren de 'intelligentie'en'coordinatie'.

Groeifactoren worden als proteine in de wonde snel 'geneutralizeerd' door enzymen. Met *gen-therapie* wordt niet het groeifactor proteine zelf, maar het DNA- sjabloon van de groeifactor in de cellen gebracht. Die cellen worden zo omgevormd tot mini-productie units van de gekozen groeifactor. Op diepe standaard wonden plaatsten we speciale *wondkamers* om een vochtig milieu te creëren wat heling intens versnelt. De keuze van het varken als proefdier stoelt op de intrinsieke gelijkenissen in huidstructuur en klinische wondheling met de mens.

In een eerste project brachten we hoge concentraties van de groeifactor VEGF rechtstreeks in de wonden met doel op snellere bloedvatvorming en heling. We gebruikten een afgezwakt griepsvirus als drager voor de VEGF. De hoogst aangewende DNA concentraties leidden tot de hoogste VEGF proteïne expressie in de wonde, maar ten koste van de snelheid van wondheling; een minimale maar efficiënte dosis leek veeleer aangewezen voor wondhelings doeleinden.

Vanuit huidgreffen cultiveerden we vervolgens *'ex vivo'* celculturen van basale (stam)cel keratinocyten (BCKs): huidcellen die 'getransfecteerd' werden met de groeifactor EGF die heling van opperhuid bevordert. De BCKs stimuleerden de wondheling en functioneerden als efficiënte dragers voor de EGF. Bij hoge EGF secretie door de BCKs, versnelde de wondheling significant.

Een zelfde strategie, maar waarbij de BCKs de VEGF groeifactor produceerden, leidde tot een intensere matrix opbouw, en een verhoogde angiogenese. We toonden ook aan dat die endotheelcellen zich in de aanwezigheid van VEGF sneller organizeerden tot 3-D bloedvaatjes.

Belangrijk gezien bloedvoorziening elementair is in elke weefselreconstructie Tevens incorporeerden we in de VEGF plasmid een genetische switch, te activeren door het antibioticum tetracycline(TC). Pas wanneer TC in de wondkamer werd geinjecteerd, startte de VEGF productie door de drager cellen. Zulke gen-switch laat ons toe de timing en sequentie van de groeifactor expressie te controleren. Belangrijk voor toekomstige toepassingen bij de mens.

Deze data leiden ons naar het benodigde platform tot de cultivatie en maatgemaakte productie van een authentieke en lichaamseigen weefsel equivalent.

3. Curriculum Vitae Jan Jeroen Vranckx

Jan was born on January 11, 1969 and raised in Turnhout. There he attended the Sint Jozef College (1-4 rd year Latin-Greek and 5-6th year Latin-Sciences).

He started his Medical studies at the LUC and continued at the KUL in Leuven. In his 6th year he worked as a trainee in Oostende (AZ-H. Hart), Bonheiden (AZ-Imelda) and in Merida, Venezuela (Clinico Universitario de los Andes).

He obtained his Medical degree in 1994. The same year he commenced the truncus communis in General Surgery at the KUL (Prof. P. Broos) and at the Clinique d'Europe St.-Michel in Brussels (Dr. J.Vandeperre). In 1997 he begins the training in Plastic & Reconstructive Surgery at the KUL Leuven University Hospitals (UZ-Gasthuisberg, Prof. W. Boeckx, P. Guelinckx and P. Brenner). In his 5th year of residency, he worked abroad at the Hospital Universitario Sao Joao in Oporto (Prof. J.Amarante and H.Costa), at the Chang Gung Memorial Hospital in Taipei, Republic of China (Prof. F.C. Wei, D. Chuang and HC. Chen) and at the Brigham & Women's Hospital in Boston, USA (Prof. E. Eriksson and J. Pribaz).

In 2000 Jan obtained the degree of Plastic, Reconstructive and Aesthetic Surgery at the Katolieke Universiteit Leuven and became Fellow of the Belgian Collegium Chirurgicum Plasticum. The following two years he returned to Boston (USA) and worked as Fellow at the Laboratory of Tissue Repair and Gene Transfer of the Plastic Surgery Division at the Brigham & Women's Hospital (Prof. E.Eriksson) for which he received grants from the Fulbright Commission (2001) and the BAEF Belgian American Educational Foundation(2002). There he starts his doctoral PhD-training.

Since October 2002, he has been working as a full time staff member at the Dept. of Plastic & Reconstructive Surgery at the KULeuven University Hospitals. His main clinical interests in the broad domain of Plastic & Reconstructive Surgery further evolved into 'head and neck' surgery, microsurgical reconstructions, breast surgery, vascular anomalies, burns and aesthetic surgery.

In 2004 he received a 2-year Clinical Research Fund from the FWO and a 3-year KOF Research Fund from the KUL University Hospital to continue his research in tissue engineering and gene transfer for tissue repair. Later that year he founded the "Laboratory of Plastic Surgery and Tissue Engineering Research", the *LOPSTER* lab in which he until now enthusiastically 'parented' 4 research fellows. Ultimate aim: the full integration of clinical work in plastic, reconstructive and aesthetic surgery, research & development.

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