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In vitro and *in vivo* studies of tissue engineering in
reconstructive plastic surgery

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List of publications

This thesis is based upon the following papers, which will be referred to in the text by their roman numerals (I-VI):

- I F.R.M. Huss, G. Kratz
Mammary epithelial cell and adipocyte co-culture in a 3-D matrix: The first step towards tissue-engineered human breast tissue.
Cells, Tissues, Organs 2001;169(4):361-367
- II F.R.M. Huss, G. Kratz
Adipose tissue processed for lipoinjection shows increased cellular survival *in vitro* when tissue engineering principles are applied.
Scand J Plast Reconstr Surg Hand Surg 2002;36(3):166-171
- III F. Huss, E. Svensson, C.-J. Gustafson, K. Gisselgålt, E. Liljensten, G. Kratz
New degradable polymer scaffold for regeneration of the dermis: *In vitro* and *in vivo* studies.
Manuscript, submitted to: European Cells and Materials
- IV F. Huss, G. Elmerstig, A. Birgisson, L. Salemark, H. Johnson, G. Kratz
Growth of cultured human ecto- and mesodermal cells on macroporous biodegradable gelatin spheres.
Manuscript, submitted to: European Cells and Materials
- V F.R.M. Huss, J.P.E. Junker, H. Johnson, G. Kratz
Macroporous gelatine spheres as culture substrate, transplantation vehicle, and biodegradable scaffold for guided regeneration of soft tissues. *In vivo* study in nude mice.
Manuscript, submitted to: Br J Plast Surg
- VI F. Huss, E. Svensson, J. Bolin, G. Kratz
***In vivo* study on the use of macroporous gelatin spheres as a biodegradable scaffold for guided tissue regeneration in human.**
Manuscript

Wiping his brow

He managed to tear himself away

The past few nights

Had left him rather tired

Returned his thoughts

To a need he found overpowering

He felt success

Was now within his grasp

Climbing the ladder

Three runs forward, two runs back

Climbing the ladder

Trying to stay on just the right track

Keeps up the pace

Tells himself that it's all worthwhile

Hard work is its' own reward one day

Could he be wrong?

Are all his dreams merely fantasies?

And would it all

Fall in on him some day?

Now he sits back

Amid all things he worked so hard for

And wonders...

Was his energy well spent?

Whatever the price

He is where he wants to be

The end has justified

The means and all

Abstract

To correct, improve, and maintain tissues, and their functions, are common denominators in tissue engineering and reconstructive plastic surgery. This can be achieved by using autologous tissues as in flaps or transplants. However, often autologous tissue is not useable. This is one of the reasons for the increasing interest among plastic surgeons for tissue engineering, and it has led to fruitful cross-fertilizations between the fields. Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and life sciences for development of biologic substitutes designed to maintain, restore, or improve tissue functions. These methods have already dramatically improved the possibilities to treat a number of medical conditions, and can arbitrarily be divided into two main principles:

> Methods where autologous cells are cultured *in vitro* and transplanted by means of a cell suspension, a graft, or in a 3-D biodegradable matrix as carrier.

> Methods where the tissue of interest is stimulated and given the right prerequisites to regenerate the tissue *in vivo/situ* with the assistance of implantation of specially designed materials, or application of substances that regulate cell functions - guided tissue regeneration.

We have shown that human mammary epithelial cells and adipocytes could be isolated from tissue biopsies and that the cells kept their proliferative ability. When co-cultured in a 3-D matrix, patterns of ductal structures of epithelial cells embedded in clusters of adipocytes, mimicking the *in vivo* architecture of human breast tissue, were seen. This indicated that human autologous breast tissue can be regenerated *in vitro*.

The adipose tissue is also generally used to correct soft tissue defects e.g. by autologous fat transplantation. Alas 30-70% of the transplanted fat is commonly resorbed. Preadipocytes are believed to be hardier and also able to replicate, and hence, are probably more useful for fat transplantation. We showed that by using cell culture techniques, significantly more pre-adipocytes could survive and proliferate *in vitro* compared to two clinically used techniques of fat graft handling. Theoretically, a biopsy of fat could generate enough preadipocytes to seed a biodegradable matrix that is implanted to correct a defect. The cells in the matrix will replicate at a rate that parallels the vascular development, the matrix subsequently degrades and the cell-matrix complex is replaced by regenerated, vascularized adipose tissue.

We further evaluated different biodegradable scaffolds usable for tissue engineering of soft tissues. A macroporous gelatin sphere showed several appealing characteristics. A number of primary human ecto- and mesodermal cells were proven to thrive on the gelatin spheres when cultured in spinner flasks. As the spheres are biodegradable, it follows that the cells can be cultured and expanded on the same substrate that functions as a transplantation vehicle and scaffold for tissue engineering of soft tissues.

To evaluate the *in vivo* behavior of cells and gelatin spheres, an animal study was performed where human fibroblasts and preadipocytes were cultured on the spheres and injected intradermally. Cell-seeded spheres were compared with injections of empty spheres and cell suspensions. The pre-seeded spheres showed a near complete regeneration of the soft tissues with neoangiogenesis. Some tissue regeneration was seen also in the 'naked' spheres but no effect was shown by cell injections.

In a human pilot-study, intradermally injected spheres were compared with hyaluronan. Volume-stability was inferior to hyaluronan but a near complete regeneration of the dermis was proven, indicating that the volume-effect is permanent in contrast to hyaluronan which eventually will be resorbed. Further studies are needed to fully evaluate the effect of the macroporous gelatin spheres, with or without cellular pre-seeding, as a matrix for guided tissue regeneration. However, we believe that the prospect to use these spheres as an injectable, 3D, biodegradable matrix will greatly enhance our possibilities to regenerate tissues through guided tissue regeneration.

So here we are
We've reached the top floor
We've pushed all the buttons
There aren't any more

Do you think it's time
That we all took a break
Or maybe we should cross our fingers
And sit here and wait

Now that you're here
Did you think there was more
Hey! Get those grubby fingers
Away from the door

Why are you standing there
Wearing that frown
We've got somewhere to go
We can all go down

And now that you've had
The ride of your life
Have you got any questions
I don't charge for advice

Abbreviations

2-D	two-dimensional
3-D	three-dimensional
CK	cytokeratin
DMEM	Dulbecco's modified Eagle's medium
DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
FCS	fetal calf serum
FISH	fluorescence <i>in situ</i> hybridization
FITC	fluorescein isothiocyanate
g	gram
g	gravitational force
GM	glucose monohydrate
GTR	guided tissue regeneration
H&E	haematoxylin-eosin
HEPES	N-(2-Hydroxyethyl)Piperazine-N'(2-Ethanesulfonic Acid)
HMEC	human mammary epithelial cells
IHC	immunohistochemistry
MEM	modified Eagle's medium
MGS	macroporous gelatin sphere(s)
MTT	3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide
NCS	newborn calf serum
o.n.	over night
orO	oil red O
PFA	paraformaldehyde
PBS	phosphate buffered saline
PMN	polymorphonuclear cells
PUUR	poly(urethane urea)
RT	room temperature
TE	tissue engineering
TEMP	tissue engineering manufactured product

It takes time to get to Avalon
That's why we're on this marathon

Preface

Since the beginning of time, man has nurtured the idea of recreating parts of the human body. Examples can be found abundantly in the popular press, e.g. Dr McCoy's amazing methods of treatments in the Star Trek movies and Professor Frankenstein's (in)famous monster.

Attractive solutions for the everlasting shortness of donated organs could be that we regain the knowledge of how to regenerate diseased tissues and organs *in vivo*, or by *in vitro* organ and tissue cultures. The idea is probably not as far fetched as it might seem at a quick glance. In the animal kingdom, several species have retained the ability to re-grow limbs if severed or traumatically amputated¹. For example the blindworm can lose the distal part of its tail but it will re-develop in some time. The tail lies flopping on the ground, or in the predator's mouth, distracting the harasser while the snake gets away. Another example is the salamander that can regenerate whole limbs in a matter of a few weeks if severed in a fight.

The genetic information to regenerate tissues and organs must also be retained in humans since the fetus, during the first two trimesters *in utero*, has the ability not only to heal wounds without scarring, but also to re-grow limbs if, for some reason, injured². This genetic information is, later in the pregnancy, either lost or hidden since we as full-born babies no longer possess these excellent properties.

Scientists have since long tried to find the key to the hidden treasures of tissue and organ regeneration. These early research activities predates its later invented denotation - '*Tissue Engineering*'. The term '*Tissue Engineering*' can be tracked back to a bioengineering panel meeting in Washington D.C. in the spring of 1987, held by the National Science Foundation³. In 1988, at the first meeting devoted specifically to TE, at Lake Tahoe in California, USA, one definition of the expression '*Tissue Engineering*' was coined;

'Tissue engineering is the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions.'^{3,4}

The essence of TE is to, by the use of living cells and/or components of (extracellular) matrix, manufacture trans-/implantable products or devices to restore or replace functions in the (human) body^{5,6}. Of this follows the understanding that to be able to engineer living tissues and/or organs, the structural and functional relationships of cells, surrounding extracellular matrix, tissues, and organs must be recognized. If we can understand, and hence control, these inter-behavioral relationships, it can become possible to obtain (biological) TE manufactured products for trans-/implantation.

Plastic surgeons often perform corrections of soft tissue defects by means of autologous, allogenic, heterologous, and alloplastic agents, for example by autologous fat transplantation. An intriguing alternative is to use TE techniques to regenerate tissues or to produce TEMPs for soft tissue defect corrections.

In this thesis, tissue engineering protocols to reconstruct, restore, maintain, and improve soft tissues are explored.

I sacrifice myself for my position
Dedicate my time to indecision
Spend my days in idle conversations
Spend my nights in random concentration

Every day the same old situation
Every night the same infatuation
Life is good but life can be frustrating
I'm still here, and I'm still waiting

I state my case before the judge and jury
Everybody says 'What's your hurry?'
Life's too short to spend anticipating
I'm still here, and I'm still waiting

Background

Introduction

Each year, millions of people around the world suffer from organ failure, or tissue loss for different reasons. The total health care cost of this patient-group was in 1993 approximated to exceed \$400 billion per year in the US alone⁴. The treatment of tissue or organ loss is based on surgical reconstruction (e.g. free or pedicled flaps), the use of extra- or intracorporeal mechanical devices (e.g. mechanical heart valves or dialysis), and inter-individual tissue and/or organ transplantations (e.g. cornea transplants, heart transplants). Although many lives have been saved or improved using these methods, they all have severe drawbacks such as donor(site) morbidity, the necessity of immunosuppressors, or the fact that the patient is bound to visit the clinic several times/week for treatment. Organ and tissue transplantation is further limited by a lack, or an ever-increasing shortage, of donors. In Sweden, in the 1980's, around 140-150 persons/year donated organs⁷. This number dropped to around 100 donors/year in the following two decades. In the year 2000, Sweden harvested tissues or organs from 11,1 donors/million inhabitants (PMP)/year, Belgium 21,7 PMP/year, and Spain 33,7 PMP/year⁷. There are of course a number of explanations to both the differences between countries and to the low number of donors and PMP/year, but the fact remains – the amount of patients in need of tissue or organ transplantation exceeds the amount of donated organs and tissues by far.

Mechanical devices, such as heart valves and hip prostheses, can function for long periods of time but often involve the need of medication (e.g. immunosuppressors) or the need to change the device due to material wear or malfunctioning. Furthermore, mechanical devices are not able to supply all the functions of the organ, and hence can not prevent further deterioration of the patient.

A surgical reconstruction often alleviates only the bulk, or the volume, of the tissue but seldom contributes significantly to the physical properties of the lost tissue or organ. Too often quite extensive surgery is needed, including anesthetic, surgical, and postoperative risks, as well as risk for flap necrosis and infections.

As is implied, there is a need for improvement in the field of treating tissue and organ (function) loss. One of many solutions to these problems could be the use of tissue engineering.

Tissue engineering

Even though scientists have worked in this area of research for quite some time, the term '*tissue engineering*' is stated to have been coined at a workshop in 1988 with the understanding of TE as being '*the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function.*'^{3,4}

Few fields of science are as interdisciplinary as TE. It is a necessity, in order to succeed, that TE combines such a diversity of research areas as biology, medicine, chemistry, material science, physics, and more.

With the above mentioned definition of tissue engineering, this field of research is quite vast. In this thesis I have focused on the regeneration of soft tissues, using the third strategy (open system) of TE (*vide infra*).

Langer and Vacanti⁴ described, in 1993, three general strategies for recreation of tissues;

1. **Isolated cells or cell substitutes.** Supplementation of only those cells that resolve the lost or needed function, permitting manipulation of cells before injection. This strategy evades the need for surgery. The shortfall is, among others, that the cells might not retain their functions when injected, or that the host rejects the cells.
2. **Tissue-inducing agents.** Appropriate signal substances, such as cytokines and growth factors, are supplied to the tissue of interest leading to an auto-regeneration of the tissue.
3. **Cells placed on or within matrices.** This strategy can be used in an open or closed system.
 - **Closed system** – Cells are contained within a membrane that allows passage of nutrients, waste, and the wanted cell function(s), but prevents destructive factors such as e.g. antibodies from reaching the cells. The closed system can be implanted in the host or used as an extracorporeal device.
 - **Open system** – Cells are seeded, or attached, to a scaffold and implanted into the host to be incorporated with the surrounding tissue. The scaffold material can be synthetic or biologic, permanent or biodegradable. By the use of autologous cells rejection is avoided.

The rationale for the open system is based on *in vivo* observations stating that every tissue endures a never-ending remodeling. Under optimal conditions (e.g. in 3-D matrices) cells in culture tend to reform, or mimic, the appropriate *in vivo* tissue structure. As an example; endothelial cells cultured in collagen gels spontaneously form capillary-like tubes.

Cells transplanted in a suspension start without any intrinsic organization and do not have a template that guides the reconstruction as is the case if a scaffold is used. Furthermore, if the cells/tissue is implanted in large volumes, the nutritional requirements can become a problem since the distance to the nearest capillary is too great. Hence, the open systems are designed so that the scaffold guides the cell organization and proliferation, but also allows diffusion of nutrients to the cells. When the cell number expands by proliferation after transplantation, the matrix is vascularized either as a host response to the material or induced by the release of angiogenic factors from the matrix and/or the transplanted cells⁴.

Yet another approach to regenerate tissues, also based on the rationale of tissue remodeling (*vide infra*), is by guided tissue regeneration, where a 3-D (biodegradable) scaffold provides an ECM analogue which functions as a needed template for host cell infiltration and a physical support to guide the differentiation and proliferation of cells into the matrix from the surrounding tissue. As cells infiltrate the scaffold, and start producing autologous extracellular matrix, the scaffold is degraded in the event of normal tissue remodeling⁸.

Soft tissue

During embryogenesis, gastrulation occurs in the 3rd week of development. This process establishes all three germ layers in the embryo. Cells of the epiblast form the mesoderm and embryonic endoderm by invagination, and cells remaining in the epiblast form the ectoderm⁹.

In the 3rd to 8th weeks of development (embryonic period) every germ layer gives rise to specific tissues and organs, and the main organ systems are established at the end of this period.

The ectodermal layer gives rise to organs and structures that are in contact with the outer world, e.g.; central and peripheral nervous system, sensory epithelium, epidermis, and mammary gland. The mesodermal layer forms e.g.; the dermis and subcutaneous tissues of the skin, connective tissue, cartilage, bone, muscle, blood cells, and endothelium⁹.

Regarding the ECM in developing tissues, two major continuous alterations occur; the components of the ECM change, and the cellular reactivity to specific ECM components change¹⁰. The ECM and cell surfaces interact functionally and this plays an intense role in the development and maintenance of a number of cells and tissues. Hence, the ECM is also instructive, or informational, and greatly influences cell behavior.

ECM is mainly composed of collagen, proteoglycans, and glycoproteins. Different ECMs contain different elements which define their tissue specificity¹¹.

Adipose tissue

From a developmental, anatomical, and functional perspective, the adipose tissue is an independent, but diffusely located, metabolically active organ¹². The main function of this tissue/organ is to act as an energy depot and it is subjected to hormonal control¹³. The adipocytes accumulate mainly triacylglycerol (three fatty acid molecules attached to a glycerol moiety), but is also able to accumulate and mobilize large amounts of unesterified cholesterol. The rate-limiting factor in the adipocytes' fatty-acid uptake is lipoprotein lipase (LPL)¹⁴, which is also utilized as an early marker of adipocyte differentiation (*vide infra*). Other functions of the adipose tissue are; linkages to the immune system, synthesis of alternative complement pathway components, hormone production and regulation, and its physical properties as padding and bulking agent^{15,16}. As the adipocytes themselves secrete hormones such as leptin, the obese gene product, one can consider the adipose tissue also as an endocrine organ.

Fatty tissue appears as both discrete depots and within other tissue (e.g. muscle). The adipocytes have a characteristic round, unilocular shape with an eccentrically placed cytoplasm and nucleus due to the large lipid containing vacuole that constitutes most of the cell.

For a long time it was believed that new fat cells could not be formed in man after puberty, and that childhood-onset adipositas was associated with adipocyte hyperplasia, whereas the adult-onset obesity was due to adipocyte hypertrophy only¹⁴. Even though Smith¹⁷ probably was the first to describe the preadipocyte as 'a fibroblast-like cell' grown in tissue cultures of human adipose tissue it was Poznanski¹⁸ et al in 1973, and again later in 1976, Van¹⁹, who demonstrated that the adipocytes indeed developed by proliferation and differentiation of cells located in the stromal-vascular fraction of fatty tissue, and that the fully differentiated, or mature, adipocyte has no capacity of proliferation^{20,21}. Several others have later reproduced these findings^{16,22}, whereas others debate the conception that unilocular adipocytes do not divide²³.

Hence it became possible to culture adipocyte precursors *in vitro* after isolation. Usually preadipocyte cultures are set up mainly by collagenase digestion of adipose tissue samples, thereby releasing cells with a fibroblast-like morphology, the preadipocytes²⁴. These precursors are able to proliferate (with a doubling time of about 20-96h^{25,26}) and differentiate into mature adipocytes²⁷. The committed preadipocyte maintains the capacity for replication but has to withdraw from the cell cycle before adipose conversion¹⁶.

In research, primary preadipocyte cultures offer several advantages over preadipocyte cell lines; 1) They are diploid and reflect the *in vivo* situation better, 2) Primary cells can be isolated at various stages of differentiation and from different depots which is important since different physiological properties have been described in preadipocytes from different depots. One should bear in mind though; preadipose cell lines and preadipocytes are already committed to the adipocyte lineage and primary cultures are often highly heterogeneous in their cellular population¹⁶.

Roncari²⁸ and co-workers showed that not only do obese individuals have more adipocytes in their adipose tissue, but also their preadipocytes replicate at a higher speed than do lean persons' preadipocytes. As these experiments were performed on subcultures of preadipocytes this indicates an aberration inherent in the cells.

Also today, only little is known about what commits a cell to the adipogenic lineage. Preadipocytes are believed to originate from a pluripotent stem cell, of unknown origin and nature¹⁵. Preadipocytes behave and look like fibroblasts early in cell culture and it may be that the fibroblasts from the stromal-vascular fraction have the potential to become preadipocytes¹⁹.

When the preadipocytes differentiate (5-90% of a given preadipocyte cell culture converts to adipocytes; strongly dependent on the inoculation density, donor depot, and age of the donor^{16,21,22,24,29}) chronological changes in the expression of numerous genes are observed by the appearance of early, intermediate, and late mRNA/protein markers.

Differentiation is further characterized by an elevated lipogenic capacity and a switch from the fibroblast-like shape to the unilocular shape of a mature adipocyte (terminal differentiation)²⁴. This occurs *in vitro* when the cells reach confluence, but is not triggered by cell contact. Instead it is believed to be due to growth arrest at the G₁/S stage of the cell cycle^{16,21,30}. Induction and elevated expression of several specific mRNAs (such as the transcription factors C/EBP- α and PPAR- γ) and the accumulation of lipids characterize the process³¹. Smaller lipid vesicles fuse ultimately into a single globule 2-4 weeks post-confluence.

Markers of preadipocyte differentiation^{16,21,22,26}

Abbreviation	Marker	Specificity	Period
A2COL6/pOb24	Leptin		Early
C/EBP	CCAAT/enhancer binding protein		Early
LPL	Lipoprotein lipase	Not adipocyte specific	Early
PPAR- γ	Peroxisome proliferator-activated receptor- γ	Largely adipocyte specific	Early
SREBP-1c/ADD1	Sterol regulatory element binding protein-1c/adipocyte determination and differentiation factor 1		Early
	Clone 5		Early
IGF-I	Insulin-like growth factor-I		
	Acetyl coenzyme A carboxylase		Late
ALBP/aP2/P442	Adipocyte lipid binding protein	Adipocyte specific	Late
	Fatty acid synthase		Late
G-3-PDH	Glycerol-3-phosphate dehydrogenase		Late
PLA ₂	Phospholipase A ₂		Late
	Adipsin	Adipocyte specific	Very late
	Phosphoenolpyruvate carboxykinase		Very late

Growth factors and hormones regulating proliferation and differentiation of preadipocytes^{16,21,22,26}

Abbreviation	Growth factor/hormone	Proliferation	Differentiation
EGF	Epidermal growth factor	Stimulates	Inhibits
aFGF	Acidic fibroblast growth factor	Stimulates	Unknown
bFGF	Basic fibroblast growth factor	Stimulates	Unknown
	Insulin (the effect via IGF-I receptor)	Stimulates	Stimulates
IGF-I	Insulin-like growth factor-I	Stimulates	Stimulates
PDGF	Platelet derived growth factor	Stimulates	Unknown
TGF- α	Transforming growth factor- α	Stimulates	Inhibits
TGF- β	Transforming growth factor- β	Stimulates	Inhibits
TNF- α	Tumor necrosis factor- α		Inhibits

Dramatic changes also occur in cellular morphology, cytoskeletal components, and the level and type of ECM components. Markers of differentiation are turned on and off at different specific times. One of the earliest ones is pOb24, its expression increases rapidly during the early stages of differentiation and decreases when later markers are turned on. By the use of different polyadenylation sites, two mRNAs are derived from the same gene; pOb24 and A2COL6. Expression of these two mRNAs is not specific for adipose tissue.

The differentiation of preadipocytes can be postponed (indefinitely) if one holds the cells in a continuously proliferative state²¹ and long-term preadipocytes cultures are hence possible³².

As the adipose tissue is intricately linked to the hormone production and regulation in the body, several growth factors regulate the proliferation and differentiation of preadipocytes and adipocytes. Of course, the adipose tissue's own hormone and growth factor production also affects a number of organs, tissues, and systems in the body. It is likely that a number of these factors also have a role in altering the energy balance e.g. during infections.

Fetal calf serum is one of the strongest adipogenic factors known. Other adipogenic factors used in adipose cell culture are adipose conversion factor (ACF), bovine pituitary extracts, dexamethasone (glucocorticoids), IBMX/MIX (3-isobutyl-1-methylxanthine), insulin, and IGF-I. The adipogenic effect of these factors can further be amplified by cAMP-elevating agents^{21,22}. Apart from soluble factors and hormones, the ECM has been shown to play an intricate part in the preadipocyte differentiation. The ECM interconnects adipocytes and gives rise to fat cell clusters *in vitro* and fat lobules *in vivo*. Early in the differentiation process, the deposition of collagen at the cell-ECM border is seen and it has been shown that an active collagen synthesis is required for adipocyte differentiation¹⁶. ECM components probably modulate the differentiation process, perhaps by release of cell-cell adhesions and thereby allowing changes in the cell morphology and volume.

Green and Kehinde³³ were probably the first to employ 'TE thinking' to the regeneration of adipose tissue. They demonstrated soft tissue regeneration in nude mice, over the course of six weeks, after having transplanted cells from an established preadipocyte cell line. This idea was again explored by Van and Roncari in 1982 who collagenase treated epididymal fat pads from rat, cultured the cells from the stromal-vascular fraction, and transplanted these intramuscularly to the same rat. They showed that the transplanted preadipocyte suspension gave rise to adipose tissue, stable for more than six months, while fibroblasts treated and injected in the same way did not³⁴.

As Billings and May²⁵ stated already 1989; ‘...the role of the preadipocyte in free fat transplantation can be postulated. The graft of mature adipose tissue with its connective-tissue stroma, when implanted, goes through an initial period of ischemia and inadequate nutrition. This could cause many of the mature fat cells to either necrose or dedifferentiate to preadipocytes. When the blood supply adequately supplies oxygen and nutrients to the graft, the adipocyte precursor pool of immature preadipocytes could differentiate into mature adipose tissue, albeit of less volume.’ Hence, we, as well as others^{25,35,36}, have postulated the train of thoughts to skip the event of mature adipocytes suffering from ischemia, and instead directly transplant preadipocytes, in order to gain a larger volume of viable adipose tissue as the preadipocytes differentiate *in vivo* when regenerating adipose tissue (*vide infra*).

Connective tissue

Connective tissue is a diverse group of tissues, with different functions and characteristics, and it contains cells that noticeably are separated from one another. Generally it consists of cells and extracellular fibers embedded in a matrix of ground substance and fluids. It develops from the mesenchyme (embryonic connective tissue). The ever-present cell is the fibroblast, which is responsible for the production of extracellular fibers and ground substance. The process occurs both within the cell and outside. A few basic events happen in the fibroblast’s cytoplasm;

1. Polypeptide chains are produced and simultaneously discharged into the cisternae of the rough endoplasmatic reticulum.
2. Post-translational modifications of the poly-peptide chains, e.g. cleavage of the signal peptide, hydroxylation of prolysine and lysine residues, addition of O-linked sugar groups, which results in the formation of pro-collagen.
3. Pro-collagen is moved to the exterior by secretory granulae.

When pro-collagen is secreted into the extracellular space, enzymes cleave amino acid residues from the terminals and thereby form tropo-collagen which aggregates to form the collagen fibril³⁷.

The integumentary system

The integument (skin, cutis) and its derivatives make up for the integumentary system that covers the entire body. It consists of two main layers; epidermis and dermis, and contains several associated structures (appendages) such as sweat glands, hairs, sebaceous glands, and nails.

The function of the cutis is to; provide protection against physical, chemical, and biological injuries, regulate fluid and temperature balance, be the interface to the surroundings (sensation receptors), and finalizing vitamin D.

Dermis

The dermis develops from the mesenchyme and the dermal papillae are formed during the 3rd and 4th months of gestation⁹. The papillae which project into the epidermis usually contain a capillary or sensory nerve end organ. The dermal papillae assure an extensive surface area interface between the epidermis and dermis, strengthening the attachment of the epidermis to the connective tissue. The deeper layer of the dermis, subcorium, contains large amounts of fat.

The dermis is a highly specialized and complex structure upon which the epidermis is functionally and anatomically dependent, both in our daily existence and in situations of wound healing³⁸.

The dermis is responsible for the strength and pliability of the skin. It can not regenerate in adult mammals but instead heals with repair and scar formation. Collagen is the main structural component of the ECM and the collagen bundles in normal human skin show a complex, but well defined 3-D structure of patterns. In the absence of an organized matrix, the fibroblasts initially synthesize an immature collagen matrix which is remodeled into scar tissue due to an abnormal deposition of the ECM^{8,38}.

Epidermis and dermis can not be allografted; they are ultimately rejected due to immunogenic responses directed primarily against the cells of the epidermis (Langerhans cells and keratinocytes) and the dermis (endothelial cells and fibroblasts) that express foreign major histocompatibility complex (MHC) antigens³⁸.

Several attempts have been made to reconstruct, or culture, the dermis *in vitro*³⁹. Alas, no one has succeeded completely this far. A more successful route has been shown to be by GTR. The dermis can be looked upon as a collagen scaffold that guides the fibroblasts to deposit ECM in an organized fashion. Hence, if a biologic or synthetic construct is applied to a wound bed, the host fibroblasts migrate into the scaffold to populate it while depositing autologous ECM and degrading the scaffold⁴⁰⁻⁴³.

Several different compositions (material, porosity, degradation speed, etc.) of scaffold materials have been described for GTR and TE of the human skin^{38,44-47}. Some authors advocate the inclusion, or pre-seeding of the scaffold with autologous or allogenic cells (fibroblasts and/or keratinocytes) in order to gain a quicker and more thorough regeneration of the dermis (and epidermis). It is assumed that the presence of fibroblasts in the scaffold accelerates the regenerating process by releasing cytokines and other biologically active substances⁴⁸.

Epidermis

Epidermis originates from the surface ectoderm of the embryo. The embryo is covered by a single layer of ectodermal cells which, in the beginning of the 2nd month, divide and develop the periderm. The basal cells proliferate further and by the end of the 4th month the epidermis acquires its definitive arrangement as a stratified squamous epithelium⁹. The epidermis then consists of five layers where the deepest, stratum basale, is adjacent to the basal lamina and contains the dividing cells.

The epidermis holds many physiological functions such as temperature and fluid regulation, vitamin synthesis, is a barrier to the outer world, and more.

Contrary to the dermis, the epidermis heals by regeneration. The regeneration relies on remnants of keratinocytes residing deep within dermal structures (sweat glands, hair follicles) and from the wound edges. From this follows that if the wound is full-thickness and too large (more than a few centimeters across) the ingrowth from the wound edges will be insufficient and spontaneous healing will not occur⁴⁹.

To treat larger wounds that can not be closed primarily, the technique of skin grafting can be employed (*vide infra*).

In 1975, Rheinwald and Green⁵⁰ developed a reliable method to culture epidermal cells by the use of lethally irradiated 3T3 mouse fibroblasts as feeder cells. A cutaneous biopsy is cut into fragments and digested by trypsin to separate the epidermis from the dermis. Proteolytic enzymes are then used to further dissociate the epidermis to a single-cell suspension of keratinocytes before culturing. The plating efficiency of the primary explant is low since only about 10% of the keratinocyte population are able to proliferate (the remainder is terminally differentiated)⁵¹. In subsequent passages the plating efficiency increases to about 30% as fewer cells are terminally differentiated.

The method described by Rheinwald and Green⁵⁰ is based on irradiated, non-proliferating fibroblasts. The function of the 3T3 cells is still somewhat unclear but they favor growth of the keratinocytes, as well as they inhibit the growth of human fibroblasts which otherwise quickly contaminate and take over the cultures.

Autologous cultured epidermal sheets are now available to plastic surgeons as a complement to split-thickness skin grafts when treating major burns or other large wounds. However, as no dermal component is transferred along with the keratinocytes the epidermis becomes quite unstable and is prone to blistering upon minor trauma. Nevertheless, autologous cultured skin grafts do have a place in burn treatment. Since Dispase[®] is often used to release the keratinocyte sheet-grafts from the culture surface, it may remove surface proteins from the cells and reduce their adhesive potential. This understanding has led to a progressive development of skin culture techniques. The use of Dispase[®] can be avoided by transplanting the cells in a suspension, rather than as a sheet. Further advantages of suspension transplantation are the reduced time needed for culture, it produces cells that have not undergone phenotypic changes (differentiation) associated with contact inhibition, and the tedious manual labor of attaching keratinocyte sheets to a transplantation vehicle can be avoided, hence reducing the costs of the procedure⁵². The cultured keratinocytes can then be spray-painted on the wound surfaces by means of for example fibrin-glue (Figure 1 and 2)⁵³.

Still, only the epidermal part of the skin is transplanted and the problem of blister-prone and fragile skin is still present due to insufficient epidermo-dermal junctions and mechanical instability.



Figure 1. Cultured keratinocytes in transport vial.



Figure 2. Cultured keratinocytes spray-painted on wound surface with fibrin-glue.

Breast tissue

Mammary glands have evolved as milk-producing organs to feed the offspring. During embryogenesis both sexes show breast growth and development. Glands develop along the milk lines on the ventral aspect of the thorax. In females, the breast glands are subjected to further development under hormonal influences, and are also influenced by changes in the ovarian hormone levels during menstruations. The secretion and milk production is regulated by prolactin from the pituitary gland and somatomammotropin from the placenta. At menopause the glandular portion of the breast involutes and is replaced by connective tissue and fat³⁷.

The adult gland comprises 15-20 irregular lobes of branched tubuloalveolar glands separated by fibrous bands of connective tissue. The lobes radiate from the nipple and are divided into several lobules. Abundant adipose tissue is intermingled in the dense connective tissue of the interlobular spaces.

The tubuloalveolar glands are derived from modified sweat glands of the epidermis and each gland ends in a lactiferous duct opening onto the nipple³⁷. The lining of the ducts is composed of two-layered cuboidal epithelium in the lactiferous sinus transient into a single-layered columnar epithelium in the rest of the ducts. Myoepithelial cells, having contractile properties, are intermingled in the epithelium in the secretory portion of the gland. In the inactive gland the glandular portion is sparse, consisting mainly of ducts, whereas the active gland show dramatic proliferation and development during pregnancy; fat and connective tissue portion decreases and ducts and alveoli develop, secretory cells hypertrophies³⁷. The epithelial tissue of the inactive gland could be described as consisting of a dynamic, immature epithelium which is continuously renewed during menstrual cycles.

To protect the integrity of the epithelium, any loss of cells needs to be compensated by cells with identical phenotype. This can be achieved by mitosis within a population of differentiated cells, or by *de novo* replacement through selective differentiation of progenitor cells. Boecker and Buerger⁵⁴ has shown that a single progenitor cell of the resting breast epithelium gives rise to both the glandular and myoepithelial cell lineages, and that these progenitor cells (approximately 4% of all epithelial cells) are located in the luminal epithelium of the double-layered breast epithelium.

Human mammary epithelial cells are located in branched ducts that terminate in lobules, are surrounded by a basal membrane, and are embedded in connective tissue and fat. The HMEC are subdivided into two categories; the luminal cells that border the lumen and myoepithelial cells that are located between the basal membrane and the luminal epithelium⁵⁵. A third cell type, the alveolar cell, lines the large distended ductules or alveoli during lactation, and is responsible for the production and secretion of milk. In histological sections the cell types can usually be distinguished by their positions⁵⁶.

When setting up cultures of HMEC, enzymatic digestion of breast tissue is a successful route. The preparation of organoids allows separation of ductal and stromal breast elements and the organoids can further be digested to single-cell suspensions. As this is a heterogeneous cell population, purification of the culture is necessary. Unlike other organ systems, HMEC in culture are not obtained from fully differentiated tissues.

As for keratinocytes, cell culture studies of HMEC were hampered for a long time due to inadequate culture techniques, even though this could be overcome by the use of feeder cells.

Among the first to describe long-term cultures of HMEC without feeder cells was Stampfer⁵⁷ who, in 1980, described a technique to culture HMEC from reduction mammoplasty tissue using conditioned medium and growth factors to yield reproducibly active epithelial cell growth for several months and passages. This novel technique opened the door for studying cellular physiology of HMEC in monolayer cultures.

The heterogeneous nature of the breast tissue have troubled cell culturers, e.g. Stampfer⁵⁷ used selective medium to obtain nearly pure cultures of HMEC, whereas Gomm⁵⁸ used immunomagnetic separation with Dynabeads[®] to part epithelial and myoepithelial cells in cell cultures from the normal human mammary gland. A major problem has been the lack of markers that distinguish the two classes of epithelial cells. Antibodies to the human milk fat globulin have been employed to detect luminal epithelium⁵⁵.

Initially, focus of HMEC *in vitro* cultures was on studying properties of normal and pathological mammary epithelial cells in e.g. cancer, developmental, and endocrine research⁵⁹⁻⁶¹, but later on also TE scientist gained interest in HMEC cultures to regenerate the female breast⁶².

When HMEC is cultured on plastic surfaces they rapidly lose their differentiated characteristics. When cultured in collagen gels or other stromal substrates, and in the presence of lactogenic hormones, HMEC retain their differentiation ability and will accumulate and secrete casein (milk protein synthesis) which is a specific molecular marker of HMEC differentiation^{63,64}. The mechanisms involved are not fully understood but changes in cell shape and proteoglycan compositions on the surface of HMEC have been suggested.

Besides lactogenic hormones, Levine, and others, have shown that cell-cell interactions between mammary epithelial cells, adipocytes, and fibroblasts have a potent growth-promoting activity for mammary epithelium, and subsequently several techniques have been developed to co-culture HMEC and stromal cells⁶³⁻⁶⁵. It is believed that the co-culture system of HMEC and adipocytes is similar to the developing mammary gland milieu, where growth and morphogenesis occurs⁶⁶. In embryogenesis, a single layer of ectodermal mammary cells invades the fat pad precursor tissue to form the complete ductal tree.

When HMEC are cultured on biomatrices or co-cultured with adipocytes the cells undergo morphogenesis in that much as ductal structures with lumina and secretion of milk components occur, mimicking the *in vivo* structure of the breast gland^{61,62,64,67}. In the development of ductal structures seven distinguishable steps can be noticed; 1) Pseudopodia are sent out in different directions, 2) Cells gain directionality and move or send processes towards other cells, 3) Cells line up with each other, 4) Cells move closer to one another, 5) Rows of cells become interconnected, 6) Rows become thicker and duct-like, 7) Alveoli-shaped spheres of cells develop at the ends of the duct-like structures⁶⁷. By neutralizing bFGF the duct formation can be completely inhibited, and by neutralizing TGF- β , duct formation is stimulated. TGF- β is one of the most potent of the negative regulators of epithelial cell growth⁶⁸. It is also a multifunctional regulator of cell development and differentiation. It stimulates ECM formation, supports wound healing, and influences differentiating processes.

Cartilage

Articular cartilage is a metabolically active tissue, but the chondrocytes (corresponding to about 1% of the volume of hyaline cartilage) have a relatively slow turnover⁶⁹. The tissue itself lacks a vascular system that could support repair and remodeling. Furthermore, chondrocytes are not required to proliferate to maintain the cartilage tissue, also, there is no direct access to progenitor cells, as is the case in many other tissues (e.g. skin). Hence, cartilage has a limited capacity of self-renewal, and because of the limited capacity for spontaneous repair, even minor injuries may lead to progressive damage and degeneration⁷⁰.

Tissue engineering approaches could offer novel possibilities for restoration of damaged or lost tissue. But cartilage TE brings certain requirements that need to be considered;

- > To functionally and mechanically restore the defect, the implant probably needs to include reparative cells that are capable of synthesizing hyaline cartilage (or elastic cartilage) specific ECM⁷¹.
- > The cells need to be supported by a biodegradable matrix that is equivalent to the mechanical properties of the surrounding tissues.
- > Donor tissue is scarce since most articular cartilage is weight bearing and cell harvest from these areas results in donor site morbidity.
- > Elastic cartilage is somewhat easier to obtain as a biopsy from the external auricle or the sterno-costal region and does not bring about that heavy donor site morbidity.

As millions of people around the world are affected by arthrosis, and other cartilage disorders, a great amount of research is dedicated towards reconstructing cartilage. Thus, a TE based reconstruction of cartilage could have a tremendous impact on available medical treatments and cost of treatments.

Today there are cell culture based techniques, to treat cartilage injuries, available to the clinic. From a non-weight bearing area of cartilage, a biopsy is taken and the chondrocytes are isolated from the tissue. Chondrocytes are then numerically expanded *in vitro*. Upon transplantation, the wounded area is debrided, a periosteal flap is sutured as a lid over the defect and the chondrocyte cell suspension is injected in the periost-cartilage cavity⁷². This autogenous chondrocyte implantation with a periosteal graft has shown encouraging results, but the predictability and reliability of hyaline or fibrocartilage formation is still questionable.

One of the problems with *in vitro* expansion of chondrocytes is that during the culture process (in 2-D cell cultures) the chondrocytes lose their spherical shape and attain a fibroblast-like appearance (probably due to dedifferentiation). Subsequently the expression of hyaline cartilage markers such as aggrecan and collagen type II decreases (collagen type II represents 90-95% of the collagen in hyaline cartilage ECM⁶⁹), whereas the expression of non-hyaline cartilage specific collagen type I increases.

Three-dimensional cell culture matrices and MGS have been described to have a favorable impact on the *in vitro* culture of chondrocytes as the cells retain their chondrocytic phenotype to a higher extent^{70,73,74}.

In vivo studies using the open-system approach (*vide supra*) have been performed with various results^{75,76}.

Cell culturing

The era of cell culturing may have started with Harrison⁷⁷ almost 100 years ago (*vide infra*). Traditionally, anchorage dependent cells have been cultured on plastic or glass surfaces. Several limitations with these substrates have been observed; failure of cells to adjust to culture, polarity and morphology changes of the cells, excessive proliferation, loss of differentiating capacity, and shortened or limited lifespan¹¹. Since most cells bind to/with specific adherence proteins rather than directly to plastic or glass surfaces, these proteins have to be supplied (e.g. by coating the surfaces with gelatin, fibrinogen, fibronectin, or laminin) or the production of these proteins by the cells themselves has to be stimulated. By electrically charging the culture surfaces, cellular attachment and growth can be enhanced. Pretreating culture surfaces with components of the ECM can improve the culture of many cells. The ECM components that cells are in contact with *in vivo* also give the best response when cells are cultured *in vitro*¹¹.

The 2-D cell culture system is sufficient for most research applications, but to increase cell yield, microcarriers have been developed. With microcarriers the culture surface can be many-folded and anchorage dependent cells can be cultured on the carriers in bio-reactors or spinner flasks. To further increase culture surface area, and hence cell yield, porous spheres can be used. On these carriers not only the surfaces can harbor cells, but cells can also attach and migrate into the spheres. As some porous spheres are biodegradable, they can also be used as transplantation vehicles (*vide infra*).

Non-anchorage dependent cells (e.g. blood cells) are usually cultured in suspensions in bio-reactors or spinner flasks.

To mimic *in vivo* tissue milieus, cells can be cultured in different gels or matrices such as collagen gel or Matrigel. By doing this, cells can grow in 3-D structures that closer resembles the *in vivo* architecture^{11,62}.

Storage

Live cells can be stored almost indefinitely in liquid nitrogen (down to -180° C) and for very long times in high performance freezers. The use of dimethylsulphoxide (DMSO) as cryoprotectant prevents intracellular ice crystal formation, and subsequent detrimental osmotic effects. A quick and steady freeze rate of -1° C/minute is optimal and is performed by the use of specialized freezing containers.

When wanted, cells can be thawed and culture resumed. Thawing should be performed rapidly, without heating, and the DMSO should be diluted, or rinsed of, quickly for optimal recovery of the cells.

Cell harvest techniques

Explants

Tissue culture was probably first performed in 1907 to resolve a neurobiological dispute⁷⁷. Small pieces of spinal cord were placed on clotted tissue fluid in a moist, warm chamber and observed. In time, individual nerve cells were seen extending into the clot. The method used was later termed explant technique.

Explant technique is still widely used to harvest cells for cultures. It provides an easy technique to harvest cells from tissue biopsies and is sometimes the only successful technique e.g. for certain kidney tumor cell cultures and other fragile cells.

The drawback is sometimes very heterogeneous cultures, as tissues usually contain more than one cell type. Separation can later be performed to purify the cultures in subsequent passages.

Single cell suspension

With this method cells are obtained for culture by conversion of the tissue to a single-cell suspension. This is usually performed by disruption of the ECM and intercellular junctions that holds the cells together; typically this is performed by treating the tissue with an (proteolytic) enzymatic digestion mixture containing trypsin or collagenase. Cell-cell adhesions depend on Ca^{2+} and the disruption of the adhesions is enhanced if the tissue is subjected to chelating agents such as EDTA. By gentle mechanical disruption the cells are then dissociated into a single-cell suspension⁷⁸.

Any given tissue contains more than one cell type, and the separation of cell types and the selection of the type of interest can be performed by different approaches, e.g.;

- > **By physical properties** – large cells can be separated from small cells, and dense cells from light cells by centrifugation.
- > **By adhesion properties** – some cell types adhere strongly to glass or plastic and can hence be separated from cells adhering less strongly. Some cell types detach easily from culture surfaces when subjected to e.g. trypsin and can be separated from cells that endure trypsin longer before detachment.
- > **By binding properties of antibodies** – antibodies, binding specifically to surface antigens of the cell type of interest, can be coupled to e.g. collagen, or magnetic or other types of beads to form a surface that only the cells of interest will adhere to. Bound cells can subsequently be released by gentle mechanical shaking or by degrading the matrix.
- > **By fluorescence-activated cell sorter (FACS)** – cells are labeled with antibodies coupled to fluorescent dyes, and labeled cells can then be separated from unlabeled cells by an electronic FACS.
- > **By nutritional properties** – cell types of interest can be singled out in a culture if they have specific nutritional requirements. By feeding the cultures the right medium, cells of interest will proliferate but not others.

Cell visualization

Cell authentication is a necessity in cell culturing. A number of molecular assays can be used for this, e.g. morphological analysis and cell protein expression analysis.

Routine histology

Routine histology examination can be performed using for example H&E staining procedures. This procedure is adequate to display morphological features, but not for examining chemical characteristics. Usually H&E is performed on fixed, paraffin embedded, and sectioned, tissue samples. The nucleus is stained blue and the cytoplasm pink.

To specifically stain or label certain cell types, functions of cells, or other chemical characteristics, other selective methods must be used. To stain neutral lipids (as in (pre)adipocytes) oil red O or one of the Sudans staining methods may be used. Immunohisto(cyto)-chemistry utilizing selective and specific antibodies for the structure or functional unit of interest may be employed either on tissue sections or directly on cultured cells.

By raising mono- or polyclonal antibodies it is possible to use them on cells or tissue sections where they selectively will bind to their antigens. If the antibody is conjugated with a fluorescent dye, the reaction (and hence the antigen of interest) can be visualized using a fluorescence microscope, i.e. direct immunohisto(cyto)chemical labeling.

If the antibody instead is bound to enzymes (e.g. peroxidase) the antibody will still bind to its antigen, and when the appropriate enzyme substrate is supplied, the reaction may be visualized with a light microscope.

3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-staining

An enzyme active in the respiratory chain, located in the mitochondria of living cells, is responsible for the cleavage of MTT. This process generates MTT-formazan, which is a dark blue, obvious, staining of the cell⁷⁹. Living cells can then easily be observed by ordinary light microscopy.

Adipocytes

Oil red O

Using oil red O, neutral lipids (mainly triglycerides), are stained with an orange-red color. Using oil red O staining requires fixation and handling of the tissue that does not release the lipids, such as e.g. using frozen sections⁸⁰. Sections can be counterstained for example by Mayer's haematoxylin to visualize the nuclei. Oil red O can also be combined with immunofluorescence staining to automate quantification of lipids⁸¹.

Perilipin

Perilipin is a major intracellular phosphoprotein of adipocytes. It is a unique protein associated with the periphery of intracellular lipid vesicles and appears in two forms; 56 kDa – Perilipin A (the most abundant form), and 47 kDa – Perilipin B. The function is mostly unknown but is suggested to have a role in lipid metabolism⁸². Perilipin seems further not to be found in other triacylglycerol synthesizing cells such as lactating mammary gland cells or liver cells of newborn mice, but indeed in steroidogenic adrenal cortical and Leydig cells⁸².

Human mammary epithelial cells and epithelial cells

Several different staining techniques to label HMEC are available. α -lactalbumin, β -, and γ -casein are considered selective markers for fully differentiated, and milk protein producing, HMEC and immunohistochemical staining with monoclonal antibodies is available⁸³. (Cyto)keratins, even though not specific to HMEC but to cells of epithelial origin, may be used as they indirectly show that cells in the culture are of epithelial origin⁶⁴.

The different HMEC types (luminal, myoepithelial, and alveolar cells) can be distinguished by their different CK expressions. Luminal cells are positive for CK 7, 8, 18, and 19, myoepithelial cells are positive for CK 5, 14, and 15^{56,60,84,85}. Cytokeratin 19 positive cells are the dominant cells in the lactating breast and can be seen shed into milk.

Cytokeratin 14 is expressed by basal cells in stratified epithelia, and CK 7, 8, 18 are normally associated with simple epithelia.

Surgical options

There are a number of surgical options available to treat tissue defects of different origins and kinds. They all have their pros (e.g. autologous tissue, easily performed) and cons (e.g. donor site morbidity, technical challenges, use of foreign material). Tissue engineering aims at manufacturing autologous tissues, and tissue constructs, that supersedes and/or complements the available surgical techniques in order to alleviate drawbacks of existing options.

Skin grafts

A skin graft consists of epidermis and some portion of the dermis. A skin graft can be full-thickness or split-thickness, depending on how much dermis is included in the graft. Full-thickness grafts have a tendency to contract immediately after harvest due to the elastin fibers in the dermis. Also split-thickness grafts contract primarily; the extent depends on the amount of dermis included in the graft. The real problem with skin grafting though, is the secondary contraction which is seen in healed grafts. This effect is probably due to myofibroblast activity⁸⁶. The thinner the graft is the more secondary contraction and the thicker the graft is the more primary contraction.

Full-thickness grafts have the best quality and endurance after transplantation, but the donor site has to be either sutured primarily or covered with a split-thickness skin graft. Hence full-thickness grafts are saved for functional and aesthetical important areas, whereas the majority of wound surfaces can be covered with split-thickness grafts (Figure 3). If the wound surface is too great, as is often the case in e.g. major burns, there might not be enough healthy skin, to use as donor sites, to be able to cover all the wounds⁸⁷. In the 1950s one tried to overcome this problem by seeding the wounds with skin particles that had been reduced mechanically. Meshing the skin grafts proved a more consistent alternative though.

Enzymatic treatment to dissociate the epidermal cells in order to seed them on the wound as single cells was then evaluated and abundant research was devoted to find a way to culture autologous keratinocytes for grafting on wounds (*vide supra*).

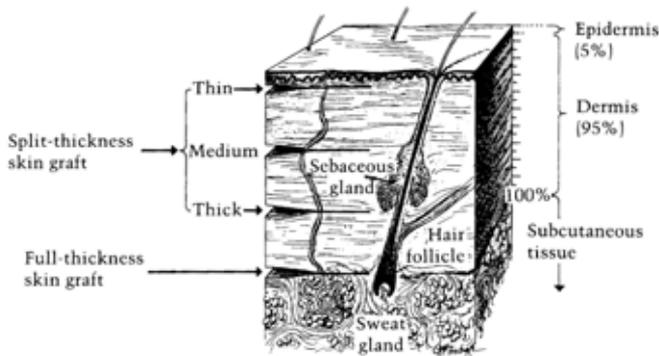


Figure 3. Drawing of normal human skin with adnexa and skin graft thicknesses indicated. (From 'Grabb & Smith's Plastic Surgery' 5th ed.⁸⁶ © Lippincott Williams & Wilkins)

Pedicle flaps

A flap is tissue that is transferred or transplanted with intact circulation⁸⁶. Historically, skin and subcutaneous tissues were raised as 'random' flaps, i.e. not based on a specified blood vessel. To reach longer distances these flaps had to be attached to a temporary recipient site before being further transplanted to the target site. In some areas specified vascular pedicles were identified, and since flaps could be raised centered on these vessels (axial flaps), it became possible to raise larger flaps with longer reach.

When it was discovered that muscles could be used as sources of tissues for flaps, it opened tremendous possibilities for reconstructions of defects since muscles are available almost anywhere on the body⁸⁶. When the vascular pedicles to muscles were identified, it became possible to detach the muscle's origin or insertion (or both), and to transfer the muscle to a new site as a flap maintaining circulation with the vascular pedicle. With the identification of the vascular connections between the skin and underlying muscles it became obvious that also a skin segment was possible to be transferred along with the muscle flap. Eventually the free tissue flap was developed.

Free flaps

Development within the field of tissue transplantation laid the grounds for the technique to transfer tissues with a blood supply that was possible to reconnect by vascular anastomoses. Autotransplantations were satisfactory with large sized vessels, but quite difficult when handling vessels of less than 3 mm in diameter⁸⁸. When Nylen⁸⁹, in 1921, introduced the operating microscope, small-diameter vessels became possible to anastomose with a high degree of patency. Some 40 years later, plastic surgeons turned their attention to the possibility of transferring tissue-blocks to treat tissue defects with the use of microsurgically anastomosed tissue transfers.

As of today, there are a number of free flaps available for treatment of virtually any large or small tissue defect known on the human body. The technique puts heavy demands on many factors; long intraoperative time, technically challenging, long learning curve, the need of several different technical apparatus (microscope, monitors, etc.), fairly high donor site morbidity, and more. But the technique also displays many advantages such as the possibility to reconstruct a female breast, out of autologous tissue, that is natural to the eye and touch.

Implants

Already 3000 B.C. Incas of Peru used gold and silver to treat trephination defects, and in 1565 Petronius described the use of alloplastic material such as gold to treat a cranial defect⁸⁶. In the 1940s when advances in biomaterial science brought forward many new materials suitable for implantation, the use of synthetic implants became widespread. Augmentation and reconstruction of tissues became possible by the use of implant material.

The use of implant material offers several advantages such as avoidance of operative time for graft harvest, absence of donor site morbidity, and an unlimited supply. On the other hand, the disadvantages of material wear, foreign body reaction, capsule formation, and risk of infection follows.

Implant material can be designed to stimulate tissue ingrowth with incorporation of the implant material, e.g. polyethylene or polypropylene materials (Medpor[®], Marlex), or collagen matrices^{47,90}. Or it can be designed to be as inert as possible and exert its function by just withholding its physical shape as in silicone breast implants.

The use of implants surely holds its position in reconstructive surgery, but very few of the available materials, if any, can compete with normal tissue.

Fillers

Man has been quite inventive in finding ways to correct soft tissue defects. Several so called 'fillers' have been developed over the years. Usually they are injectable materials of synthetic polymers, allo-/xenogenic tissues, or biodegradable polymers. Many of them have been shown to elicit immunological reactions, granuloma formation, and migration of the material away from the initial site of deposit. Others, such as the degradable fillers, have only a limited (in time) effect as the injected material is degraded (quickly) over time. In developing new fillers, one often combines the knowledge from TE/cell culturing with the experience of the older fillers, and by doing so minimizing or overcoming such issues as transmission of diseases and immunogenicity.

An example of a permanent filler is Polyacrylamide (PAAG) which is a homogenous, stable, and non-biodegradable material used as a filler substance. Some studies have proven this filler to be effective and easily used without any negative effects^{91,92}, whereas others indeed find negative aspects of the material⁹³. Among Lempere's findings were; that the implants showed no reaction and were palpable after nine months, but had decreased in size. Upon histology the PAAG was difficult to detect already after one month and the gel gave rise to a fine fibrocellular capsule. After 6-9 months, the material had dispersed into the skin and was found to be surrounded by macrophages and fibroblasts. The gel appeared to be slowly absorbed⁹³. Furthermore, concentrations of acrylamide monomers that could be toxic is reported to be <10 ppm. Found side effects are enlarged lymph nodes, migration of the material, and edema formation. The U.S. Environmental Protection Agency classifies acrylamide as a medium-hazard probable human carcinogen (SIC!)⁹³.

It is commonly believed that superficial contour defects such as rhytids and dermal depressions are best treated with 'like material', such as dermal collagen, thereby restoring structural integrity, volume, and texture. Collagen is abundantly found in all human tissues. Bovine collagen has for a long time been the collagen type most readily available, other species used are e.g. porcine⁹⁴. To render the bovine collagen non-immunogenic, techniques like cleaving the telopeptides from the central portion of the molecule have been developed. However, treated bovine collagen still elicits immunologic reactions, including tachyphylaxia in more than 3% of a population^{94,95}.

In the 1980s scientists investigated how to extract intact human collagen fibers from skin. In this way autologous or allogenic collagen could be used to treat dermal defects without the risk of immunologic reactions as for xenogenic collagen⁹⁵. Autologous and allogenic human collagen for filling purposes is now commercially available; a skin biopsy is removed or surgical waste (skin) from aesthetic or other surgical procedures is sent to a laboratory where the collagen is processed and sent back for treatment, or banked for later use^{96,97}. Typically 0,6 ml collagen (Autologen[®]) per gram skin can be obtained, equaling the need for approximately 1,28 square inches of abdominal skin⁹⁸. The clinical experience is that the effect of human collagen fillers lasts longer than reconstituted bovine and degraded porcine collagen fillers (i.e. beyond 12 months).

A further development along the TE approach of the fillers is the advent of treating dermal depressions with autologous cells (fibroblasts) (*vide infra*).

Fat grafting

Autologous fat grafting was first described in the late 1800 when Van der Meulen grafted a free omentum and fat autograft between the liver and diaphragm. Fat grafting for aesthetical reasons were first described by Neuber in 1893^{25,99,100}. Neuber quickly understood the limiting factor of the size of the transplant. He only used very small pieces and later reported that '*grafts larger than an almond would not give good results*'. For nutritional reasons individual cells can not be placed further away than a few hundred micrometers of nearest capillary.

Over the years one can see an evolution of free fat grafting, where the pioneers started with fairly large pieces of tissue that were transplanted, to later publications where smaller and smaller 'parcels' of fat are transferred¹⁰¹⁻¹⁰³, following the theoretical biologic benefit of graft participation enhancing the revascularization, cellular nutrition, and total graft survival¹².

Fat grafting has also been employed when reconstructing the female breast where Czerny²⁵ was the first to describe a successful case in 1895. Many have followed, and a great number of papers report good results even though they most often find that the graft shrinks and becomes hard to the touch. It is quite obvious that the authors really describe scarification of fat tissue that has become necrotic.

Adipose tissue is often referred to as '*the surgeon's best friend*' as it shows extremely versatile usage. To just mention a few; orthopedists have used fat grafts to fill bony defects and to treat joint ankylosis. Neurosurgeons have used fat to treat skull-, dura-, and brain defects. Nerves can be surrounded by fat after neurolysis or chest wall and pleural defects can be filled¹⁰⁴. Fat has also been used to treat enucleation of the eye, defects after mastoid surgery, and vocal fold (recurrent laryngeal nerve) paralysis¹⁰⁵. This illustrates that fat autografting provides a useful material for soft, as well as hard, tissue defect corrections.

The main problem with fat transplantation is the variable 'take rate'; a gradual but imminent resorption of 30-70% of the transplanted volume is often seen^{94,106,107}.

In their inclusive review article of free fat grafting, Billings and May²⁵ describe the microscopic fate of free fat autografts; an extensive host cellular infiltration of the graft involving PMN, lymphocytes, and more, is seen in the first four days after transplantation. After four days some anastomoses have occurred between smaller graft vessels and host blood supply. The cellular infiltration shows a high number of eosinophils and foreign-body-type giant cells. The time hereafter typically shows a further adipose cell breakdown and an increasing number of host histiocytes. All microscopy studies showed an early adipocyte breakdown with the formation of cyst-like deposits and the presence of a large host histiocyte infiltration.

With the introduction of liposuction surgery in the late 1970's, free fat autotransplantation has gained increased popularity as, with liposuction, donor material became easily and rapidly available¹². Illouz¹⁰⁸ was probably the first to attempt autotransplantation of the liposuction aspirate¹⁰⁹. Many plastic surgeons thereafter believed the liposuction material to be optimal for grafting due to the liposuction's particulation of the fat (*vide supra*), the reality however, is that the harvesting procedure incur cellular disruption and fragmentation, releasing free fatty acids that are cytotoxic^{110,111}. Liposuction is commonly preceded by instillation of local anesthetics and adrenalin that in themselves are cytotoxic. The particulation of the lipoaspirate is also rather low, and the small fragments of adipose tissue that are transplanted are in fact bundles of thousands of adipocytes. This severely violates the theoretical axiom of that any given cell needs to be within a few hundred micrometers from a capillary. Furthermore, lipoaspirate fat grafting may be a potential dangerous procedure; severe complications such as blindness, aphasia, sensorimotor hemiparesis, and life-threatening sepsis have been described^{112,113}.

Some authors have described the potential benefit of bioactive peptides (e.g. b-FGF) or pharmaceutical agents (e.g. insulin, steroids, vitamin E) to improve fat graft survival by encouraging recruitment and replication of preadipocytes within the graft after transplantation^{12,101,114}. Others have focused on preserving the integrity and purity of the adipocytes or the transplantation techniques, by certain cleansing and transferring modalities, to increase graft survival^{102,103,115-118}. Since the adipose tissue is a rather poorly perfused recipient bed it has been suggested that lipoinjection should be performed in tissues with richer vasculature, such as muscle, to increase the take rate of the transplant¹¹⁹⁻¹²¹. Obviously, no consensus exists on how to harvest and handle fat grafts.

However, the future evolution of fat transplantation seems to be by the use of the more resilient preadipocyte (with, or without, a transplantation vehicle) which is allowed to proliferate and differentiate *in situ*^{36,122-124}.

Cell transplantation

Nutrition *in vivo*

In vivo, any given cell is located closer than a few hundred micrometers from a capillary. This is essential due to nutritional prerequisites. This fact also has an impact on the transplantation of cells as any transplanted cell is dependent on diffusion of nutrients until neovascularization has occurred. Hence the distance to the nearest blood vessel must not be too great.

As of today, the tissues that have successfully been tissue engineered in a more routinely fashion are tissues that have fairly low nutritional requirements and an uncomplicated vascular system; epidermis, cartilage, and bone.

The revascularization of the recipient area can be improved by the simultaneous implantation, or supplementation, of angiogenic factors. It has been demonstrated that the slow release of angiogenic factors from the construct harboring the cells increases the capillarization in the transplanted area, and hence the survival and growth of the transplanted cells increases¹²³.

Adipose tissue

The idea of using autologous preadipocytes cultured *in vitro* for transplantation and regeneration of soft tissue has been postulated by several authors^{25,29,106,123}. The preadipocyte is assumed to be more resilient and resistant to trauma than mature adipocytes. One approach could be that from a small inconspicuous adipose biopsy the preadipocytes could be obtained and cultured (expanded in number) *in vitro*, transplanted back to the patient, and *in vivo* slowly differentiate into lipid-laden adipocytes. As the cells are injected in a single-cell suspension and slowly differentiate, it is further assumed that the recipient area capillarizes adequately^{29,106}. For any engineered cellular construct, it will be imperative to be delivered in a manner that optimizes cell survival and that initiates and supports cell engraftment. This will likely involve the use of biodegradable matrices, or scaffolds^{106,125}. Initially the scaffold functions as a vehicle during transplantation, and upholds the volume of the transplanted construct. As the transplanted cells proliferate, differentiate, and start producing autologous ECM the scaffold is degraded, resulting in a regeneration of the adipose tissue.

Halbleib¹²⁶ and co-workers presented a study comparing different seeding techniques and culture conditions for the TE of adipose tissue. They demonstrated that human preadipocytes successfully and reproducibly can be inoculated and cultured on hyaluronic acid-based scaffolds and they also found clear evidence that these preadipocytes are able to undergo full maturation into adipocytes *in vitro*. The authors also stated that there is a problem with the limited source of preadipocytes as the yield of precursor cells from 1 g adipose tissue ranges in $2-3 \times 10^5$ cells of whom not all will attach to the matrix¹²⁶. Hence, some expansion in cell number *in vitro* is necessary before seeding of scaffolds and transplantation.

Fibroblasts

Injectable collagen has been a popular method for correcting soft tissue defects all from its introduction in the late 1970s. Even though different ways to decrease the immunogenicity have been described, it is still a problem. Soft tissue augmentation with autologous cells should be able to evade problems like allergic reactions and rejection. In the case of fibroblasts, it is possible to obtain cells from an inconspicuous skin sample and to expand the number of cells *in vitro*. Living fibroblasts can then be transplanted (injected) back to the patient in a single-cell suspension and they take part in a long-term protein repair process to sustain the correction¹²⁷. This method has shown good results, especially when treating acne scars^{97,128}. The technique is now commercially available, and studies show good long-lasting effects without any major complications or rejections, even though injections have to be repeated to gain full correction of the defect^{96,127}.

Skin

With the development of techniques to culture autologous keratinocytes, a number of novel approaches to transplant cultured cells emerged.

Among the first clinical uses of autologous cultured keratinocyte sheet-grafts was the work described by Gallico¹²⁹ and co-workers in 1984. Thereafter the use of cultured epithelial autografts has been available, and used, in burn centers around the world. A common procedure is to pre-treat the wound with cadaver skin (fresh, cryo-preserved, or glycerol-preserved) to stimulate vascular ingrowth from the underlying wound bed and to provide a better bed on to which to transplant the cultured autologous keratinocytes^{130,131}.

The invention of glycerol- or cryo-preserved human donor skin for use as a biological dressing for burn treatment has further been developed into decellularized dermis. Removal of all cells and cell-fragments from the dermis renders the resulting collagen lattice immunological inert. The cell-free dermis can then be applied to a wound surface and act as a biodegradable matrix leading to GTR of the dermis^{41,49}. To further promote healing, and as an alternative way to cover major burn wounds, the decellularized dermis can be applied with cultured autologous keratinocytes seeded on the top, as a bi-laminar permanent skin-substitute^{87,132}.

The drawbacks presented earlier in this text have led to further development and research to heighten the quality of sheet-grafts by combining the cultured epithelial cells with a biodegradable matrix (e.g. cell-free dermis or Integra[®]), either by transplanting keratinocytes on/in the matrix^{133,134} or by pre-treating the wound surface with the matrix before transplantation of keratinocytes⁴⁹.

Today the single-cell suspension transplantation of keratinocytes, spray-painted on the patient in a tissue glue, and overgrafted with meshed allogenic donor skin, is a common approach^{52,135}.

However, also the single-cell suspension transplantation of keratinocytes has its drawbacks. It still takes a long time (2-3 weeks) to reach an adequate number of cells when cultured 2-dimensionally on feeder cells, or in a serum-free environment. To circumvent this time-lag, the use of bioreactors have proven successful. Clinical studies on culturing keratinocytes on biodegradable macroporous gelatin spheres (*vide infra*) for transplantation on chronic leg ulcers have been performed¹³⁶ and show that transplantation can be performed 9-15 days after cell harvest. The MGS provide a good culture substratum for anchorage-dependent keratinocytes, and the cell number rapidly increases when cultured suspended in a bioreactor. The keratinocytes can be directly transferred to the MGS, expanded *in vitro*, and further expanded upon transplantation onto the wound surface. This technique further minimizes the stress on the cells since no enzymatic detachment of the cells (which might alter the anchoring proteins of the cells) from the carrier/culture surface is necessary as it degrades rapidly on the wound surface¹³⁷.

More sophisticated TEMP's, or skin equivalents, containing live autologous and/or allogenic fibroblasts and/or keratinocytes, have been developed and subjected to *in vitro* and *in vivo* studies with variable success^{49,138,139}.

Scaffold materials

Carrier materials (scaffolds, matrices) can be used for GTR or to transplant cells back to the patient, either in an open or a closed system (*vide supra*). Several different techniques to seed cells on matrices are described in the literature e.g.; one can basically just drip the cell suspension on the matrix, spin cells and scaffold in spinner flasks, centrifuge or inject the cells into the matrix, mould the matrix and by doing so include cells in the matrix^{36,140-142}.

Scaffold materials can be synthetic or biologic, degradable or non-degradable, depending on the intended use. Scaffold materials should promote attachment, migration, and proliferation of cells, be easy to use, and retain its physical properties for the wanted time-period. It is obvious that both the chemistry and the topography of the material surface may directly influence the cells' behavior through adsorbing the ECM molecules and altering their conformation that, in turn, regulate cell-substrate interactions. Surface characteristics such as hydrophilicity, surface charge density, surface micromorphology, free energy, and specific chemical groups affect the cell adhesion, spreading, and signaling. Hence surface characteristics regulate a wide variety of biological functions including cell growth, migration, differentiation, synthesis of ECM, and tissue morphogenesis. Cells prefer to attach on a hydrophilic surface over a hydrophobic surface.

Synthetic polymer scaffolds are favored by some as their properties (e.g. degradation time, porosity, and mechanical attributes) can be tailored for specific applications. Synthetic scaffolds can also be manufactured with a high degree of reproducibility and a long off-the-shelf storage possibility. Biologic materials are favored by others as they are expected to mimic *in vivo* milieu better and as they are biologic. Thus synthetic and biologic materials all have their pros and cons.

Common synthetic scaffold material components are poly(lactic-co-glycolide) (PLG), which are hydrolytically degradable polymers with high mechanical strength. Alas, they are hydrophobic and generally processed under harsh conditions making factor incorporation and entrapment of cells difficult. Hydrogels (highly hydrated polymer materials of synthetic or biologic origin) can be viewed as an alternative since they are processed under mild conditions and have structural and mechanical properties similar to different tissues and ECMs¹⁴³, and can be delivered in a non-invasive manner. Examples of synthetic hydrogels are poly(vinyl alcohol), poly(ethylene oxide), and polypeptides. Examples of biologic hydrogels are chitosan, collagen, gelatin, and hyaluronic acid.

The degradable scaffold materials are expected to function as reinforcement bars and scaffolds for tissue regeneration, and as these regenerative processes move along, the matrix is degraded and replaced by autologous tissue¹⁴⁴. For hydrogels there are three basic degradation pathways: hydrolysis, enzymatic cleavage, and dissolution. Most synthetic hydrogels degrade through hydrolysis. As this hydrolysis takes place at a constant speed *in vivo* and *in vitro* the degradation speed can be controlled by the composition of the material but not the surrounding¹⁴³. The speed of enzymatic degradation of scaffold materials depend on both the number of cleavage sites in the material, and the level of enzymes in the surroundings. Ionically crosslinked materials dissolve at a speed dependent on the ionic surrounding.

A plethora of scaffold materials have been described and also used *in vitro* and in clinical applications^{40,46,122,142,143,145-147}. An appealing method to use carrier materials, which has been pursued in this thesis, is the use of a macroporous gelatin sphere that functions as cell culture substrate, transplantation vehicle, scaffold, and an ECM analogue for GTR. Macroporous gelatin spheres can thus be viewed upon as an injectable solid, but porous, scaffold material.

Several other types of microcarriers (solid, porous, degradable, nondegradable) have also been described in the literature¹³⁷. However, solid microcarriers have the disadvantages of a low surface-area-to-volume ratio, which minimizes the possible number of cells attached to each carrier¹⁴⁰. The cells are also vulnerable to damage caused by carrier collision or hydrodynamic forces in the culture. Porous carriers have a larger surface area/carrier and the internal pores may function as safe havens for cells, protecting them from damage due to carrier collision.

Macroporous gelatin spheres

Porous carriers can be described as preformed particles with a porous internal structure into which cells can grow and be maintained in suspension while culturing. The advantages of a porous carrier are that they can support a higher cell number than solid carriers, and that the structure protects the cells against (mechanical) damage such as shearing forces and air-liquid interfaces in agitated or suspended cultures¹⁴⁸.

As the cells are protected against shear, an increased agitation can be used, which in turn facilitates an increased cell density since the mass transfer coefficient of oxygen can be elevated. Anchorage dependent cells that do not form multilayers in culture flasks may behave quite differently in the microcarrier and completely fill the interior of the carriers¹⁴⁸.

The CultiSpher macroporous gelatin microcarrier is a non-ionic microcarrier, based on a highly crosslinked (with glutaraldehyde) type A porcine derived gelatin matrix¹⁴⁹. They are manufactured from an 8% gelatin solution by a double emulsion technique. The average internal pore size is 10-20 μm when rehydrated in PBS. A wide range of sizes are available from 40-400 μm \O . Other physical characteristics of the carriers are; density 1,04 g/cm^3 , loading capacity 0,34 ml cells/ml carrier, and carrier volume 14 ml/g.

A plethora of different human and animal, primary and secondary, cell (lines) have proven successfully cultured on macroporous gelatin spheres^{73,79,136,140,150}.

Furthermore, since gelatin is the degradation product of collagen, it is hypothesized that gelatin retains informational signals such as the Arg-Gly-Asp sequence (RGD – a specific cell attachment domain binding to integrin) which facilitates cell attachment, thus being an informational matrix⁷⁰.

The porous spheres provide a large 3-D habitat for the anchorage and propagation of cells. Furthermore, 3-D growth of the cells is possible in the porous network of the carriers¹⁴⁸. As cells readily grow on, and in, the spheres, and a large number of spheres can be applied or injected, a fairly uniform distribution of cells within the matrix can be attained. This is otherwise a difficult obstacle to overcome in other types of scaffolds. A non-uniform cell distribution in the matrix results in an uneven regeneration of the tissue with non-functioning areas.

The MGS' properties of being a biodegradable 3-D matrix, which when rehydrated, can be injected, allows for the use of the carrier also as a matrix for GTR. We have in this thesis explored different approaches for the use of MGS in TE; to culture human primary cells upon and to inject pre-seeded MGS and naked MGS in mice and human to regenerate soft tissues.

Aims of the present study

To regenerate soft tissues *in vitro*

To evaluate whether earlier postulated theories of transplanting single-cell suspensions to treat soft tissue defects could have a bearing as evaluated by *in vitro* and *in vivo* studies

To evaluate possible biodegradable scaffolds for tissue engineering of soft tissues

To appraise *in vitro* and *in vivo* behavior of cells and matrices, and the regeneration of soft tissues

Think, when was the last time
You, stumbled on rest time
Go, try to relax now
Or, did you forget how

You've been very busy
Now, you feel awfully dizzy
Why, is it so vital
You, insure your survival
Slow motion

Stop, remember the way back
Now, now you can lay back
They'll get by without you
For, they're not about to

Slow motion
Let everybody else run around
Slow motion
Maybe you have forgotten how

Material

Cell types

Mammary epithelial cells

Papers I and IV

Biopsies of human breast tissue were obtained from healthy patients undergoing routine reduction mammoplasty. Fresh biopsies were transported to the laboratory in sterile saline-soaked gauze and processed within 24h. Tissues were washed repeatedly in sterile PBS. Visible blood vessels, connective tissue, and adipose tissue were removed sharply, with scissors, leaving clean mammary epithelial areas. Epithelial tissue was then minced mechanically, with scissors, into fragments of about 1 mm³ in size and incubated in a tissue digestion mixture of DMEM and crude collagenase (1 mg/ml, type I, 312 U/mg) in a spinner flask for 60 minutes at 37° C. Obtained cell suspension was centrifuged for 5 minutes at 400g and the pellet resuspended. The procedure was repeated twice. Cell suspension was then serially filtered through 100- and 70 µm cell-strainers to remove connective tissue fragments and mammary epithelial organoids, and to obtain a single-cell suspension. Human mammary epithelial cells were then cultured in 75 cm² culture flasks.

Upon subconfluence, cells were passed by discarding culture medium, washed twice with 2 ml EDTA (0,02%) and detached enzymatically by incubation with 4 ml EDTA (0,02%)/trypsin (0,1%) solution (1/1, vol/vol) at 37° C for approximately 15 minutes.

Preadipocytes

Papers II, IV, and V

Adipose tissue biopsies were obtained from routine abdominoplasty or reduction mammoplasty operations on healthy patients. Fresh biopsies were transported to the laboratory in sterile saline-soaked gauze and processed within 24h. Biopsies were washed repeatedly in PBS. Visible blood vessels and mammary epithelial tissue were removed by sharp dissection. Tissue was mechanically minced into fragments of about 1 mm³ in size and incubated in a spinner flask for 30 minutes at 37° C in a tissue digestion mixture of DMEM and crude collagenase (1,5 mg/ml, type I, 312 U/mg). The cellsuspension was then centrifuged for 5 minutes at 200g and the pellet was resuspended in DMEM and again centrifuged for 5 minutes at 200g. Cell pellet was resuspended in culture medium before inoculation. Where applicable, cells were passed as stated above.

Dermal fibroblasts

Papers III, IV, and V

Cells were acquired from normal human skin obtained from routine plastic surgery operations. Subcutaneous fat was removed, as was as much of the epidermal component as possible. Remaining dermis was minced with scissors to fragments <1 mm³ in size and enzymatically digested in a tissue digestion mixture of DMEM, crude collagenase (0,5 mg/ml type I, 312 U/mg), and Dispase[®] (2,5 mg/ml, 1,04 U/mg) supplemented with 2% NCS o.n. in an incubator at 37° C, 5,0% CO₂, and 95% humidity. Tissue suspension was then triturated repeatedly with a pipette to dissociate tissue fragments. Suspension was centrifuged at 400g for 10 minutes, supernatant removed, and cell pellet resuspended in culture medium. Cells were passed as stated above.

Keratinocytes

Paper IV

Skin biopsies from healthy patients undergoing routine reduction mammoplasty was processed within 24h. Tissue was washed repeatedly in PBS, subcutaneous fat and as much of the dermal component as possible were removed with scissors. The tissue was cut into fragments of about 1 mm³, placed in a spinner flask with EDTA (0,02%)/trypsin (0,1%) (1/1, vol/vol), and incubated at 37° C for 30 minutes. The supernatant was collected and fresh EDTA/trypsin was added and tissue again incubated in the same spinner flask for another 30 minutes. The supernatant was collected and the process repeated yet a third time before discarding remaining tissues. The pooled supernatants were centrifuged for 5 minutes at 400g. The resulting supernatant was discarded and the cell pellet resuspended in culture medium. Cells were passed as stated above.

Chondrocytes

Paper IV

Cartilage biopsies were obtained from routine nasal septoplasties. Fresh biopsies were transported to the laboratory in DMEM and processed within 24h. Cartilage was rinsed repeatedly in PBS, cut into fragments of about 1 mm³, and incubated at 37° C o.n. in a tissue digestion mixture of DMEM, crude collagenase (1,5 mg/ml type I, 312 U/mg) and Dispase® (2,5 mg/ml, 1,04 U/mg) supplemented with 2% NCS in an incubator at 37° C, 5,0% CO₂, and 95% humidity. The cell suspension obtained was centrifuged for 5 minutes at 400g, and cell pellet was resuspended in culture medium. Cells were passed as stated above.

Culture conditions

All cell cultures were incubated at 37° C, 5,0% CO₂, and 95% humidity at atmospheric pressure. Culture media were changed 3 times/week. During numerical expansion of cells, morphological inspections were performed when medium was changed. Only healthy patients' tissues were used, i.e. no tissue came from patients with known malignancies, infectious diseases, metabolic disorders, or treated with immunomodulating drugs.

Growth medium

- > DMEM (with sodium pyruvate, glucose 1000 mg/l and pyridoxine) supplemented with 10% FCS and antibiotics/-mycotics (penicillin 50 U/ml and streptomycin 50 µg/ml) - **Paper I** (expansion of cell number and initially in collagen gel cultures) and **Paper IV** (chondrocytes).
- > DMEM/Ham's F12 (1/1, vol/vol) supplemented with insulin (3,6 µg/ml), transferrin (3,6 µg/ml), triiodothyronine (1,4 x10⁻⁹ M), hydrocortisone (0,3 µg/ml), cholera toxin (0,7 x10⁻¹⁰ M), EGF (6,25 ng/ml), 7% FCS, 1,5% adenine, and antibiotics/-mycotics (penicillin 50 U/ml and streptomycin 50 µg/ml) - **Paper I** (cultures in collagen gels when cells reached subconfluence) and **Paper IV** (HMEC).
- > DMEM/Ham's F12 (1/1, vol/vol) supplemented with 10% FCS, and antibiotics/-mycotics as above - **Papers II, IV, and V** (preadipocytes).
- > DMEM supplemented with 10% NCS, and antibiotics/-mycotics as above - **Papers III, IV, and V** (fibroblasts).
- > Serum-free keratinocyte medium with bovine pituitary extract (25 µg/ml) and recombinant EGF (0,2 ng/ml) - **Paper IV** (keratinocytes).

Scaffold materials

Three-dimensional collagen gel cultures (Paper I)

Three-dimensional collagen gels were prepared by mixing 7 ml Collagen R (2 mg/ml in 0,1% acetic acid) with 1 ml 10x modified Eagle's medium (MEM), 1 ml HEPES buffer (0,02 M) and 1 ml of cell suspension (approximately 10^6 cells). For gelation to occur, pH was adjusted by adding small increments of NaOH (1 M).

Portions of 1 ml cell-containing gels were incubated in 12-well culture plates. The gels (building approximately 3 mm in height) were covered with 1 ml culture medium.

Poly(urethane urea) (Paper III)

The poly(urethane urea) was synthesized in solution using a two-step polymerization method. Scaffolds of the various PUURs were prepared either by packing of fibers or by a solvent casting/particle leaching process. Fibers were formed by extrusion of a 30% w/w polymer solution through a spinneret (120 holes, diameter 80 μm) submerged in a coagulating bath containing hot water (80° C). The fibers were packed in a cylindrical mould (diameter 12 mm) and a solution of 92% DMF and 8% water was allowed to pass through the packed fibers to cause binding between the fibers. The fibrous scaffold was then washed with water, dried, and sliced into 2 mm thick discs.

For the solvent casting/particle leaching process, the polymer solution was diluted with DMF to a concentration of 9% w/w or 12% w/w. The polymer solutions were mixed with glucose or GM. The 12% w/w solution was mixed either with glucose in a ratio of 33:40 (porous scaffold), or with sieved GM with a particle size ranging from 150 to 250 μm (scaffold A). The 9% w/w solution was mixed with unsieved GM particles (~10-500 μm , ~5% <53 μm , ~10% >250 μm) (scaffold B). The resulting mixture formed a gel, which was soaked in a water bath at 40° C to remove glucose and DMF. The washing was continued until the weight of the dried scaffold was constant. The scaffolds were then sliced to 2 mm thick discs. All scaffolds were sterilized by electron beam radiation (28 kGy).

Macroporous gelatin spheres (Papers IV, V, and VI)

The CultiSpher-S macroporous gelatin microcarrier

The CultiSpher-S macroporous gelatin spheres used had a diameter of 133-321 μm – in **Paper IV**, and 70-170 μm – in **Papers V and VI**. The sphere is based on a highly crosslinked type-A porcine derived gelatin matrix. The average internal pore size is 10-20 μm when rehydrated in PBS. All microcarriers used were prepared according to the manufacturer's instructions; dry microcarriers were rehydrated in Ca^{2+} - and Mg^{2+} -free PBS, 50 ml/g dry carriers, for a minimum of 1h in RT. Without removing the PBS, microcarriers were sterilized by autoclaving (121° C, 20 minutes, 80° C cooling temperature, 2,8 bar support pressure) and later stored at 4° C until use. Sterilized microcarriers were finally prepared upon use by washing twice with fresh PBS (microcarriers were left to sediment after resuspension to allow removal of the supernatant) and once with respective culture medium before inoculation with cell cultures.

Loading of cells on MGS – Papers IV and V

One hundred milligram dry MGS were rehydrated and washed as above. Cells were prepared and enzymatically detached as above and washed twice in culture medium by centrifugation/resuspension. Cells were transferred to a spinner flask along with the microcarriers in 50 ml culture medium. Suspension was continuously stirred at 35 rpm (just enough to prevent cells/carriers to sediment). After 24h another 50 ml culture medium was added. Medium was changed 3 times/week by allowing cells/carriers to sediment, removing 50 ml culture medium, and adding 50 ml fresh culture medium.

Loading of MGS in syringes – Papers V and VI

To load syringes with MGS the gelatin spheres were allowed to sediment and culture medium was removed. In 1 ml syringes 0,8 ml of carrier/medium suspension was aspirated, syringes turned upside-down, and left to sediment for approximately 3h before ejecting the 'supernatant' leaving 0,1 ml (**Paper V**) or 0,5 ml (**Paper VI**) densely packed microcarriers ready for injection.

Clinical studies (Papers III and VI)

Healthy volunteers were included according to;

- > Inclusion criteria - healthy volunteers, men aged between 18 and 50 years, signed informed consent.
- > Exclusion criteria - underlying diseases such as cancer, HIV, or other diseases judged by the investigators to interfere with the study, previous inclusion in another study within 3 months, scar tissue at the site of surgery.

Paper III

Four healthy male volunteers aged 30 to 42 years (mean 34), all white, were studied. A physical examination was performed and routine blood samples taken. The skin at the site of the study was chosen to be uninjured, scar free, and not exposed to friction (the upper part of right or left buttock was used in all subjects).

Operations were done under local anesthesia (Lidocaine hydrochloride 10 mg/ml). The skin was washed with chlorhexidine solution (1 mg/ml) and a 4 mm skin biopsy punch was used to create 6 semicircular intradermal pockets. The pockets were enlarged by sharp dissection with scissors. Two discs (4 mm in diameter and 2 mm thick) of each scaffold A and B were inserted (to be extracted after 2 and 8 weeks, respectively) and the pockets were closed with Steri-strips™ tape and covered with permeable non-woven tape (Micropore™). The remaining two pockets, control sites, were closed in the same manner as the test sites, with the exception that no implants were inserted. The sites were photographed immediately and every seventh day.

Changes such as swelling, redness, blistering, eczema, purulent secretion, and heat, as well as adverse events such as itching, pain, or other subjective symptoms were noted using frequency tables with “yes/no” for each finding. The study site was then again covered with Micropore™ tape. After 2 and 8 weeks, one of each implant and one control including surrounding tissues were removed (using local anesthesia as above) with a 6 mm skin biopsy punch. The wounds were either closed with a stitch or Steri-Strips™. When the last biopsy specimens had been removed the subjects were examined and blood samples drawn.

Biopsies were instantly fixed in PFA and processed for routine histology and IHC.

Paper VI

One week before planned injections the subjects were seen in the clinic for informed consent and information. Injection areas were sterilized with 70% EtOH and a 1 mm dot was tattooed on the planned injection points.

All subjects were injected intradermally according to an individual randomized injection scheme. In total, 8 wheals on the ventro-medial aspect of the upper arm were produced using a syringe and a 27 gauge needle. The following injections were performed; 1) Physiologic saline (C), 2) Restylane[®] (R), 3) Macroporous gelatin spheres (A and B). A volume of 0,5 ml/modality was injected.

Every seventh day, according to the plan of assessment, the study sites were photographed. Changes such as swelling, redness, blistering, eczema, purulent secretion, and heat were noted using frequency tables with “yes/no” for each finding. Adverse events such as itching, pain or other subjective symptoms were noted, again using frequency tables with “yes/no” for each finding.

After 2 and 8 weeks, one of each implant (A1, B1, C1, R1 and A2, B2, C2, R2 respectively) including surrounding tissue was removed. Each wheal was anesthetized using local anesthesia. A 6 mm skin biopsy punch was used to remove the samples as full-thickness skin biopsies with some subcutaneous fat. The wounds were closed with stitches and wound tape.

Animal study

A total of 30 female, 6-7 weeks old, nude mice (nu/nu BALB/cA Bom), with a body weight of approximately 20 g were used in the experiment. Mice were housed in pairs on a 12-h/12-h light/dark cycle at 25° C in the specific pathogen-free animal unit. The mice had free access to autoclaved water and irradiated low-fat standard rodent diet (R36). All animals were allowed to acclimate before the experiment commenced. The mice were individually anesthetized using ether (ether puriss, 99,5%), and the backs of the mice were swabbed with Jodopax vet. (5% iodine, 15% acetic acid, diluted 20 ml Jodopax vet./liter H₂O) before the 6 intradermal injections were made; three wheals on each side of the spine on the dorsal aspect of the mouse were produced. The mice were marked individually by ear-clipping before terminating anesthesia. Each mouse was carefully observed for approximately 10 minutes post-injection before being returned to the home cage.

All animals were injected intradermally using a syringe and a 27 gauge needle (outer diameter of 0,4 mm, BD Microlance[™] 3) according to the following schedule; 1) Physiologic saline, 2) Cultured human fibroblasts in a single-cell suspension, 3) Cultured human preadipocytes in a single-cell suspension, 4) MGS, 5) MGS pre-seeded with cultured human fibroblasts, and 6) MGS pre-seeded with cultured human preadipocytes.

A randomization scheme was used for the injection sites on each mouse. A volume of 0,1 ml/modality was injected. The animals were grouped ten individuals in three groups to be sacrificed 7, 21, and 56 days post-injection. The animals were sacrificed in groups by CO₂-inhalation.

The skin, including subcutaneous tissue, was surgically removed from the back. The nose- and tail-ends of the skin were marked before tissue was fixed o.n. in PFA.

Ethical approvals

***In vitro* studies** have been approved of by the local Ethics Committee and conformed to the Helsinki declaration of 1975, as revised in 1983.

Paper III - the study was approved of by the local Ethics Committee (Dnr 01-114) and the Swedish Medical Products Agency (Dnr 34:2001/3692) and conformed to the Helsinki Declaration of 1975, as revised in 1983.

Paper V - the experiment was approved of by the local ethical committee of animal experiments (Dnr 27-03, Linköpings Djurförsöksetiska Nämnd, Linköping, Sweden).

Paper VI - the study was approved of by the local Ethics Committee (Dnr 03-172) and the Swedish Medical Products Agency (Dnr 461:2003/69076) and conformed to the Helsinki Declaration of 1975, as revised in 1983.

Methods

Cell culture

All cultured cells used were acquired by setting up primary cultures by enzymatic degradation of healthy normal human tissues obtained from routine plastic surgery procedures (reduction mammoplasty, abdominoplasty, and nasal septumplasty).

To ascertain a high degree of original phenotypic stability, cells of the 1st-6th generation were used in all experiments.

Morphology

Cells were visualized and studied in culture flasks regularly, at least 3 times/week, using a phase-contrast microscope. If microorganism contamination of cultures were noticed (foul smell, turbid medium, slow growth, 'unnatural' behavior of cells or medium) cultures were discontinued.

Visualization/imaging

A CETI Topical light microscope (**Papers I-III**), an Olympus BX41 light microscope (**Papers IV-VI**), or an Olympus IX51 inverted phase-contrast microscope (**Papers IV and V**) was used.

Immunohistochemical- and FISH-sections (**Papers IV and V**) were examined using the Olympus BX41 epifluorescence equipped microscope (x40/0,75 - FITC and x100/1,3 - FISH) with proper filter settings (filter cube U-MWIB2; 505 nm dichroic mirror; 460-490 nm excitation filter and 510 nm barrier filter).

Images were captured using either a 270,000 pixel 1/3" IT-CCD with DiRactor Pixera Professional digital camera system (**Papers I-III**), or an Olympus DP70 CCD camera (**Papers IV-VI**).

Routine histology

Representative sections from all material were routinely stained with H&E. Culture samples were fixed in 4% buffered PFA o.n., washed in PBS o.n., and dehydrated through an ethanol-xylene series (70% EtOH o.n., 95% EtOH - 2h, 99,5% EtOH - 2h, xylene - 30-60 minutes). Dehydrated samples were soaked in warm liquid paraffin o.n. and subsequently paraffin embedded and blocked. After cooling, 7-10 μ m sections were cut with a microtome and transferred to a warm water bath before being placed on microscopy glass slides.

Immunohistochemistry

Sample preparation

- > **Cryosections (Paper I)** were prepared after snap freezing of the samples on dry ice. The sections were left to air dry for 10 minutes before being stored at -80° C until further processing. Sections were then immersed and fixed in cold PFA for 30 minutes, after which they were washed with PBS at the time of staining.
- > **IHC on cell cultures** in culture flasks were prepared by discarding culture medium and rinsing the cell culture twice with cold PBS.
- > **Paraffin sections** were prepared as above.
- > **Immunohistochemical** staining was performed on cell cultures cultivated in culture flasks or on MGS, and on paraffin sections of cells cultured in collagen gels, Artelon[®]-scaffold material, MGS, human skin, and mouse skin tissue samples.

Primary antibodies

> Human mammary epithelial cells

A monoclonal mouse-anti-human antibody raised against an epitope, which is present in a wide range of cytokeratins including keratins 5, 6, 8, 17, and probably 19 (Dako, Glostrup, Denmark) (**Papers I and IV**).

> Pro-collagen

A monoclonal rat-anti-human antibody raised against pro-collagen I (MAB 1912, Chemical International Incorporated, Temecula, Ca, USA). Fibroblasts laying down new collagen synthesize pro-collagen which is used to indicate actively secreting fibroblasts and distinguish them from merely migrating or dividing fibroblasts (*in vitro* study **Paper III**).

> Endothelium

A monoclonal mouse-anti-human antibody raised against the glycoprotein von Willebrand Factor which is secreted and located in storage granulae of endothelial cells was used (clone 8/86, Dako, Glostrup, Denmark) (clinical study **Paper III** and **Paper VI**).

> Keratinocytes

A pre-diluted monoclonal mouse-anti-human antibody blend (IHC 2025-6) raised against epitopes present on 19 human epithelial keratins (acidic and basic) (clones AE1 and AE3, Chemicon Int., Temecula, Ca, USA) (**Paper IV**).

> Perilipin

A polyclonal guinea pig-anti-human antibody raised against duplicated N-terminus of perilipin. Perilipin build a family of phosphoproteins located at the surface of intracellular storage lipid droplets (Research Diagnostics Inc., NJ, USA) (**Paper IV**).

> Endothelium

A rabbit polyclonal antibody (Ab 6994) raised against the full length native glycoprotein von Willebrand Factor which is secreted and located in storage granulae of endothelial cells was used (AbCam, Cambridge, UK) (**Paper V**).

Protocols

- > **In Paper I** endogenous peroxidase activity was quenched by 30 minutes incubation in 1% hydrogen peroxide. Nonspecific protein binding was blocked with 2% normal goat serum in PBS. Subsequently, samples were incubated with primary antiserum for cytokeratin at a final concentration of 10 µg/ml for 1h at RT. Samples were rinsed in PBS and incubated with biotinylated secondary antibodies (2 µg/ml) for 30 minutes. After washing in PBS, bound antibody was localized with an avidin-peroxidase Vectastain elite ABC kit and the substrate was an avidin-horseradish peroxidase complex; Vector[®] VIP.
- > **In Paper III** the same protocol was followed using incubation with primary antisera for pro-collagen or von Willebrand Factor.
- > **In Paper IV – cytokeratin IHC** - the same protocol as in **Paper I** was used with the following exceptions; endogenous peroxidase activity was not quenched, nonspecific protein binding was blocked with 2% normal horse serum in PBS, followed by prediluted primary antiserum for cytokeratin. Macroporous gelatin spheres were repeatedly rinsed in PBS and incubated with biotinylated secondary antibodies (15 µl/ml) for 30 minutes at RT.
- > **In Paper IV – perilipin IHC** – the same protocol as in **Paper I** was used with the following exceptions; nonspecific protein binding was blocked with 2% normal bovine serum in PBS followed by incubation with primary antiserum for perilipin (dilution 1:200) for 45 minutes at RT. Sections were then rinsed repeatedly in PBS and incubated with FITC-conjugated donkey anti-guinea pig antibodies (dilution 1:400) for 30 minutes at RT.

- > **In Paper V – endothelial cell IHC** - the same protocol as in **Paper I** was used with the following exceptions; antigen retrieval was performed by subjecting the sections to 0,1% trypsin for 15 minutes before quenching endogenous peroxidase activity with 0,3% hydrogen peroxide for 30 minutes, nonspecific protein binding was blocked with 2% normal goat serum in PBS. Subsequently the sections were incubated with primary antiserum for von Willebrand Factor at a final concentration of 12 µg/ml for 30 minutes at RT.
- > **In Paper VI – endothelial cell IHC** – the same protocol as in **Paper I** was used with the following exceptions; endogenous peroxidase activity was not quenched, nonspecific protein binding was blocked with 2% normal horse serum in PBS, followed by incubation with primary antisera for von Willebrand Factor at a final concentration of 9,5 µg/ml for 30 minutes at RT.

Control for the immunohistochemical staining included omission of primary antibody.

Viability assays

Crystal violet, MTT, and trypan blue stainings were used for direct visualization of cells cultured on plastic or MGS, indicating viable state of studied cells.

Oil red O

Stock solution of orO was prepared by dissolving 0,5 g orO (EEC No 215-295-3) in 200 ml isopropyl alcohol by heating the solution for 1h at 56° C. Working solution was prepared by mixing 6 parts of orO stock solution at RT with 4 parts distilled water and filtrated through filter paper (Munktell Filter No. 1002, filtration speed according to Hertzberg 250 ml/min).

Cryosections were fixed and rinsed serially in tap water and 60% isopropyl alcohol before staining with orO for 10 minutes. Sections were then washed briefly in 60% isopropyl alcohol, counterstained in Mayer's haematoxylin, washed, blued, and mounted.

Cell counting

Cells were counted by three different methods;

- > Culture wells were washed repeatedly with PBS and once with EDTA (0,02%) after which cells were detached by incubation in EDTA (0,02%)/trypsin (0,1%) (1/1, vol/vol) before counting using a VDA 140 Coulter Counter.
- > Direct visualization using crystal violet. Working solution was prepared by dissolving 1 mg crystal violet in 10 ml 99,5% EtOH and further dissolving in 20% EtOH (1/5). Crystal violet was added culture wells that were then left to air dry o.n. and mounted with glycerol.
- > By adding 0,4% trypan blue to single cell solution (1:1) after trypsinization, viable cells were then counted using a Bürker chamber.

Fluorescence *in situ* hybridization

To detect transplanted cells of human origin (**Paper V**), FISH analysis was performed by utilizing the commercially available Vysis Inc. Spectrum Green Female Total Human Genomic DNA probe and kit. All incubations were performed in RT unless otherwise stated.

Tissue sections, 4-6 μm thick, on Superfrost[®] Plus microscopy glass were baked o.n. on a hot plate (58° C), deparaffinized in xylene, and rehydrated in ethanol (99,5%). The sections were then incubated in 0,2 M HCl for 20 minutes, rinsed in dH₂O for 3 minutes and in neutral washing buffer for an additional 3 minutes. The sections were incubated with the pre-treatment solution for 30 minutes at 82° C, rinsed in dH₂O for 3 minutes and subsequently in washing buffer for 2 x 3 minutes. Sections were further incubated in protease solution (pH 2,0) for 15 minutes in 37° C, rinsed in washing buffer 2 x 3 minutes and dehydrated using an ethanol series (70%, 95%, and 99,5% EtOH). A mixture of 1,5 μl probe, 7 μl Vysis hybridization buffer, and 1,5 μl dH₂O was applied to the sections and a cover slip mounted using DPX mounting agent. The hybridization process was carried out for 16h in a Vysis HYBrite. Cover slips were thereafter removed and the sections transferred to a washing solution containing 2 x SSc and 0,3% NP40 for 2 minutes in 82° C. The sections were allowed to air dry and subsequently mounted using fluorescent mounting medium containing 0,75 nM DAPI.

Statistics

In **Paper II**, all data is shown as mean (SEM) unless stated otherwise. The number of cells and colonies were normalized to the initial tissue weight i.e. expressed as number/g tissue. Significance was determined by using the two-tailed paired Student's *t*-test. Probabilities of less than 0,05 were considered significant.

Results

Paper I

Human mammary epithelial cells and preadipocytes were harvested by enzymatic degradation of normal female breast tissue. The cells were cultured in flasks separately and in co-cultures. Human mammary epithelial cells showed a typical growth pattern with characteristic cobblestone appearance and were identified using IHC. Preadipocytes grew initially in fibroblast-like shapes but acquired a more rounded shape as they started to accumulate lipid, and with time turned into spherical, monolocular shapes as they differentiated into mature adipocytes. We had difficulties staining the preadipocytes with orO in a significant manner when cultured on plastic.

Human mammary epithelial cells and preadipocytes were subcultured to increase the number of cells and were subsequently proven to withstand freezing and thawing as cells were banked between numerical expansion and 3-D co-culture experiments. This is noteworthy in a clinical situation where tissue biopsies and numerical expansion of cells precedes the possible transplantation at a later time point.

That HMEC thrive in collagen gel had been reported previously and we could confirm and reproduce many of the six steps of HMEC growth patterns; formation of cell protrusions, cell movements towards each other, lining up of cells, rows interconnecting before thickening into duct-like structures, having rounded swellings at the ends.

When co-culturing HMEC and preadipocytes in collagen gels, cells show a pattern of ductal structures of HMEC embedded in clusters of adipocytes mimicking the *in vivo* architecture of human breast tissue. This indicates that human autologous breast tissue, regarded as HMEC and adipocytes, can be regenerated *in vitro* by co-culture in 3-D matrices.

Paper II

In vitro comparison of two clinically used fat transplantation methods (lipoaspirate cleansed with centrifugation (LC) and lipoaspirate cleansed in syringes (LS)) to one following TE principles (enzymatic degradation of an adipose biopsy (B)) showed that enzymatic degradation of adipose tissue led to a significantly increased cellular survival.

The mean number of preadipocytes/g tissue after 48h of culture were 81, 49, and 56 in the enzymatic degradation group (B), liposuction/centrifugation washing group (LC), and liposuction/syringe washing group (LS) respectively. Indicating significantly more cells in group B compared to group LC ($p < 0,05$) and a tendency to more cells than the LS group ($p = 0,09$). After 120h of culture the differences were more obvious as B (211 cells/g) showed significantly more cells than LC (66 cells/g, $p < 0,01$) and LS (77 cells/g, $p = 0,01$). Hence enzymatic degradation of adipose tissue gave rise to 320% and 274% more cells after 120h of culture when compared to LC and LS.

When cultured cells were stained with crystal violet and the number of cell colonies formed/g tissue were counted after 48h group B (7 colonies/g) had significantly more cell colonies than LC (0,1 colonies/g, $p < 0,05$) and LS (0,2 colonies/g, $p < 0,05$).

At the second time point (120h of culture) the differences between the groups were striking. Group B (19 colonies/g) showed significantly more colonies than LC (0,6 colonies/g, $p < 0,001$) and LS (0,2 colonies/g, $p < 0,01$).

Furthermore it was shown that cells obtained by enzymatic degradation increased significantly between 48 and 120h of culture regarding both number of cells/g tissue and number of cell colonies/g tissue. This was true also for LC and LS regarding number of cells/g tissue but not regarding number of cell colonies/g tissue.

Paper III

Normal human fibroblasts were seen to have attached to the surface of the porous PUUR matrix after one week in culture. Cells migrated deeper into the scaffold with time and after 2 weeks fibroblasts were present halfway to the center of the matrix. The entire thickness of the scaffold was populated with cells after 4 weeks. The considerable increase in cell number and abundant cell divisions indicated a proliferative activity.

The fibrous PUUR scaffold was populated in all parts after only one week. With time the number of fibroblasts increased and cell-aggregates connected the fibers after 4 weeks.

The fibroblasts were actively producing collagen in both matrices after 6 weeks of culture as indicated by IHC examination of pro-collagen production by the cells.

The porous scaffold was experienced being more apt to the clinical situation and was thus surgically introduced to the dermis of healthy volunteers. All four subjects completed the investigation, having normal laboratory results at start and end of the study. No adverse events were reported. A slight redness was noticed around all incisions including control sites. All but one subject had low-grade inflammation with minor turbid secretion from study sites. The skin covering the scaffolds contracted partially exposing parts of the scaffolds in all study sites. The B scaffold (9% Artelon[®]) elicited a slightly more pronounced inflammation.

Histology after 2 weeks implantation time showed a large number of cells in the scaffolds, mostly neutrophils and lymphocytes. The number of inflammatory cells decreased after 8 weeks in all subjects. Spindle-shaped cells, judged being fibroblasts, occupied the entire scaffold material and grew in thick bundles after 8 weeks. Using IHC, cells occupying the scaffolds, were noticed synthesizing pro-collagen in all scaffolds at week 8. IHC stained sections for von Willebrand Factor showed capillary formation in two of the subjects after 8 weeks, indicating neo-angiogenesis. The other two showed endothelial budding but was not developed enough to be judged as capillaries.

Paper IV

The handling and preparation of the macroporous gelatin spheres proved easy and convenient. The spheres could be prepared and stored in the fridge for up to a month before being seeded with cells.

Cells cultured in serum-supplemented medium as the preadipocytes, HMEC, fibroblasts, and chondrocytes adhered to the surface of the spheres within 24h, whereas keratinocytes (cultured in serum-free medium) needed 48h to adhere as visualized by using MTT-staining. With increased culture time, the number of cells/sphere increased and cells were noticed to have migrated deeper into the spheres occupying also the interior surfaces of the spheres as visualized by H&E.

The different cell types showed a peak in cell density at somewhat different time points; preadipocytes (day 6-10), HMEC (day 8), fibroblasts (day 6), chondrocytes (day 6), and keratinocytes (day 6-8). At the day(s) of peak cell density the number of cells/sphere seemed to reach a 'steady-state', or plateau, as no further increase in cell number/sphere was noticed.

After 10 days of culture, keratinocytes were verified being keratinocytes on both paraffin embedded sections and directly in culture vials by positive IHC staining. Preadipocytes were verified being preadipocytes on paraffin embedded sections in the same manner by IHC.

All cell types could easily be stained with MTT in culture vials. Cells attached to the microcarriers could be stained with H&E or IHC (either directly in culture vials or after dehydration, paraffin embedding, and sectioning of the microcarriers).

Paper V

All animals tolerated the injections well and recovered rapidly and fully from anesthesia. All mice, but two, survived to planned sampling time with good health. As the mice had very thin skin, it was difficult to ascertain that injections were intradermally. Routine histology did also show that most injections were indeed subcutaneous. Wheals from injected saline and single-cell suspensions had disappeared within 24h, whereas injected MGS with/without cells detained their wheals even though the size decreased somewhat over time. No local or systemic reactions were seen from any injection site. No measurement of original wheal size or reduction in wheal size was performed.

Routine histology

No scarring or inflammatory reactions were seen from the saline injections. No fibroblast or preadipocyte accumulations or increase in population of the cells from the injected single-cell suspensions could be retrieved from any mouse. Nor was any increase in dermal or subcutaneous thickness noted. Furthermore, no scarring or signs of inflammation were seen. On all mice, every injection of MGS and pre-seeded MGS were retrieved. Overall, the tissue accepted the MGS very well. No signs of inflammation, rejection, encapsulation, or multinucleated giant cells were seen.

By day 7, 'naked' MGS were densely populated with cells in the outer vicinity of the injected material (approximately 300-500 μm from the border). Connective tissue around and within the pores of the MGS was also visible in these areas. Macroporous gelatin spheres were populated with cells also in the center but more scarcely so. Around the injected material, a slightly denser connective tissue structure was seen. It did not appear fibrous as if being capsule-formation. At day 21, dense populations of cells were seen deeper towards the center of the injected material. By day 21, and even more so by day 56, the MGS became populated with cells completely throughout. Neocollagenesis was seen as the material was near completely occupied by cellular connective tissue.

The MGS pre-seeded with cultured human fibroblasts and preadipocytes showed the same pattern as the naked MGS with the exception of a population of cells in the center of the material already by day 7. The pre-seeded MGS did also show a quicker and more thorough cell population throughout the material with denser connective tissue formation. In MGS pre-seeded with fibroblasts or preadipocytes clear signs of neoangiogenesis through the whole of the injected material were visible. The naked MGS were noted to collapse to some degree as time progressed, whereas the pre-seeded MGS kept their initial volume to a higher extent.

Neoangiogenesis was not as well developed among the naked MGS. An overall impression is that the preadipocyte pre-seeded MGS showed a more natural looking neoformation of connective tissue (with neoangiogenesis) on and within the MGS than did the fibroblast pre-seeded MGS which in turn had a better result than the naked MGS.

Fluorescence *in situ* hybridization (FISH)

By employing FISH with the Spectrum Green Female Total Human Reference Genomic DNA probe, and DAPI stain to visualize all nuclei, the presence of cells of human origin, injected as single-cell suspensions, were noticed at all time points. The amount of human cells seemed to decrease with time. Macroporous gelatin spheres pre-seeded with fibroblasts or preadipocytes were also demonstrated to contain cells of human origin at all time points. With increased *in vivo* time the total number of cells occupying the MGS was noticed to increase (as seen on routine histology) but was not paralleled by an increased amount of human cells (as indicated by FISH). Human fibroblasts and preadipocytes remained viable also after 56 days implantation time.

Paper VI

All injections were easily performed and the subjects tolerated the injections well. No signs of inflammation, infection, rejection, itch, or pain from any of the injected substances could be detected, neither in the acute nor in the late phase of the study. The Restylane[®] was difficult to inject as a defined wheal as it had a tendency to diffuse out in the dermis, creating a wider, but less elevated, wheal compared to MGS and saline. With MGS and saline, distinct wheals were created. All wheals reduced in size with time, Restylane[®] retained the initial volumes the most. The saline wheals were resorbed completely within 24h.

Routine histology day 14

Routine histology of the saline controls revealed normal human skin as expected.

The Restylane[®] was seen exerting its volume-creating effect by simply physically occupying space. The surrounding tissue was noted to be compressed by the substance as cells and the ECM were densely packed, or squeezed, together at the interface between the Restylane[®] and dermis. No signs of cellular ingrowth into the substance were seen. A slight inflammatory, or encapsulating, rejection process was noted with a small number of multinuclear giant cells and PMN around the deposits.

The MGS were observed to be upholding the physical properties of the dermis as the wheals were still present. A cellular ingrowth had begun, and the MGS were completely populated by rounded cells, interpreted as inflammatory cells and migrating fibroblasts. As no clinical signs of inflammation (rubor, tumor, calor, or dolor) were present, it is highly unlikely that these cells would all be PMN or lymphocytes. At the interface between dermis and MGS a regeneration of dermis was present since dense connective tissue-like tissue was migrating into the spheres closest to the dermis.

Routine histology day 56

Routine histology of the saline controls still revealed normal human skin as expected.

Again, the Restylane[®] was seen exerting its volume creating effect by occupying space. The surrounding tissue was still compressed by the substance. After 56 days there was still no indication of cellular ingrowth into the substance, nor any signs of material degradation. The slight inflammatory response had resolved and no capsule formation was present.

In all subjects, the MGS were completely occupied by fibroblasts and connective tissue. The degradation of the spheres had begun as the internal pore sizes had increased and the spheres had collapsed somewhat. No inflammatory reaction or capsule formation was noted around the spheres.

Discussion

In **Paper I** we showed that human preadipocytes and HMEC can be harvested from breast tissue biopsies in a single session. The cell harvesting procedure is straightforward and biopsy harvest can be conducted as an outpatient procedure in a clinical setting. We did also show that it was possible not only to co-culture these two cell types, but also to co-culture them in a 3-D matrix. When cultured in a 3-D matrix, cells show growth patterns mimicking the *in vivo* architecture of normal human breast tissue.

This study indicates that human autologous breast tissue, regarded as HMEC and adipocytes, can be regenerated *in vitro* by co-culture in 3-D matrices. Theoretically, a small breast tissue biopsy could, after subcultures and expansion in cell number, generate enough cells to seed a biodegradable scaffold which could be re-implanted in a breast reconstructing purpose.

Tissue engineered autologous breast tissue produced *in vitro* could have wide clinical applications in reconstructive plastic surgery and compete (or complement) with artificial prostheses and tissue flaps when reconstructing the breast after mutilating surgery.

In this paper we used collagen gel as 3-D matrix since it is cheap and readily available. For future purposes, collagen gels are probably not the most optimal scaffold material. In order to become clinically interesting the volume of the tissue engineered breast tissue construct has to be in the deciliter-range which necessitates a vascular circulation also within the construct. This problem can be approached in different ways;

- > By introducing the vascular architecture in the culture *in vitro* (e.g. co-culture also with endothelial cells).
- > By sparse seeding of the chosen 3-D scaffold and, after implantation, as the matrix is degraded *in vivo*, the seeded cells expand in number and neo-vascularization occurs from the periphery.
- > By co-culturing HMEC and preadipocytes in a matrix that is injectable and over a period of time intermittently enlarge the breast with increments of co-cultured HMEC, preadipocytes, and matrix. Thereby allowing vascularization to occur in between.

One must bear in mind though, that introducing HMEC in tissue engineered breast tissue could constitute an oncologic problem, at least in theory. Spontaneous mutations are very rare in ordinary cell cultures and there are methods available to screen cultures for transformed cells. Human mammary epithelial cells will probably be omitted for this technique to reconstruct breasts to be clinically accepted. With increased knowledge and advanced culture techniques it may be possible though, that functional, lactating, tissue engineered breast tissue could be developed in the future.

The adipose tissue is important in the reconstruction of the mammary gland as well as usable in other reconstructive purposes. A common procedure to correct soft tissue defects is to transplant fat. In **Paper II** we studied lipoaspirate as the source for donor material.

A vast number of techniques for correcting dermal and soft tissue defects have been described in the literature. Their main drawbacks being the need to overcorrect or repeat the procedures, migration of implant material, inflammation, and granuloma formation. One of the more common procedures is the lipoinjection technique where autologous adipose tissue is in different ways harvested and transplanted back to the patient. A great number of techniques are available, many necessitating special instruments and gadgets. Some authors stress the need to wash/clean the material before transplantation.

Usually 30-70% of the injected material is resorbed. This is thought to be because mature adipocytes do not have the ability to replicate, and that the adipocytes depend on diffusion to survive until vascularity has developed and they will die if this does not occur in time. Furthermore, it has previously been described that liposuction is highly traumatic to adipocytes and that the use of e.g. saline to wash the aspirate with can damage adipocytes. Preadipocytes are understood to be less fragile, withstand nutritional leanness better, along with the inborn ability to replicate, and hence are probably more useful than mature adipocytes for reconstruction of soft tissue defects.

In this paper we have shown that by using TE/cell culture techniques, significantly more pre-adipocytes could survive and proliferate *in vitro* for up to 120h in culture compared with the clinically used techniques of washing liposuction material in syringes or with centrifugation.

The small fragments of adipose tissue that are aspirated by liposuction are in fact bundles of thousands of adipocytes that all have to depend on diffusion for nourishment. Mature adipocytes may perhaps survive liposuction and injection, but will not replicate and eventually die while generating inflammation after transplantation.

Theoretically, a small biopsy of adipose tissue could, after subculture and expansion in number of cells, generate enough cells to either be injected directly to treat a defect, or to seed a biodegradable matrix that could be implanted to reconstruct a soft tissue defect. Preadipocytes have the ability to replicate and can be seeded in/onto the scaffold in a single-cell suspension and hence have the possibility to survive through nutritional diffusion and replicate at a rate that parallels the vascular development, until the whole 3-D matrix has been occupied by regenerated, vascularized adipose tissue *in vivo*.

We think that the use of cultured autologous preadipocytes seeded in a biodegradable 3-D matrix could improve the technique of lipoinjection and transplantation.

In the quest for an optimal scaffold material for soft tissue regeneration we performed an *in vitro* and a subsequent *in vivo* study of a synthetic polymer in **Paper III**. This material had shown interesting properties in previous studies in reconstruction of the cruciate ligament of the knee.

Synthetic polymers are interesting in TE-research as their properties (e.g. porosity, degradation time, and mechanical characteristics) can be tailored for specific applications. Synthetic polymers are often cheaper than biologic scaffolds; they can also be produced in large, uniform quantities, and have a long shelf-time. The Artelon[®] material, poly(urethane urea), has a vast deformability and elasticity which makes it interesting also for soft tissue reconstruction.

The *in vitro* part of the study investigated whether the Artelon[®] material could host dermal fibroblasts and if the cells would proliferate, migrate, and lay down new connective tissue on/in the material.

Two macro-structurally different scaffolds were used; fibrous and porous. Fibroblasts were found attaching, proliferating, and migrating on to and within both scaffold materials. In the porous scaffold, cells migrated deeper in to the material with time, eventually occupying the whole scaffold. Whereas on the fibrous material no ingrowth into the fibers were seen but cells proliferated on and between the fibrous strands interconnecting them with thick cellular bands.

The *in vivo* part of the study was performed using the porous scaffold since this scaffold was experienced easier to handle and more clinically feasible.

Semicircular pockets were created in subjects' dermis to harbor the implants. The pockets were later found to be too shallow for the implantation study to be flawless. The sterical hindering of the scaffolds led to lid contraction and protrusion of the scaffolds with inflammatory reactions as a result. However, an obvious ingrowth of dermal fibroblasts into the scaffolds was seen along with the production of connective tissue and neoangiogenesis.

The Artelon[®] material has a quite lengthy degradation time *in vivo* (several years). It is often claimed that a TE scaffold should have a degradation time paralleling the turnover time of the tissue of interest. We hypothesize that this is not necessary true. This could be explained by the fact that the scaffold then functions as reinforcement, upholding the physical properties of the tissue while the autologous tissue regenerates slowly with more natural properties and less scarring than would be the case if the scaffold degrades too quickly.

Further along the quest for an optimal scaffold material for TE and reconstruction of soft tissue defects we stumbled upon an interesting macroporous gelatin sphere that showed several positive characteristics which were explored in **Paper IV**.

Many of the scaffold materials for TE described in the literature are in the form of blocks, or sheets. Even if these materials function very well in regenerating tissues and organs, they come with the drawback of necessitating invasive surgery in order to bring them to the place of interest. An injectable scaffold material has several advantages over a solid material; invasive surgery is not necessary, easier to deposit 'right amount' of scaffold, 'topping off' possible, trans/implantation can be performed in (as an outpatient) sessions. Usually a more even distribution of cells within the scaffold can be performed in a shorter *in vitro* culture period.

In this paper we investigated several human ecto- and mesodermal primary cell cultures' behavior on MGS. The CultiSpher microcarrier has been described in the literature as a good scaffold for bioreactor culturing of a number of animal cells and human cell lines. The culture area is several-fold increased when using macroporous microcarriers as culture substrate compared to solid microcarriers or 2-D surface culture. Being porous, cells can proliferate within the carriers and avoid being subjected to shearing forces which otherwise could detach or crush the cells.

The gelatin spheres proved easy to handle and prepare. Human primary cells were proven to quickly attach to, and proliferate on, the microcarriers. In a spinner flask cells attached to the carriers within 48h and proliferated to reach a plateau after about 6 days. The attachment and proliferation was easy to follow with vital-staining (MTT), and spheres could be IHC-stained in culture vials or after paraffin embedding and sectioning.

As the microcarriers could be loaded in a syringe and cells/microcarriers injected, the gelatin spheres hold promise to fulfill several of the characteristics that we look for in a soft tissue regeneration scaffold material, and this was pursued in the following papers.

The gelatin spheres investigated in **Paper IV** have been used to transplant human dopamine-producing cells to the brain of patients suffering from Parkinson's disease. The same carriers seeded with cultured autologous keratinocytes have also been transplanted to patients with leg ulcers in order to improve and speed up wound healing.

With our positive experience of culturing human mesodermal cells on these microcarriers we continued the exploration of this material in **Paper V** where we designed a study investigating the *in vivo* behavior of MGS as is, or pre-seeded with either human fibroblasts or preadipocytes, when injected subcutaneous in nude mice. As described in the literature, soft tissue defects can also be treated by injection of harvested and/or cultured fibroblasts and preadipocytes in a single-cell suspension. Hence as controls to the MGS with/without cells, we injected saline and single-cell suspensions of fibroblasts and preadipocytes.

All mice received six subcutaneous injections, one for each treatment modality and the animals withstood the injections well. The wheals from saline and single-cell suspensions disappeared within 24h whereas the MGS wheals were prominent for up to 56 days.

The ‘naked’ MGS did show tissue ingrowth, but MGS pre-seeded with cells showed a more thorough neoangiogenesis and tissue regeneration. ‘Naked’ MGS had a tendency to collapse and begin to degrade earlier than pre-seeded MGS, indicating that by including cells with the scaffold, the duration of the physical properties of the scaffold could be prolonged.

The cells and the MGS were possible to inject, leading to a clinically acceptable way to bring the scaffold to the site of interest.

By employing FISH-analysis we could show that cells occupying the MGS after transplantation indeed were human cells. Of course, mouse fibroblasts had migrated into the microcarriers as expected and wanted, but cells pre-seeded in microcarriers were still present and alive after 56 days in the mice.

Since this preliminary animal study we have taken the next step and implanted macroporous gelatin spheres in human subjects.

After having shown that the MGS very well function as culture substrate for human primary cells, and also show good characteristics when implanted in nude mice, we wanted to bring the spheres all the way and performed in **Paper VI** an *in vivo* study on human subjects. As previously described, the handling and preparation proved to be easy. None of the subjects showed any adverse events after treatment and the injections were performed with ease. Adequate wheal sizes were retained also after 56 days implantation time. Routine histology showed some (expected) inflammatory reaction after 2 weeks implantation time. After 56 days the injected MGS were completely occupied by regenerating dermal tissue and the degradation of the material had begun. The MGS were compared to Restylane[®], which can be considered the golden standard of fillers today, and were inferior regarding retaining the wheal size over time. However, Restylane[®] exerts its effect by physically occupying space whereas MGS induce a tissue regeneration. The effect of Restylane[®] is known to wane after 3-6 months and the treatment must be repeated. Macroporous gelatin spheres are considered, and to some extent proven in this paper, to induce GTR of the dermis, obviating the need to repeat treatments as the filler effect becomes permanent.

In the light of the results presented in this paper we venture to hypothesize that MGS could be used as a filler to treat smaller soft tissue defects such as rhytids or creases. The MGS uphold the structural properties of the dermis as a regeneration of the dermis is taking place.

We further believe that the approach of using autologous fibroblasts seeded on MGS can constitute a novel and highly effective way to treat larger soft tissue defects. Further *in vivo* studies are needed to evaluate the filling effect proper, and to investigate whether the suggested superiority of pre-seeded spheres holds true.

Concluding remarks and future perspectives

In order to correct soft tissue defects we have pursued the idea of combining autologous cells and scaffold materials, i.e. utilizing the 'open system' approach of TE. We have learned how to harvest, culture, and store human soft tissue cells, as well as how to culture the cells on different scaffold materials.

The material quest brought us to the use of macroporous gelatin spheres, which showed several appealing characteristics. The MGS withstood the animal study test showing that pre-seeded MGS indeed could be used to regenerate soft tissue defects. We have since continued this strategy and taken the step to implant MGS in human subjects. Preliminary data are positive, showing a regeneration and neoangiogenesis of the tissue of interest at a pace paralleling the degradation of the spheres.

Tissue engineering is a fairly young field of research that has shown huge potential thus far. In only about twenty years, the groundbreaking pre-clinical findings of Rheinwald and Green⁵⁰ of culturing sheets of keratinocytes *in vitro* have become an established principal to treat severe burns. The technique has progressed, in that keratinocytes now are sprayed as solitary cells on the patient. In continuation; other recent advances in TE, which most likely will prove to be important landmarks are the invention of *in vivo* tissue culture chambers and the use of gene therapy in wound healing^{151,152}.

It is imperative to be humble enough to realize that TE combines several different fields of research, in themselves very complicated, as biology, medicine, chemistry, material science, and more. TE is a teamwork task. The biologists can show us how the cells should be treated, the medics show us what is needed in the clinic, the material scientists can bring us scaffolds and materials that may, or may not, function as wanted, but no single person have the knowledge to do it all⁵.

The main obstacle in the progress of TE today is the vasculature system. Any TEMP of sizes larger than a couple of hundred microns in diameter need a circulatory system for nutritional reasons. This is mirrored in the fact that the tissues that have found their ways to the clinical setting are all tissues and organs with the least complex blood circulation; skin, cartilage, and bone. In order to succeed also with more complex organs and tissues, this problem has to be resolved.

Ways to overcome this obstacle are to co-culture also endothelial cells and/or blood vessels in the TEMPs, to follow the route of GTR, i.e. to provide the body/tissue with the right prerequisites to regenerate the organ/tissue by itself, or by intermittently deliver cells and scaffold material in a speed that allows neoangiogenesis to occur in between.

Today we can culture virtually any cell of the human body. Even the long-thought non-proliferating cells of the central nervous system can be cultured *in vitro*. Many TE scientists stood by the tissue specific adult 'stem cells' as a cell source for TE, even when embryonic stem cells were looked upon with awe as the solution to many of the adult stem cells' shortcomings. But let's face it, it is a quite rigid way of views that one specific tissue can only be regenerated by the use of the same specific cell type. Human DNA contains all the information used during embryogenesis and evolution of all tissues and organs. Nothing is lost; hence, the information is still contained within the cells' DNA, no matter if the cell be an adipocyte or a fibroblast. When we learn to unlock the secrets of the silent genetic information, when we learn to trans-differentiate cells, any given cell can be transformed in to any other of choice. This brings us closer to true TE. Then we can start growing organs and tissues for the broader masses of patients dying on our transplantation waiting lists.

I've got an urgent appointment
And I can't be late
I've got a million things
That have to wait
Just hang on a minute
I'll try to hurry back
I hate to make you wait
But I'm going and I'm going fast

Sammanfattning på svenska

Tissue engineering är en ung, men snabbt växande forskningsgren som syftar till att bibehålla, återställa eller förbättra vävnaders och organs funktioner. Vanligtvis menar man med TE att man kombinerar autologa celler som odlas utanför kroppen på någon form av 3-dimensionellt matrix, eller mall, och transplanterar denna cell/matrix enhet tillbaka till patienten. I teorin har TE många kliniska applikationsmöjligheter som t.ex. produktion av reservdelsvävnader/organ för de flesta organsystemen. Inom plastikkirurgi används idag t.ex. odlad hud i den kliniska vardagen.

Inte sällan kan kirurgi ge konturdefekter p.g.a. mjukdelsförluster, vilket ger estetiskt dåliga resultat. Man kan korrigera dessa m.h.a. t.ex. fettransplantation. Där har erfarenheten dock visat att resultaten är nedslående. Ofta resorberas 30-70% av transplanterad fettvävnad och man måste således överkorrigera eller komplettera med flera ingrepp. Vidare ger fettransplantation ofta upphov till hård och knölig vävnad då det mesta av fettets omvandlas till ärrvävnad. En, via TE, regeneration av mjukvävnad skulle kunna vara till stor hjälp inom rekonstruktiv plastikkirurgi.

Inom den estetiska plastikkirurgin har man sedan länge kunnat erbjuda behandling av rynkor och hudfäror. Man använder s.k. 'fillers' som kan vara allt mellan patientegen fettväv till plastkuler eller silikonolja för att fylla ut konturdefekterna. De fillers som är nedbrytningbara försvinner efter en viss tid emedan de permanenta fillers inte sällan ger upphov till materialvandring eller granulombildning. Idag finns det ingen riktig bra filler som har långvarig effekt utan att riskera de komplikationer som de permanenta fillers ger. Inom estetisk plastikkirurgi finns en enorm marknad för tissue engineered mjukdelsvävnad för korrektion av konturdefekter.

Syftet med denna avhandling har varit att utveckla laborativa metoder och tekniker, samt utvärdera olika matrix för TE av human mjukdelsvävnad.

Avhandlingen bygger på sex delarbeten som refereras till med romerska siffror:

I

Det kvinnliga bröstet kan drabbas av cancer och den primära behandlingsmetoden är kirurgiskt avlägsnande av bröstet. De flesta kvinnorna önskar rekonstruera sitt förlorade bröst vilket kan ske genom implantation av en silikonprotes eller via extensiv lambå-kirurgi där man rekonstruerar bröstet m.h.a. hud och mjukdelar från annan plats på kroppen.

I detta första arbete tog vi fram en metod där man genom att ta en biopsi av bröstvävnad kan selektera fram förstadiet till fettceller och bröstkörtelepitelceller. Dessa celler visade vi att man kunde odla *in vitro* och således expandera i antal. Cellerna kunde frysas och tinas, och på så sätt kan man förvara cellerna under en längre tid. Genom immunohistokemi och rutinfärgningsmetoder kunde vi påvisa att de celler vi odlade verkligen var preadipocyter och mammapitelceller. Genom att co-kultivera båda celltyperna i ett 3-dimensionellt matrix (kollagen gel) visade vi att preadipocyter växer i god harmoni med mammapitelcellerna och att kulturerna uppvisade liknande utseende som normal human bröstvävnad. Det vill säga att bröstkörtelepitelet växte i gångformationer med alveoli och omkringliggande fettvävnad.

Kontentan av studien är att humana celler från bröstbiopsier kan vid co-kultivering i ett 3-dimensionellt matrix fås att efterlikna normal human bröstvävnad, således är det första steget mot TE av bröstkörteln taget.

II

I den kliniska vardagen används ofta autolog fettransplantation för korrektion av kontur- och mjukdelsdefekter. Metoden är behäftad med flera tillkortakommanden. Flera studier har publicerats där man använder sig av speciella metoder för att tvätta fettcellerna före transplantation eller speciella instrument vid skördning/transplantation. Dessa metoder sägs förbättra resultaten.

Vi önskade i detta arbete testa hypotesen att selektionen av preadipocyter som sedan odlas *in vitro* för att expandera cellantalet ger upphov till högre cellulär överlevnad vid transplantation än de idag kliniskt använda metoderna.

I samband med andra plastikkirurgiska operationer togs en fettbiopsi samt en mindre fettsugning utfördes. Fettsugningsaspiratet behandlades enligt två kliniskt gångbara metoder där fettcellerna tvättas antingen i centrifug eller i spruta före transplantation. Fettbiopsin behandlades med enzymer för att åstadkomma en singelcell suspension av preadipocyter. Cellerna från respektive behandlingsmetod odlades sedan i sedvanlig fettcellskulturskultur. Antalet celler och cellkolonier, normaliserade till gram ursprunglig vävnad, analyserades efter 48h och 120h odlingsperiod.

Den enzymatiska behandlingen av fettvävnad gav upphov till ett klart signifikant högre antal celler och cellkolonier/gram ursprungsvävnad än övriga två behandlingar vid båda tidpunkterna.

Resultaten indikerar att vid en transplantation av fett torde en högre cellulär överlevnad erhållas om fettcellerna behandlas enzymatiskt före transplantation än med sedvanlig tvättning, vilket i sin tur indikerar att resultaten vid fettransplantation torde kunna förbättras om singelcell suspension av preadipocyter transplanteras.

III

I arbete tre utvärderade vi ett syntetiskt 3-dimensionellt matrix för regeneration av dermal vävnad. Materialet Artelon[®] har i kliniska studier använts för rekonstruktion av rupturerade främre korsband i knän. Vid implantation i knäled migrerar fibroblaster in i vävnaden som successivt, över ett antal år, bryts ned och slutligen helt ersatts av patientegen bindväv.

I en *in vitro* studie utvärderade vi hur humana dermala fibroblaster uppträdde vid odling på två makroskopiskt olika former av materialet (flätad fiber eller porös svampliknande). Vi såg att med tiden migrerade fibroblaster in i materialet (det porösa) under det att de prolifererade. Fibroblasterna visades, medelst immunohistokemiska markörer för pro-kollagen, producera kollagen indikerande att de var aktivt fungerande fibroblaster. I det fibrösa materialet växte inte cellerna in i fibrerna men beklädde snabbt ytan av dem och växte i förband mellan fibrerna.

Det porösa materialet upplevdes som mer kompatibelt för ett eventuellt kliniskt bruk och gick vidare i en pilotstudie på friska frivilliga försökspersoner. Med en hudstans skapades semi-cirkulära fickor i dermis och materialet implanterades. Material med omgivande frisk vävnad avlägsnades med en hudstans efter 2 och 8 veckor. Man noterade en låggradig inflammation kring implantaten men det var svårvärderat om det var materialet som sådant som gav upphov till den inflammatoriska reaktionen eller om det var att fickorna var för trånga. Man noterade även att hudlocket på fickorna kontraherades och blottade materialet.

Vid den histologiska analysen sågs efter 2 veckor inflammatoriska celler ockupera större delen av det implanterade materialet. Efter 8 veckor sågs inte längre några inflammatoriska celler utan materialet var helt genomsatt av fibroblaster som växte i tjocka förband. Den histologiska bilden var mycket lik normal human dermis. Med immunohistokemi påvisades ånyo pro-kollagen, indicierande aktivt secernerande fibroblaster, liksom även celler som uttryckte von Willebrand Faktor. von Willebrand Faktor är ett protein som produceras i endotelceller varför immunohistokemin kombinerat med det tubulära växtsättet indicerade neoangiogenes i materialet.

Sammanfattningsvis förefaller Artelon[®] kunna utgöra ett matrix för GTR av dermis.

IV

Det är sedan tidigare visat att genom bruket av microcarriers kan expansionsgraden av celler mångfaldigt ökas då odlingsytan ökas genom många små kulor som cellerna fäster på i odlingen. En makroporös gelatinkula ökar odlingsytan än mer, då cellerna även kan adherera och proliferera inuti kulorna. Då dessa microcarriers är tillverkade av gelatin, som är en nedbrytningsprodukt av kollagen, innebär det att de är biologiskt nedbrytbara *in vivo*. Man kan således odla en stor mängd celler på dessa microcarriers och sedan transplantera cellerna direkt med den odlingsyta som använts. Detta tillvägagångssätt skulle innebära ett stort steg framåt för TE av mjukdelsvävnader.

Från en vävnadsbiopsi selekteras önskade celler fram, expanderas i antal på makroporösa gelatinkulor vilka sedan kan injiceras på platsen som skall regenereras/rekonstrueras.

I denna studie utvärderade vi hur ett antal olika ecto- och mesodermala humana celler uppträder vid odling på makroporösa gelatinkulor.

Humana preadipocyter, mammarepitelceller, kondrocyter, keratinocyter och fibroblaster var alla lätta att odla på dessa kulor. Cellerna adhererade inom 48h och uppvisade ett proliferationsmaximum efter c:a 6 dagar.

Respektive celltyp verifierades med immunohistokemi och rutinfärgningar. Tillväxtmönstret analyserade medelst vital-färgningar.

V

Då odling av humana celler på makroporösa gelatinkulor uppvisat positiva resultat gick vi vidare med en djurstudie för att utvärdera beteendet av de makroporösa gelatinkulorna *in vivo*.

Humana fibroblaster och preadipocyter odlades på gelatinkulorna och injicerades intradermalt på ryggen på nakenmus (nude mice) samtidigt med singelcell suspensioner av fibroblaster, preadipocyter, och natriumklorid.

Djuren tolererade injektionerna väl och på samtliga sex injektionspunkter skapades kvaddlar i huden av de injicerade lösningarna. Redan efter ett dygn hade kvaddlarna av singelcell suspensioner och natriumklorid resorberats emedan de kvaddlar som skapats av gelatinkulorna med/utan celler kvarstod under hela försökets längd, d.v.s. 56 dagar.

Djuren avlivades efter 7, 21 respektive 56 dagar och mössens rygghud togs till histologisk analys. Vid rutinfärgning sågs en inväxt av bindvävsceller i det injicerade materialet som blev mer uttalad ju längre djuret hade gått med injektionerna. Vid de senare tidpunkterna föreföll de gelatinkulor utan celler att ha fallit samman något varför volymen av det injicerade materialet minskat något. De kulor som för-odlats med celler kvarhöll sin volym i större utsträckning. Efter 56 dagar var kulorna i det närmaste helt genomsatta av bindvävsceller.

Kulor med preadipocyter visade bäst regeneration följt av kulor med fibroblaster och bara kulor. I de cellbeklädda kulorna sågs efter 56 dagar en neoangiogenes med kapillär inväxt in i det injicerade materialet. Kapillärerna verifierades med immunohistokemi för von Willebrand Faktor.

På injektionspunkterna för singelcell suspensioner och natriumklorid kunde ingen cellanhopning eller annat från omkringliggande vävnad avvika.

Med FISH-teknik kunde vi påvisa att bland de celler som ockuperade gelatinkulor efter 56 dagar implantation fanns fortfarande de initiala humana cellerna vid liv.

Denna studie visar att medelst en vävnadsbiopsi kan vi således selektera fram de celltyper som är av intresse, expandera dem i antal *in vitro* på makroporösa gelatinkulor och därefter injicera dem på önskvärd plats för att åstadkomma en regeneration/rekonstruktion av den vävnaden.

Att kunna odla celler på samma transport-vehikel som används vid transplantation är av stor vikt och öppnar upp fältet för humanstudier för korrektion av mjukdelsdefekter med hjälp av denna teknik.

VI

Våra laboratorieförsök visade att primära humana celler med god framgång kunde odlas på gelatinkulor. Djurstudien gav tydliga indikationer om att gelatinkulorna gav upphov till s.k. guided tissue regeneration av vävnad där de implanterades. Effekten förstärktes när kulorna 'laddades' med celler före implantation. Studier på nakenmus kan kritiseras då djurens immunförsvar är kraftigt nedsatt/upphävt och därmed kringgår man en eventuell negativ effekt om det implanterade materialet ger upphov till en inflammatorisk reaktion som kan omöjliggöra bruket av materialet. Vi gick därför vidare med en pilotstudie på fyra friska frivilliga försökspersoner. På insidan av överarmen injicerades, intradermalt, gelatinkulor, koksalt och Restylane® (non-animal stabilized hyaluronic acid). Stansbiopsier av implantat och omkringliggande vävnad togs efter 14 och 56 dagar. Ingen av försökspersonerna uppvisade några reaktioner på injektionerna (inflammation, klåda, irritation, el. dyl.).

Resultaten visade att koksaltinjektioner resorberades inom 24h och histologin visade normal hud. Restylane® visades utöva sin effekt genom att fysiskt 'ta plats', vävnaden närmast Restylane® var hoppresad. En antydning till inflammation skönjades kring implantaten efter 14 dagar, där PMN och enstaka jätteceller sågs, inflammationen var upphävd efter 56 dagar. Ingen inväxt av celler i Restylane® sågs, inte heller någon regeneration av vävnaden.

Gelatinkulorna var enkla att injicera och gav distinkta kvaddlar. Efter 14 dagar var gelatinkulorna genomsatta av celler som tolkades som dels migrerande fibroblaster och dels enstaka inflammatoriska celler. Efter 56 dagar var kulorna helt genomsatta av fibroblaster och nybildad dermis. Nedbrytningen av kulorna hade börjat vilket indicerades av att kulornas porer har ökat i storlek och att kulorna hade fallit samman i viss utsträckning.

Resultaten från denna studie tyder på att gelatinkulor mycket väl kan användas för att behandla mindre substansdefekter och att dessa kan fyllas ut med regenererad vävnad *in vivo*. Mot bakgrund av djurstudien vågar vi föreslå att den regenererande effekten av gelatinkulorna kan förstärkas genom att de 'laddas' med humana celler före injektion och att de därmed kan användas till att behandla även substansdefekter av större volymer.

Slutsats

Denna avhandling har inneburit att metoder och matrix material för TE av mjukdelsvävnad har utvecklats.

Cellskördning kan ske på ett minimal-invasivt sätt genom en mindre vävnadsbiopsi, vilket kan utföras vid ett vanligt mottagningsbesök. Aktuella celltyper kan selekteras fram och expanderas i antal i laboratoriet. Cellerna kan frysas för förvaring under längre tid och sedan tinas upp inför transplantation. Genom att kombinera de patientegna cellerna med en transplantationsvehikel som samtidigt utgör ett 3-dimensionellt matrix för vävnaden att växa in i kan större substansdefekter korrigeras.

Metoderna är relativt enkla och billiga att genomföra. Humana studier har initierats och steget till klinisk praxis, efter effektutvärdering, torde inte vara långt.

Another late night cameo
Some other place, another show
But I'm not gettin' tired yet
Have you heard a word I've said?
I just dropped in to say hello

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Don't be fooled by what you see
I'm just an ordinary man
So many years I've sacrificed my needs
So give the gentleman a hand
I can make you look the other way
I know exactly what you see
It never ceases to amaze
What you're watching is my dreams