Linköping Studies in Health Sciences
Thesis No. 99

Differentiation of Human Dermal Fibroblasts

a New Tool in Vascular Tissue Engineering

Lisa Karlsson

Linköping University
FACULTY OF HEALTH SCIENCES

Division of Surgery
Department of Clinical and Experimental Medicine
Faculty of Health Sciences, SE-58185, Linköping, Sweden

Linköping 2009

Printed by LiU-Tryck, Linköping 2009

ISBN: 978-91-7393-617-0
ISSN 1100-6013: 99

There are only two things to worry about:

Either you are sick or you are well.

If you are well then there is nothing to worry about.

But if you are sick then there are two things to worry about:

Either you will get well or you will die.

If you get well then there is nothing to worry about.

But if you die there are two things to worry about:

Either you will go to heaven or you go to hell.

If you go to heaven then there is nothing to worry about.

And if you go to hell you will be so damned busy shaking hands with friends so you won't have time to worry.

So why worry!

SUPERVISORS

Gunnar Kratz, MD, PhD, Professor
Department of Clinical and Experimental Medicine
Faculty of Health Sciences
University of Linköping, Sweden

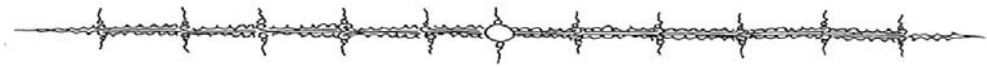
Magnus Grenegård, PhD
Department of Medical and Health Sciences
Faculty of Health Sciences
University of Linköping, Sweden

COMMITTEE BOARD

Eric Wahlberg, MD, PhD, Professor
Department of Medical and Health Sciences
Faculty of Health Sciences
University of Linköping, Sweden

Kristofer Rubin, PhD, Professor
Department of Medical Biochemistry and Microbiology
Faculty of Medicine
University of Uppsala, Sweden

ABSTRACT



Tissue engineering is an expanding field, which focuses on the development of functional substitutes for damaged tissues. A limitation in this field is difficulties with obtaining autologous cells. Recent research has shown the presence of cells with multilineage-potential within the connective stroma of the skin. In line with this, a potential plasticity inherent in human dermal fibroblasts has been demonstrated. The overall aim of this study was to investigate if human dermal fibroblasts can be used as a cell source for vascular tissue engineering. Differentiation towards an endothelial cell-like phenotype was induced by culturing dermal fibroblasts in endothelial growth medium. By utilizing *in vitro* cell culture models, the capacity of different types of serum and serum constituents in inducing a phenotypic shift in fibroblasts was investigated. To clarify the mechanisms behind this phenotypic shift and to eliminate the risk of having growth of residual endothelial cells in the cultures, both normal dermal fibroblasts and single-cell clone fibroblasts were used. Our results demonstrated that presence of human serum caused fibroblasts and single-cell clone fibroblasts to express vWf, to incorporate fluorochrome-labeled low-density lipoprotein, and to start forming capillary-like networks. As an initial step in using these cells in tissue engineering, their ability to endothelialize a surface *in vitro* was studied. Cells cultured in either fibroblast- or endothelial growth medium

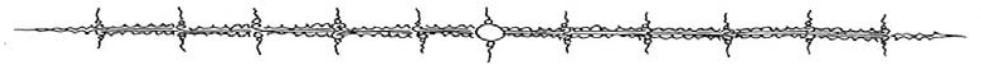
were seeded on scaffolds. Differentiation was confirmed by western blotting and immunohistochemistry using antibodies directed towards vWf, ve-cadherin, eNOS, and bradykinin receptor B_2. The results revealed that endothelial differentiated fibroblasts cultured on scaffolds showed histological resemblance to endothelial cells and expressed molecules indicative of an endothelial phenotype. In conclusion, the results presented in this study indicate a possibility to induce differentiation of human dermal fibroblasts towards an endothelial cell-like phenotype. Consequently, these data suggests that human dermal fibroblast may be a novel cell source for vascular tissue engineering.

ABBREVIATIONS

bFGF	Basic fibroblasts growth factor
cAMP	Cyclic adenosine monophosphate
CD31	Cluster of differentiation antigen 31; Pecam-1
DAPI	4', 6-diamidino-2-phenylindole
DiI-Ac-LDL	1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethyl-indocarbocyanine perchlorate low-density lipoprotein
ECM	Extracellular matrix
EGM	Endothelial growth medium
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
FCS	Fetal calf serum
FGM	Fibroblast growth medium
hESC	Human embryonic stem cell
IBMX	Isobutyl-metylxanthine
MSC	Mesenchymal stem cell
NO	Nitric oxide
Ve-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

vWf	Von Willebrand factor
PDGF	Platelet-derived growth factor
TGF-β	Transforming growth factor beta
LDL	Low density lipoprotein

LIST OF PAPERS

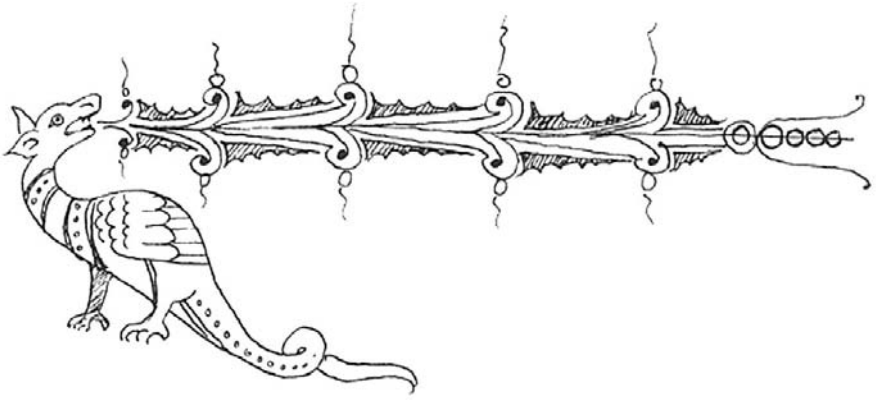


This licentiate thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Karlsson LK,** Junker JPE, Grenegård M, Kratz G. Human Dermal Fibroblasts and Single-Cell Clone Fibroblasts Have the Capacity to Alter Their Phenotype Towards an Endothelial-Like Cell Type. *European Cells and Materials Journal*; Under revision 2009.

II. **Karlsson LK,** Junker JPE, Kratz G. Human Dermal Fibroblasts: a Potential Cell Source for Endothelialization of Vascular Grafts**.** *Annals of Vascular Surgery*; In press 2009.

TABLE OF CONTENTS



BACKGROUND

Tissue engineering

Loss or failure of tissues or organs is one of the most frequent, devastating, and costly problems in human health care. A common way of replacing lost tissue is by autotransplantation, a procedure described in the literature as early as 600 BC [1, 2]. However, current methods of transplantation are among the most costly therapies available and the lack of good immunosuppression, and the shortage of organ donors has opened the door for alternative options [1, 3]. A relatively new branch of science combining the principles of engineering and life science is tissue engineering. In 1993, Langer and Vacanti published an article wherein tissue engineering was defined as "*an interdisciplinary field that applies the principles of engineering and life science towards the development of biological substitutes that restore, maintain, or improve tissue function*" [3]. The idea of tissue engineering is to create living, physiological, three-dimensional tissues and an organ by the use of a combination of cells, biomaterials, and stimulating signals (biochemical and mechanical). These three basic components are often referred to as the triad in tissue engineering (Fig. 1). Investigators have attempted to engineer almost every type of tissue within the human body.

Mainly, there are two different strategies used in tissue engineering; 1) harvest of cells, seeding, and *in vitro* culture of the cells in a scaffold resulting in the creation of an autologous tissue construct or 2) *in vivo* stimulation of cells to form new tissues, known as guided tissue regeneration [4, 5].

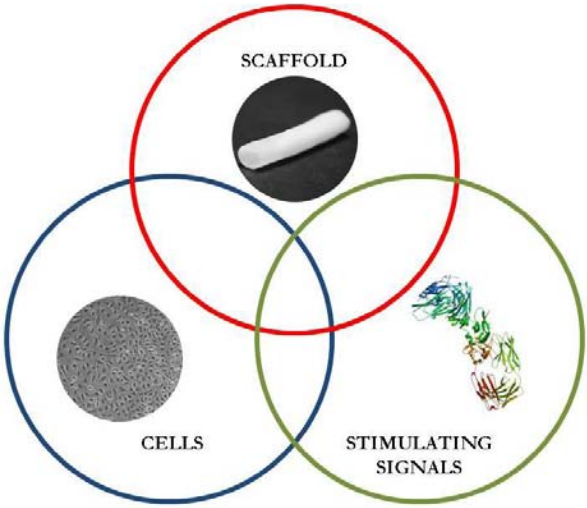


Fig 1. *The triad in tissue engineering. A combination of cells, biomaterials, and stimulating signals are often used to create engineered tissues.*

Vascular tissue engineering

Cardiovascular disease, which includes disorders of the heart and the blood vessels (e.g. hypertension, heart failure, stroke, and coronary heart disease), is one of the leading cause of mortality and a major cause of disability (www.who.int). Today vascular and coronary diseases are often treated surgically using bypass procedures, whereby autologous vessels (e. g. saphenous vein or internal mammary artery) or synthetic grafts (usually Dacron® or Polytetrafluroethylene) are used [6-8]. However, diseased vessels, problem with occlu-

sion of synthetic grafts, or previous use of the patient's own vessels have encouraged researchers to look beyond these alternatives towards the field of tissue engineering [6]. In broad outlines, the field of vascular tissue engineering can be divided into two main research areas. The first, aims at creating artificial blood vessels for the use as either vascular grafts in treating cardiovascular diseases, or as models to use when studying vascular biology. Creating artificial vessels is achieved either by endothelialization of vascular grafts or by tissue engineering of complete blood vessels. The second important research area focuses on one of the major issues in today's tissue engineering, namely the lack of vascularization in engineered tissues [9]. These various applications for vascular tissue engineering will be further discussed.

Cell types for vascular tissue engineering

During the past decades, much effort has been put in finding the optimal cell source for vascular tissue engineering. A variety of different cell types, such as various kinds of stem cells, have been investigated [10-13]. However, one should keep in mind that the optimal cell type for one tissue engineering application might not be suitable for another. In general, cells used in the development of a tissue should be easy to harvest, be highly proliferative, and have the ability to differentiate into application-specific cell types with specialized functions in a controlled and reproducible way [4]. Cells can be obtained either from the host itself (autologous), from individuals of the same species (allograft), or from another species (xenograft). Autologous cells are often to prefer since they are not rejected by the immune system and therefore do not require the use of immunosuppressive drugs. However, a problem with using autologous cells is often the lack of sufficient quantities of healthy cells [14].

Differentiated cells

Most tissues and organs in the human body are composed of differentiated cells expressing a variety of cell specific molecules. It has been discussed whether cells used in tissue engineering should be isolated as fully differentiated cells of the tissue they are meant to create or be manipulated to produce desired functions when isolated from other tissues or from stem cell sources.

The composition of a blood vessel is different depending on its position in the vascular tree. Larger vessels (arteries or veins) consist of three different cell types: endothelial cells, smooth muscle cells, and fibroblasts, while the smaller vessels (capillaries) are composed of a monolayer of endothelial cells supported by a basal membrane. Endothelial cells are a crucial component of the blood vessel wall, providing an interface between the blood stream and surrounding tissues. In an attempt to create hemocompatible surfaces, these cells are an important component in most vascular tissue engineering applications [15]. Through the expression and secretion of various molecules, endothelial cells play an important role in several physiological events including vascular tone and permeability, fibrinolysis, thrombosis, and angiogenesis [16-19]. However, the ideal endothelium for vascular tissue engineering should have a number of other desired features as well. Firstly; it should be adherent for a proper interfacing between the blood flow and underlying smooth muscle cells, secondly; it should be confluent to ensure a non-thrombogenic surface, and thirdly; quiescent to prevent activation of platelets and other clotting factors [19-21]. Various molecules associated with these endothelial-specific functions are frequently utilized to characterize endothelial cells [22, 23]. Here follows a brief description of some of the most commonly used markers used to identify endothelial cells:

Von Willebrand factor

Von Willebrand factor (vWf) is a large multimeric glycoprotein that was first described in 1964 by Webel and Palade [24]. Expression of vWf is restricted to endothelial cells and megakaryocytes. It is both released into the circulation and stored in vesicles, so called Webel-Palade bodies [25, 26]. The von Willebrand protein plays an important role in both primary and secondary hemostasis by recruiting platelets to sites of injury and associate with factor VIII to prevent bleeding. The physiological importance of vWf is demonstrated by the inherited bleeding disorder von Willebrand disease, appearing in people carrying mutations within the vWf gene [27].

Vascular endothelial cadherin

Endothelial cells form a continuous monolayer of cells connected to each other by different types of adhesive structures and cell-to-cell junctions [23, 28]. These structures consist of transmembrane adhesive molecules linked to a network of cytoskeletal proteins. These molecules are commonly divided into three groups: tight junctions, gap junctions, and adherens junctions. Vascular endothelial cadherin (ve-cadherin) is an example of an adhesion molecule, which is anchored in the cell membrane. Rearrangement of ve-cadherin on the cell surface is important for the control of vascular permeability to circulating cells.

Platelet endothelial cell adhesion molecule-1

Another molecule expressed at the border between confluent endothelial cells is platelet endothelial cell adhesion molecule-1 (CD31/Pecam-1). CD31 is expressed on platelets, monocytes, neutrophils, and endothelial cells and it serves in establishing cell-cell contact. This is important in for example regulating transmigration of leukocytes through the endothelium [23, 29].

Endothelial nitric oxide synthase

Endothelial nitric oxide synthase (eNOS or NOS3) is an enzyme responsible for synthesis of the nitric oxide (NO) found in the cardiovascular system [30]. Binding of for example bradykinin to its G-protein-coupled bradykinin B_2 receptor, leads to an increase in the intracellular calcium concentration. Calcium binds to calmodulin and this complex activates eNOS, which then can convert the amino acid L'arginine to citrulline and NO [30]. NO plays an important role in a variety of biological processes. However, most relevant for this thesis is the effects it has on vessel homeostasis by inhibiting vascular smooth muscle contraction, platelet aggregation and adhesion, and leukocyte adhesion to the endothelium [30, 31].

Fully mature endothelial cells are the ideal cell source in the regard that they have the desired phenotype. However, they have a slow expansion rate and limited proliferation capabilities, which makes it difficult to culture them in large quantities for therapeutic use [15]. Additionally, obtaining human autologous endothelial can be difficult, why finding alternative cell sources is of great interest.

Stem cells in vascular tissue engineering

Stem cells have been proposed to have a great therapeutic potential. During the last decade, different kinds of stem cells have been investigated as possible cell sources in vascular tissue engineering [10-13]. The classical definition of a stem cell requires that it is able to undergo self-renewal, meaning the ability to go through numerous cycles of cell division while maintaining an undifferentiated state, and have the capacity to differentiate into specialized cell types. Stem cells can be obtained from discarded embryos, fetal tissue, or from adult tissues. They are thought to be organized in a hierarchical way from the most primitive

(totipotent) to already differentiated tissue-committed (monopotent) cells [32]. The totipotent stem cell is the result of fusion between the oocyte and the sperm. These cells have the ability to give rise to both the embryo and the placenta. Totipotency is thought to be lost in the 32-cell stage and the cells are thereafter referred to as being pluripotent. Embryonic stem cells (ESCs) are derived from the inner cell mass of an early stage embryo, known as a blastocyst. These cells are pluripotent and can give rise to all three germ layers: mesoderm, ectoderm, and endoderm [32]. On the other hand, adult or fetal stem cells are multipotent cells thought to be restricted to either one of the germ layers. Upon cell division these cells are thought to give rise to monopotent stem cells. This is the common way to think about adult stem cells. However, during the last years the strictly hierarchic classification of adult stem cells and their ability to differentiate has been questioned [33-36].

Embryonic stem cells

In 1981, the first ESCs were isolated from mouse blastocyst, and in 1998 Thomson and colleagues established the first human embryonic stem cell-lines (hESC) [37, 38]. Among the various cell sources suggested for tissue engineering hESCs are considered to be one of the most promising candidates. The unlimited self-renewal capacity found in ESCs enables the generation of sufficient amount of cells for cell-based tissue engineering applications. They have been shown to have the ability to differentiate into cells from all three embryonic germ layers *in vitro* and *in vivo* [11, 39]. For instance, both murine and human ESCs have been shown to have the capacity to differentiate along the endothelial line [40, 41, 42, 43]. However, there are still many obstacles that must be overcome before ESCs can be used in the clinic. For example, defined conditions for derivation and expansion need to be identified. Stem cells are often purified by cell sorting, using specific surface markers. Many ESC-populations lack

unique surface markers that might be used to isolate pure populations. Furthermore, if the goal is differentiation, well-defined differentiation processes needs to be developed [15, 44]. However, one of the greatest limitations in using hESCs as a cell source in tissue engineering is the ethical concerns associated with the fact that obtaining hESCs requires the destruction of embryos. Because of these issues and the controversy associated with using cells derived from embryos, many researchers have instead started to investigate the possibility to use adult stem cells.

Adult stem cells

In the past, many tissues in the adult were assumed to be incapable of self-renewal. However, the ability of many tissues to repair or renew indicates the presences of stem- or progenitor cells. A common view is that most adult stem cells are lineage-restricted (multipotent) and they are generally referred to by their tissue origin, for instance mesenchymal stem cells (MSCs), adipose-derived stem cells, or endothelial progenitor cells (EPCs) [34, 45-47].

Human MSCs were first identified by Friedenstein *et al.* in 1966 [46]. These multi-potent stem cells mainly residing in the bone marrow stroma, but have also been found in low frequency in peripheral blood [48]. Stem cells are often isolated and identified by the use of specific markers expressed on their surface. Labelling these markers makes it possible to sort cells with for example a fluorescence activated cell sorter. MSCs do not possess a unique marker that can be used for isolation, therefore characterization of these cells is based on the presence and absence of various cell surface markers [10]. The fact that MSCs have the capacity to, upon stimulation, differentiate into mesoderm-derived tissue, such as adipocytes, chondrocytes, osteocytes, amongst others, makes them attractive as a cell source for tissue engineering [10, 49-57, 58]. Studies have also

shown that MSCs can be differentiated along the endothelial cell linage when given the right culture conditions, such as addition of vascular endothelial growth factor (VEGF) [59].

EPCs represent a rather heterogeneous population of cells mainly located in the bone marrow. Despite a large amount of published data concerning EPCs, the exact definition still remains inconsistent. They share several markers with both endothelial cells and hematopoietic stem cells (e.g. AC133, VEGF receptor-2), why it has been suggested that EPCs maybe can be viewed as being in the middle of the differentiation spectrum, between hematopoietic stem cells and mature endothelial cells [10]. EPCs have been demonstrated to have the potential to differentiate into mature endothelial cells [45, 60, 61]. However, issues limiting the usefulness of cells like MSCs and EPCs in cell-based therapies are the difficulty associated with their harvest and culturing [34]. They are all mainly isolated from aspirated bone marrow, which is a rather invasive procedure with a relatively low yield of cells [35]. In 1997, Asahara and co-workers opened up new opportunities for the use of these cells when they reported the presence of EPCs in human peripheral blood [45].

Adult stem cells are promising candidates as cell sources for vascular tissue engineering. However, there are several problems to solve. For instance, the lack of sufficient numbers of available cells, and the difficulties in maintaining and expanding long-term cultures in sufficient numbers for treatment of patients, needs to be overcome before these cells can be used in clinical applications.

Stem cell plasticity

A common view of adult stem cells has been that their differential potential is strictly limited to the cell lineages found within the tissue of origin. However, a number of studies published the last years have challenged this view by demonstrating the possibility for stem cells from one tissue to give rise to to several lineages that are distinct from their tissue of origin [33-36]. This is a phenomenon referred to as stem cell plasticity [58, 62, 63]. The findings on stem cell plasticity have been received by a degree of controversy and skepticism and the specific underlying molecular mechanisms behind the phenotypic shift of populations of cells have been and are still in focus of intense debates. One hypothesis relies on fusion of two different cell types to generate a new cell type [62, 64-66]. Another theory is based on the switch of profiles of gene expression, leading to trans-differentiation of the cell [58, 62, 63].

Lately, it has been suggested that stem cell plasticity also can be found in peripheral autologous cells [35, 67]. For example, a number of studies have reported that cells with abilities to change their phenotype have been found within the connective stroma of several tissues [68-72]. This indicates that a plausible alternative source of cells for tissue engineering may be found in human skin. Dermal fibroblasts are found in large numbers in skin. These cells can be harvested with minimal invasion, with a high yield of cells, and they can be easily cultured *in vitro* by using well-known standard laboratory protocols. These are features that make them interesting as a cell source for vascular tissue engineering. Additionally, recent studies have shown plasticity inherent in human dermal fibroblasts, indicating a possibility to differentiate fibroblasts towards several mesenchymal lineages, such as bone, cartilage, and fat [68, 69, 73, 74].

Biomaterials

Tissue engineering techniques often includes the use of some kind of biomaterial (scaffold) to achieve the desired three-dimensional structure of growing tissues [4, 75-77]. A scaffold is a material upon which cells can attach, proliferate and differentiate. The scaffold guides the cell growth and offer the ability to form tissues *in vitro* and *in vivo* relatively rapid in a shape and with properties similar to the native tissue [77, 78]. The ideal scaffold for tissue engineering should be biocompatible, compliant, biodegradable into non-toxic products, easy to process and handle in clinical applications [79]. Additionally, the scaffold should have a structure (e.g. porosity, pore size, mechanical stability and degradation time) that promote attachment, migration, and proliferation of cells [4]. A plethora of biomaterials have been described in the literature [79-89]. In general, they can be divided into three classes: naturally derived materials, acellular tissue matrices, or synthetic materials.

Synthetic materials

Synthetic materials are for example different kinds of synthetic polymers (e.g. polyglycolic acid, polylactic acid, and poly(lactic-co-glycolic acid)).These type of materials have been widely used as scaffolds for cell transplantation and tissue engineering [90, 91]. Synthetic polymers are advantageous in that their chemistry and material properties can be well controlled, leading to a high degree of reproducibility. Another advantage is the possibility to store synthetic biomaterials off-the-shelf for a long period of time. The major disadvantages of current synthetic scaffolds lies in the fact that they tend to elicit a foreign material type of response in the host and their inability to grow, remodel, or repair *in vivo* [92].

Acellular tissue matrices

Acellular tissue matrices are often prepared by removing cellular components from native tissues. All kind of tissues have been used such as arterial wall [91, 93], small intestine submucosa [94], acellular dermis [95], or ureter [7]. The acellular matrices have the advantage of being recognized by the body and will therefore slowly degrade after implantation. The matrices are replaced and remodelled by extracellular proteins synthesized and secreted by the transplanted cells or in growing cells from the host [39]. Countering these advantages is the fact that acellular tissues suffer batch-to-batch variations and scale-up difficulties [3]. In addition, they can give rise to immunological reactions, which derives from their similarity to naturally occurring molecules [91].

Natural materials

Natural materials used in this context are usually composed of extracellular matrix-components (e.g. collagen [96], gelatin [81, 85], fibrin, glycosaminoglycans) or natural polymers (e.g. cellulose, starch). The natural materials offer the advantage of being very similar to macromolecular substances naturally occurring in the body. They contain information, for example particular amino acid sequences, that facilitate for example cell attachment. Another positive aspect of natural materials is that they are degraded by naturally occurring enzymes after implantation [91]. The negative aspects with using natural materials are similar to the disadvantages encountered when using acellular tissue matrices.

Stimulating signals

Engineering of living, physiological, and three-dimensional tissues can be done both *in vitro* and *in vivo*. Common for both these methods are the need of appropriate signals to direct the tissue formation. The *in vivo* environment provides the cells with a complex and carefully regulated set of biochemical and

mechanical signals that regulate the cell function. These signals can come from autocrine, paracrine, or endocrine pathways, or from contact with the extracellular matrix (ECM) [97]. The *in vitro* environment used to culture cells in is generally a simplified version of the *in vivo* situation, mostly aiming at enhancing the viability and growth of cells. In an attempt to create tissues *in vitro* that are more similar to native tissues, much effort has been made to better mimic the *in vivo* environment. This includes biochemical factors (e.g. growth factors, cytokines, and hormones), mechanical components (e.g. fluid flow, shear stress, puls rate etc) as well as the ECM surrounding the cells.

Mechanical signals

One strategy to provide a growing tissue with the appropriate environment is the use of a bioreactor system. This is a technique that often improve the quality of engineered tissues [98]. In general, a bioreactor system is supposed to maintain uniformity of seeding and supply growing tissues with an appropriate physiological environment [97, 98]. Bioreactors designed to culture blood vessels should be able to provide the tissue with physiologically relevant tension, shear stress, and pulsatile flow of culture medium to improve the structure and mechanical properties of the vessel [99-101].

Biochemical factors

The term biochemical factors include growth factors, cytokines, and hormones. These are proteins with key roles in a variety of cellular processes. Growth factors act as signalling molecules between cells, giving rise to a complex combination of secondary signals, transcription factors and posttranscriptional modifications leading to proliferation, differentiation, or migration of cells [97, 102]. A number of growth factors have important roles in vascular development, and angiogenesis, or both. Among these proangiogenic molecules are VEGF, basic

fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor β (TGF- β) [103-106].

Vascular endothelial growth factor

VEGF was first identified by Ferrara in the late 1980:s and since then the VEGF family and its receptors have been studied extensively due to their prominent role in the development, maintenance, and remodeling of the vasculature [104, 105, 107]. The VEGF family consists of VEGF-A (normally referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor. The angiogenic effects of the VEGF family are thought to be mainly mediated through VEGF. The binding of VEGF to its receptor (VEGFR-2) on the surface of endothelial cells activates intracellular tyrosine kinases, triggering multiple downstream signals promoting survival in endothelial cells, leading to the induction of proliferation, migration, and tube formation [105].

Transforming growth factor-β

TGF-β is a family of structurally homologous dimeric proteins. In humans, various cell types can synthesize TGF-β and essentially all cells have high-affinity receptors on their surfaces [108, 109]. TGF-β exists in three subtypes: TGF-β1, TGF-β2, and TGF-β3. TGF-β is a multifunctional protein that acts both through autocrine and paracrine mechanisms [108]. It was first discovered for its role in stimulating cell proliferation, but has later been shown to also be a strong inhibitor of cell proliferation in several cell cultures [108, 109]. Furthermore, TGF-β has been shown to regulate migration, survival, ECM-synthesis, and differentiation in several cell types, including endothelial cells [10, 108, 110-116]. Again, both stimulatory and inhibitory results have been found. Studies have shown that TGF-β signalling is essential for vascular development and maturation, *in vivo* [116, 117]. Although numerous studies have investigated the mechanisms

through which TGF-β control angiogenesis, its interaction at cell level are not entirely understood.

Platelet-derived growth factor

The PDGF-family consists of four different PDGFs (A-D) establishing functional homodimers (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD) or heterodimers (PDGF-AB) [118]. PDGF was initially purified from the α-granules of platelets, but has later been shown to be secreted by a number of cell types found in the vasculature [119]. PDGF tends to have the most potent effects on cells of mesenchymal origin, but it has also been shown to have angiogenic effects. However, the effects are thought to be weaker than that of FGF or VEGF [119]. PDGF is not believed to be crucial for the initial formation of blood vessels, but studies have shown effects of PDGF on vascular cells *in vivo* and *in vitro* suggesting a role in angiogenesis [119]. Moreover, PDGF is known to play a key role in recruiting pericytes to form walls of newly formed blood vessels [107].

Basic fibroblast growth factor

In normal tissue, bFGF is present in basement membranes and in the subendothelial extracellular matrix of blood vessels. It is a potent angiogenic inducer, both by mediating the formation of new blood vessels and promoting differentiation of endothelial cells [103, 121]. It has been hypothesized that, during both wound healing of normal tissue and tumor development, the action of heparan sulfate-degrading enzymes activates bFGF leading to angiogenesis [103, 120, 121].

Therapeutic applications for vascular tissue engineering

Blood vessels form a branched system of arteries, veins and capillaries. They have three distinct structural features, which are most prominent in arteries: intima, media, and adventitia (Fig. 2). The intima forms the layer closest to the blood flow and consists of a monolayer of endothelial cells. The media contains smooth muscle cells and fibres of elastic tissue, and the adventitia is a collagenous ECM containing fibroblasts, blood vessels, and nerves. Veins, on the other hand, are thin-walled vessels which lack the distinct molecular and tissue organization of arteries.

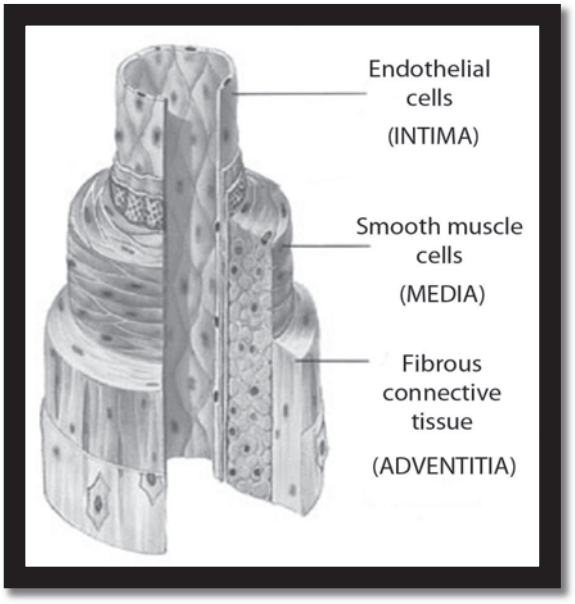


Fig. 2. *An artery have three distinct layers: the intima, which consists of a monolayer of endothelial cells; the medial layer containing smooth muscle cell; and the adventitial layer which is composed of loose fibrous connective tissue (modified from ref. [122]).*

Endothelialization of vascular grafts

About thirty years ago vascular surgeons pioneered the field of vascular tissue engineering by attempting to create endothelialized synthetic bypass grafts [123, 124]. The underlying reason was the poor results obtained when using synthetic vascular prostheses. In 1984, Herring first reported that the combination of biologically active endothelial cells and prosthetic material enhanced the survival of the grafts [125]. Since then this subject has been intensively investigated [8, 21, 86, 88, 126-131]. Endothelialization can be achieved either prior to implantation, or by accelerating the graft endothelialization *in vivo* [132]. For the *in vitro* endothelialization, both a "one-stage" procedure (adding endothelial cells to the graft at the time of implantation) and a "two-stage" procedure (harvested, followed by seeding and culturing of the cells on the graft prior to implantation) have been investigated. Due to difficulties with cell adhesion and retention, the latter approach has proven to be the more successful [127, 128, 130, 133]. Various approaches to promote endothelialization of graft surfaces *in vivo* have been explored, with particular emphasis on the incorporation of cell ligands (peptides/proteins) on the graft surface to promote cell adhesion and direct differentiation. Cells like circulating endothelial cells or EPCs could potentially be recruited onto a modified graft [132].

Tissue engineered blood vessels

In this field one usually distinguish between large-diameter vascular grafts (internal diameter > 5 mm) and small-diameter vascular grafts (internal diameter < 5 mm) [3, 76]. Large-diameter vessels have with some degree of success been replaced with synthetic prosthesis, such as Dacron® or expanded poly tetrafluoroethlene [3, 76]. Unfortunately, these synthetic biomaterials have not been successful in replacing small-diameter vessels.

The requirement for tissue engineered blood vessels with a non-thrombogenic surface, sufficient mechanical strength, and compatibility with the host is a major challenge for vascular tissue engineering. In an attempt to solve this problem a number of strategies have been presented during the past decades. In 1986, Weinberg and Bell produced the first three-dimensional vascular graft using a collagen-based construct model (Fig. 3A) [89]. Their vessel consisted of animal collagen gel layers containing bovine endothelial cells, smooth muscle cells, and fibroblasts. The collagen-based approach offers the advantages of a relatively fast *in vitro* development and simple handling, but it is limited in the mechanical strength required for *in vivo* conditions [89, 90]. In 1998, Auger and co-workers described a method whereby a complete blood vessel was generated *in vitro* without the use of a scaffold support (Fig. 3B) [134]. Viable sheets of cells and ECM were rolled over a mandrel to get a tubular configuration and were then cultured until fully matured [134-136]. This is unique since human cells make the construct completely from secreted proteins. This method, unlike the collagen-based, which to date has used animal proteins, eliminates immunological responses if autologous cells are used. A disadvantage with the cell self-assembly method is the long and complicated manufacturing process [90].

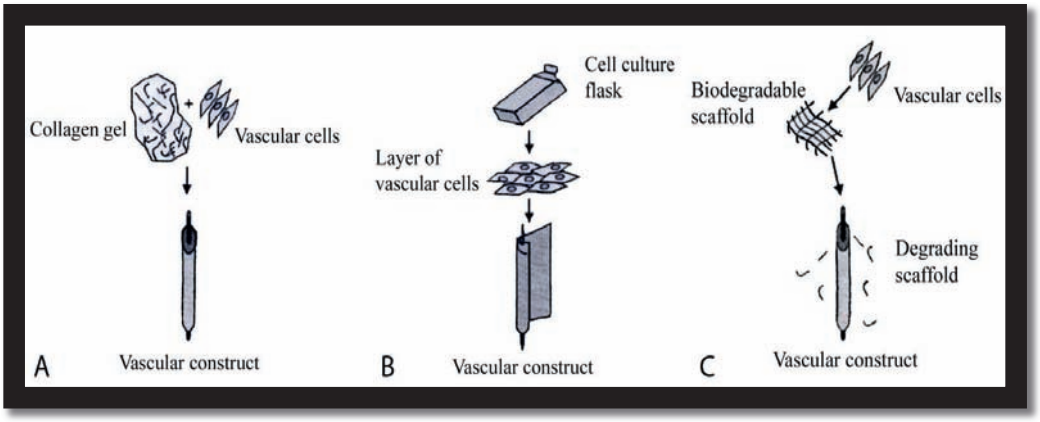


Fig. 3. *Various techniques to create a tissue engineered blood vessel: the collagen-based construct model (A), rolled sheet scaffold vascular grafts (B), and cell-seeded polymeric scaffold (C) (modified from ref. [20]).*

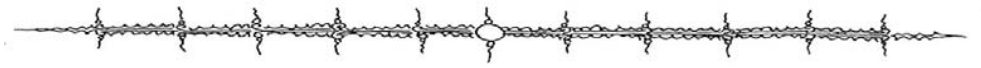
In 1999 a cell-seeded polymeric scaffold model was presented by Niklason *et al.* (Fig. 3C) [101]. In this model a biodegradable poly(glycoloc acid) scaffold and cultured vascular cells were used. The basic idea with a degradable synthetic scaffold is that when the scaffold degrades the cell population proliferates and produces its own ECM, so over the days and weeks in culture, the construct progressively transforms from a synthetic to a biological tissue analogue [101, 137]. The synthetic polymer approach has proven successful in animal models, but a limitation with this model is the long and very complicated *in vitro* development process, which also raises the manufacturing cost [90]. Despite the fact that novel approaches for producing functional small-diameter vascular grafts have been developed, there is still no long-term successful application available.

Vascularization of tissue engineered constructs

A main limitation for clinical use of tissue engineered constructs is the lack of a functional vascular network [9]. Thin tissue-constructs (100-200 μm) can survive by diffusion, while revascularization takes place[138]. However, larger complex tissues require vascularization short after implantation to satisfy the needs of nutrients and oxygen in order to survive. Currently, several strategies for engineering vascularized tissues are under investigation. These include the use of angiogenic factors, scaffold design, *in vivo* and *in vitro* prevascularization [9, 139-142]. The architecture of the scaffold, for example pore size and interconnectivity of the pores, has been demonstrated to have impact upon vascularization rate [140, 141, 143]. It is also well established that growth factors promote capillary in-growth. One approach for enhancing vascularization of tissue engineered con-structs is the delivery of angiogenic factors, such as VEGF, PDGF, TGF-β, and bFGF [144].These factors can either be added as recombinant proteins, released from biomaterials, or be secreted by cells, genetically engineered to over-express specific factors [39]. The strategies using scaffold design or angiogenic factor delivery both rely on ingrowth of blood vessels from surrounding host tissues [9]. Although blood vessel ingrowth often is noted in implanted tissues, vascularization takes too long and is too incomplete to supply the implanted tissue with enough nutrients and oxygen. The knowledge that endothelial cells cultured under right conditions and environment, start to form capillary-like structures, has encouraged researchers to investigate the possibility to introduce vascular networks inside engineered tissues already *in vitro* [9, 138, 145]. The advan-tage of this method is that the pre-vascular network formed *in vitro* connects to the host vascular system only days after implantation [143]. However, issues con-cerning culture conditions and cell source still need to be solved. A third approach for increasing vascularization is by *in vivo* prevascularization. This method is performed in two steps. The first step involves implantation of a tissue engineered construct into a region with an artery that can be led in or

around the construct. The next step is harvest of the now vascularized construct followed by re-implantation at its right position. The advantage of this technique is that the construct will be perfused immediately after implantation. However, the main disadvantage is the requirement of two operation procedures [9, 142].

AIMS OF THE STUDY



The overall aim of this licentiate thesis was to investigate if human dermal fibroblasts can be used as a cell source for vascular tissue engineering. The specific aims were:

Paper I.

* To investigate if human dermal fibroblasts can be differentiated into an endothelial cell-like phenotype.

* To investigate which factor, or factors, that are important for a possible phenotypic shift in human dermal fibroblasts.

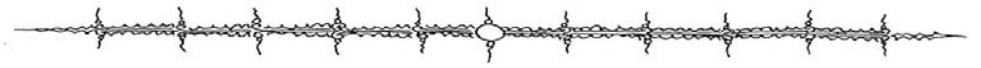
* To clarify whether cell fusion or a change in gene expression is responsible for a possible phenotypic shift in fibroblasts towards an endothelial cell-like phenotype.

Paper II.

* To elucidate if human dermal fibroblasts differentiated towards an endothelial cell-like phenotype can be used to create an endothelialized surface *in vitro*.

* To investigate the possibility to induce differentiation of human dermal fibroblasts towards an endothelial cell-like phenotype after seeded on a scaffold.

COMMENTS ON MATERIAL AND METHODS



A more detailed description of the materials and methods used in these studies can be found in the materials and methods part of each paper.

Isolation and culture of cells

There are several techniques available for the establishment of primary cultures of cells for example: explantation, mechanical disaggregation, and enzyme digestion, or a combination of these methods. One of the earliest strategies for isolation of cells is explantation of tissue fragments. Since this method rely on cells migrating from tissue pieces it normally takes longer time to establish a primary culture compared with the other methods. The use of explant or mechanical disaggregation is not effective with all tissues. Some cell types are too sensitive to forces and damage that may occur. If this is the case enzymatic treatment is a better option. However, the enzymatic treatment can have some damaging effects on certain surface molecules.

Endothelial cells

Endothelial cells can be isolated from different types of blood vessels (e.g. umbilical vein or artery, saphenous vein, pulmonary vein or artery, or micro-vessels from for example adipose tissue) [146] and from various species. However, human endothelial cells are usually preferred in vascular tissue engineering. There are various ways to isolate endothelial cells. Mechanical scraping and physical detachment is a method suitable for isolation of cells from both micro- and macrovessels. The advantages with this method are high purity and a low damage to the cells, whereas a low yield is a disadvantage. Mincing combined with enzymatic digestion is a technique often used when isolating cells from microvessels. With this technique endothelial cells are sorted out either by a filter mesh or by a cell sorter. However, the risk of having contaminant cell types is rather high when using this method [146]. In this licentiate thesis (paper I and II) endothelial cells were obtained from umbilical cord veins by enzymatic digestion, which is the preferred technique with larger vessels. Incubation with collagenase to remove endothelial cells from the basal lamina, results in a good yield of cells with a high purity.

Dermal fibroblasts

Dermal fibroblasts are often isolated either from explants, or by a combination of mechanical and enzymatic treatment of a skin biopsy. In this study (paper I and II) human dermal fibroblasts were obtained from tissue specimens from healthy patients undergoing routine plastic surgery. Primary cultures were isolated by a combination of mechanical disaggregation and enzymatic digestion (collagenase and dispase).

Single-cell clone fibroblasts

Single-cell clone fibroblasts were used in experiments in paper I and II. Primary cultures of human dermal fibroblasts were enzymatically detached and single cells were transferred from this cell suspension using an inverted microscope equipped with a micromanipulator and micropipette. These cells were transferred to separate wells and cultured there until they had reached confluence. They were then further expanded until adequate numbers were achieved for experiments to start.

Induction of differentiation

Various methods to induce cell differentiation *in vitro* have been described in the literature. One approach is to expose cells to mechanical stimuli, such as shear forces or flow [99-101]. However, one of the most commonly used techniques is by culturing cell in an induction media. Introducing cells to the right environment is thought to supply the proper conditions for differentiation. In this study normal dermal fibroblasts were cultured in a fibroblast growth medium (FGM) based on Dulbecco's modified eagles medium, supplemented with 10 % fetal calf serum (FCS), penicillin, and streptomycin (paper I and II). However, to induce a phenotypical change in dermal fibroblasts towards an endothelial cell-like phenotype, cells were cultured in Dulbecco's modified eagles medium supplemented with 30 % human serum, isobutyl-methylxanthine (IBMX), cholera toxin, penicillin, and streptomycin (paper I and II). This is an endothelial growth medium (EGM) in which mature endothelial cells maintain their differentiated state. By utilizing *in vitro* cell culture models, the capability of different types of serum (FCS and human serum) and medium constituents (cholera toxin and IBMX) in inducing a phenotypic change in fibroblasts was investigated (paper I).

As indicated above, the medium used to differentiate dermal fibroblasts towards an endothelial cell-like phenotype was supplemented with IBMX and cholera toxin. Both IBMX and cholera toxin are agents that influence the cyclic nucleotide intracellular signaling by blocking the degradation of cyclic adenosine monophosphate (cAMP) and increasing Gs-protein-coupled signaling, respectively. cAMP also activates the cAMP-response element-binding protein that participates in gene transcription and cell differentiation [147]. To investigate whether IBMX or cholera toxin were responsible for the phenotypic shift in fibroblasts, cells were cultured in EGM without IBMX or cholera toxin, or both. Fibroblasts were also cultured in FGM supplemented with IBMX or cholera toxin, or both.

Human serum is composed of water, proteins (e.g. albumins, globulins etc.), electrolytes, nutrients, enzymes, gases, waste products (e.g. urea, ammonia etc.), and hormones. In addition, it contains numerous growth factors. Both VEGF and TGF-β play important parts in differentiation of endothelial cells and angiogenesis [45, 116, 117]. To investigate whether VEGF, or TGF-β1, or both could induce a phenotypic shift in fibroblasts, cells were cultured in EGM supplemented with neutralizing antibodies directed towards these two growth factors. Blocking of VEGF with neutralizing antibodies has earlier been shown to substantially suppress tumor growth and angiogenesis [148]. Fibroblasts were also cultured in FGM supplemented with VEGF and TGF-β1.

Seeding techniques

There are several methods used for seeding of cells on scaffolds. For example, spinner flasks are a commonly used system. This is a simple method to use, which results in a rather uniform distribution of cells. Another approach is to use rotating vessels, where a uniform cell suspension, cell culture medium, and

scaffolds are transferred into a vessel with an adjustable rotation speed [99]. However, one of the most simple and most commonly used methods is static seeding. This method was used in paper II. Herein, a high concentration of cells in suspension was dropped onto pre-wetted scaffolds. When cells had adsorbed to the surface of the scaffold cell culture medium was slowly added. Scaffolds were seeded with: 1) endothelial cells; 2) fibroblasts or single-cell clone fibroblasts cultured in FGM (both before and after seeding); 3) fibroblasts differentiated towards an endothelial cell-like phenotype cultured in EGM (both before and after seeding); and 4) fibroblasts and single-cell clone fibroblasts cultured in FGM (before seeding). Differentiation of these cells was induced 24 hours after seeding by changing the growth medium to EGM. Scaffolds seeded with cells were then cultured statically for 10 days in a humidified, 37° C, 5 % CO_2 incubator.

The scaffold used in this study

In this study (paper II) cells were seeded and cultured on a biomaterial based on a highly cross-linked type-A porcine derived gelatin matrix (Fig. 4). Previous studies have demonstrated that gelatin promotes attachment and growth of various mammalian cell types, especially endothelial cells [146]. Furthermore, this matrix has earlier been used for guided tissue regeneration and cell-based therapies both *in vitro* and *in vivo* [81, 85, 149-152]. It has several attractive characteristics for tissue engineering. For example, it promotes attachment and growth of a number of mammalian cell types, it is biocompatible, biodegradable (into non-toxic substances), easily manufactured, and relatively cheap [81, 153].

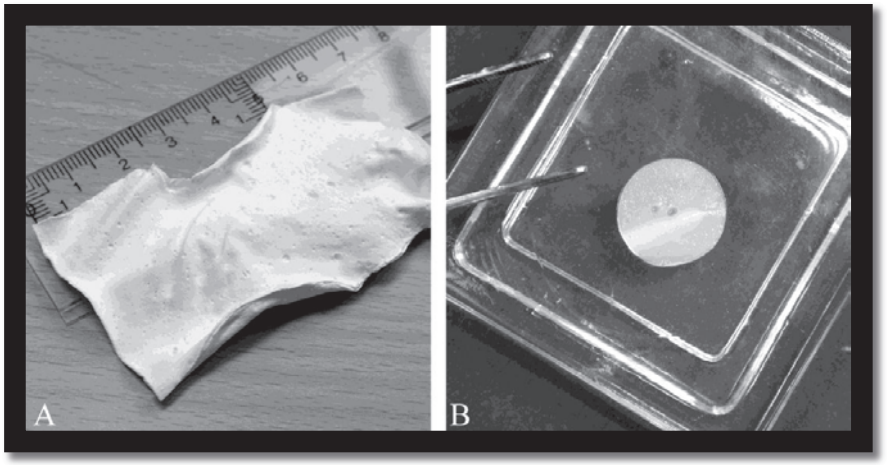


Fig. 4. *Photographs of the gelatin scaffold used in this study. In dehydrated condition (A), and hydrated in phosphate buffered saline and cut into circular pieces (B).*

Confirmation of differentiation

Several parameters are used to characterize endothelial cells in culture, for example cell morphology, cellular markers, and functional signal transduction pathways specific for endothelial cells [146]. One of the aims of this licentiate thesis was to investigate the capacity of human dermal fibroblasts to be differentiated into an endothelial cell-like phenotype (paper I). Differentiation of fibroblasts was confirmed by a combination of the criteria mentioned above: presence of the cellular markers, such as vWf (paper I and II), ve-cadherin (paper II), CD31 (paper I and II), and eNOS (paper II); incorporation of labelled acetylated low-density lipoproteins (demonstrating a functional signal transduction pathway) (paper I); and the capacity of cells to organize in capillary-like structures (cell morphology) (paper I).

Visualization techniques

Immunohistochemistry

Immunohistochemistry is a technique based on the detection of proteins in fixed cell cultures or paraffin embedded tissues with the use of specific antibodies. The antibodies can be both mono- and polyclonal, however monoclonal antibodies are generally considered exhibiting greater specificity. There are two strategies used for immunohistochemical detection of antigens in tissues, the direct method and the indirect method. The direct method of immunohistochemical staining uses one antibody, which binds directly to the antigen. On the other hand, the indirect method involves a primary antibody and a secondary antibody, which binds to the primary antibody. The fact that several secondary antibodies can react with the same primary antibody makes this method more sensitive. To visualize the position of the antibodies, these are pre-linked to an indicator substance. This may be a fluorescent substance, in which case the technique is known as immunofluorescence and the antibody are visualized in a fluorescence microscope. The antibody can also be conjugated with an enzyme that catalyses a reaction, resulting in a detectable color. A commonly used set-up is a biotinylated secondary antibody coupled with streptavidin-horseradish peroxidase.

In this study indirect immunohistochemistry and immunofluorescence were used to detect the presence of molecules commonly used to identify endothelial cells. Immunofluorescence was used when staining fresh, fixed cells since this method allows a much better localization of proteins. However, when staining paraffin-embedded cells cultured on scaffolds (paper II) we instead used a horseradish peroxidase system combined with biotinylated secondary antibodies. This system was selected due to its high sensitivity and low background staining.

Advances have been made in immunohistochemistry, particularly at the technical level, but there are still issues around interpretation and quantification, which need to be addressed. Even though immunohistochemistry is not really a quantitative technique, researchers have developed different scoring systems. These can involve counting of labeled cells to give a percentage, counting number of positive cells in 10 high-power fields, or the use of computer-assisted image analysis. Another commonly used scoring system is grading of the reactivity (weak, moderate or strong). A potential disadvantage with our studies is precisely the lack of scoring of our immunohistochemical stains. In paper I a subjective rating of the number of positively stained cells in the cells cultured for different amount of time or in different medium was used. We also looked at the presence or absence of specific staining and the localization of the staining (paper I and II). Furthermore, in all our studies we used endothelial cells as a positive control cell and fibroblasts cultured in FGM as a negative control cells and the results received when staining fibroblasts induced to differentiate towards an endothelial cell-like phenotype were compared with these (paper I and II).

Haematoxylin-eosin staining

Haematoxylin-eosin staining is the most commonly used routine staining. Haematoxylin, which is a basic dye stains acidic structures (e.g. nuclei, ribosomes) purplish blue. In contrast, eosin is an acidic dye which stains basic structures red or pink. Since most cytoplasmic proteins are basic the cytoplasm of a cell usually stains pink. Hematoxylin-eosin staining was used in paper II to evaluate the organization and migration of cells cultured on scaffolds.

4',6-diamidino-2-phenylindole staining

4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to DNA. It is used extensively in fluorescence microscopy to visualize cell nuclei. Since DAPI will pass through an intact cell membrane, it may be used to stain both live and fixed cells. All cells in this study were counterstained using DAPI both to visualize cell nuclei (paper I and II), and enable the detection of migration of cells into the pores of the scaffold (paper II).

Formation of capillary-like networks

Angiogenesis is the establishment of new blood vessels from an already existing vascular network. It is a fundamental component of a number of normal (e.g. wound healing, reproduction) and pathological processes (e.g tumour growth). Attempts to understand the molecular mechanisms that control vascular growth have led to the development of *in vitro* cell culture systems. The most successful models place endothelial cells in three-dimensional gels of native ECM-molecules. This is a relatively simple system for investigating angiogenesis in a controlled manner. Limitations of the models are the lack of other cell types and the short duration time of the assays. Since it is mostly endothelial cells that have the capability of forming capillary networks, these are models that can be used to characterize endothelial cells. In this study, the formation of capillary-like networks was utilized to confirm that the dermal fibroblasts had differentiated towards an endothelial cell-like phenotype (paper I). For this purpose we used an *in vitro* angiogenesis assay kit, which is an ECM-system optimized for maximal tube-formation. The gel used in this kit consists of various ECM-proteins (e.g. laminin, collagen type IV, heparin sulphate, and proteoglycans), several growth factors, and proteolytic enzymes.

Western blot analysis

In paper II western blot analysis was performed in order to confirm the results received from immunohistochemistry. The western blot technique combines the resolving power of electrophoresis, the specificity of antibodies, and the sensitivity of enzyme assays in order to detect a specific protein. Total protein from cells (endothelial cells, fibroblasts, and fibroblasts differentiated towards an endothelial cell-like phenotype) cultured for 10 days in cell culture flasks was loaded onto a sodium dodecyl sulfate gel. The separated proteins were then transferred onto a membrane and incubated with a primary antibody solution specific for the desired proteins (VWf and eNOS). After washing, membranes were incubated with enzyme-conjugated secondary antibodies. To visualize the proteins we used enhanced chemiluminescence, which is a method where western blot membranes are incubated with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light was detected by a charge coupled device camera. For most western blot analysis you will not only reveal proteins at one band in a membrane, but by comparing the size of the stained bands to that of a ladder will help you to find the right protein. To ensure that the same amount of protein has been loaded onto the gel you can either add a reference protein that should be equally expressed in all cell types, or you can determine the total protein content in your samples and then load the same amount of protein for all samples onto the gel. The latter method was used in this study. In this study we also added, as a positive control, a cell type known to express the desired protein.

Uptake of fluorochrome-labelled low-density lipoprotein

Uptake of low density lipoproteins (LDL) is a receptor-mediated process [154]. Acetylated LDL labelled with 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethyl-indocarbocyanine perchlorate (Ac-DiI-LDL) labels both vascular endothelial cells and macrophages. No other cell type is labelled to the same extent [154]. Cells labelled

with Ac-DiI-LDL can be visualized using a fluorescence microscope, since the lipoproteins are degraded by lysosomal enzymes and accumulated in lysosomes. An advantage with this method is that it can be used both to identify and to isolate endothelial cells from mixed cell populations without affecting the viability of the cells. Another advantage is that the labelling process is in one step, and once the cells are labelled the fluorescent dye is not removed by trypsin, which makes it possible to sort cells with a fluorescence activated cell sorter [154]. Uptake of fluorochrome-labeled LDL was used in paper I to confirm that human dermal fibroblasts have the capacity to change into an endothelial cell-like phenotype.

RESULTS AND DISCUSSION

Detailed descriptions of the results obtained in this study can be found in the results part of each paper.

Differentiation of human dermal fibroblasts towards an endothelial cell-like phenotype

In the present study the possibility to induce differentiation of human dermal fibroblasts towards an endothelial cell-like phenotype was investigated. This was achieved by culturing fibroblasts in EGM *in vitro*, a medium that is normally used to maintain the differentiated state of mature endothelial cells. A change in phenotype was confirmed by studying the presence of various markers commonly used to identify mature endothelial cells [22, 27, 146, 154]. All experiments included primary cultures of human endothelial cells, dermal fibroblasts, and single-cell-clone fibroblasts. The latter cell type was obtained by clonal expansion using a micromanipulator. Throughout the study endothelial cells displayed positive immunostaining when using antibodies directed towards vWf (paper I and II), Ve-cadherin (paper II), CD31 (paper I and II), and eNOS (paper II), whereas dermal fibroblasts and single cell clone fibroblasts did not.

Fibroblasts and single-cell clone fibroblasts cultured in EGM started to express vWf immunostaining already four days after starting the culture (paper I). However, a majority of fibroblasts and single-cell clone fibroblasts induced to differentiate for 10 days, displayed both cytoplasmic and extracellular immunoreactivity to vWf (paper I and II), and cytoplasmic localization of eNOS (paper II). Incubation with antiserum to ve-cadherin resulted in a less pronounced immunostaining (paper II), while cells incubated with antibodies directed towards CD31 did not show any immunoreactivity (paper I and II). In addition, the ability of these cells to start forming capillary-like networks and to incorporate fluorochrome-labelled LDL was studied (paper I). Indeed, our results showed that endothelial cell-like fibroblasts both incorporated fluorochrome-labelled LDL, and started to form capillary-like structures in an endothelial cell-like fashion. These results are of particular interest since they not only confirm an up-regulation of surface molecules, but also indicate the presence of functional signal transduction pathways. The endothelial differentiated fibroblasts did not express the CD31 marker, suggesting that these cells are not fully differentiated. To what extent dermal fibroblasts can change their phenotype is most likely dependent on the signals provided by the surrounding environment. Herein, an EGM was used to induce a phenotypic change of dermal fibroblasts cultured *in vitro* under static conditions. A better knowledge of the factors causing this phenomenon and a more optimal environment, resembling the *in vivo* situation, will hopefully lead to a more efficient differentiation of these cells.

Single-cell clone fibroblasts were used in this study (paper I and II) for various reasons. The main reason to use single-cell clone fibroblasts was to clarify whether cell fusion or a change in gene expression is responsible for the shift in

phenotype in fibroblasts towards an endothelial cell-like phenotype. Our results showed that it was possible to induce differentiation of single-cell clone fibroblasts in a similar manner as normal dermal fibroblasts. Based on these results we draw the conclusion that transdifferentiation may be the main mechanism associated with the altered phenotype seen in fibroblasts, and that the role of fusion between different cell types can be ruled out. However, the usage of single-cell clone fibroblasts also served another purpose. Presence of residual endothelial cells in the primary culture of fibroblasts could possibly render a false positive result in regard to differentiation capacity. This issue was addressed in various ways, firstly; endothelial cells are not favoured by FGM, secondly; endothelial cells seldom adhere to uncoated surfaces, thirdly; before any experiments were done the phenotypes of fibroblasts were confirmed by indirect immunohistochemistry, and finally; adding single-cell cloned fibroblasts to our experiments, which greatly reduced the risk of false positive results due to the possible presence of a few endothelial cells in the primary culture.

The importance of human serum for the phenotypic shift in dermal fibroblasts

To achieve a more efficient differentiation of dermal fibroblasts towards an endothelial cell-like phenotype it is of importance to elucidate which are the essential factors responsible for the differentiation. In this study we hypothesized that the type of serum added to our growth medium were of importance for induction of the phenotypic shift (paper I). Indeed, the results received in paper I showed that addition of human serum, but not FCS, play a decisive role in the differentiation of dermal fibroblasts along the endothelial linage. Human dermal fibroblasts and single-cell clone fibroblasts cultured in EGM containing 10 or 30 % human serum, displayed immunostaining after incubation with anti-vWf antibodies. However, dermal fibroblasts cultured in growth medium con-

taining FCS did not display any immunostaining to vWf. Consequently, factors presented in human serum but not in FCS are crucial for inducing a phenotypic shift in human dermal fibroblasts.

The EGM used in this study was supplemented with cholera toxin and IBMX. Both these drugs influence on the cAMP-signaling system by blocking the degradation of cAMP and by increasing the Gs-protein-coupled signaling. Furthermore, the cAMP signaling pathway has been shown to be involved in gene transcription and cell differentiation [147]. We investigated the roles of these drugs to find out if they are of importance for the alteration in phenotype in human dermal fibroblasts (paper I). However, our results showed that neither the exclusion of cholera toxin or IBMX in EGM, nor the addition these drugs to FGM, have any effect on the differentiation of fibroblasts towards an endothelial cell-like phenotype. Based on these finding we draw the conclusion that cholera toxin, IBMX, or the cAMP-signaling system has no impact on the phenotypic change of fibroblasts into endothelial cell-like cells.

Numerous growth factors are present in human serum, among them VEGF and TGF-β. Both VEGF and TGF-β play important roles in differentiation of endothelial cells and in angiogenesis. Based on this knowledge we hypothesized a possible role of these growth factors in the induction of differentiation of fibroblasts towards an endothelial cell-like phenotype (paper I). The results however, showed that the presence of neutralizing anti-VEGF or anti-TGF-β1 antibodies in the EGM did not affect the phenotypic shift. Moreover, supplementing the FGM with VEGF or TGF-β1 did not induce differentiation of fibroblasts. Consequently, we conclude that neither VEGF nor TGF-β1 is crucial for inducing a phenotypic shift in fibroblasts towards an endothelial cell-like phenotype.

Dermal fibroblasts as a cell source for endothelialization of vascular grafts

The intriguing results received in paper I encouraged us to continue to investigate the capacity of fibroblast differentiation. As an initial step in using these cells in tissue engineering their ability to form an endothelial cell-like layer on a scaffold *in vitro* was studied (paper II). Long culture times are a problem with most vascular tissue engineering techniques. In paper II scaffolds were seeded with human dermal fibroblasts which subsequently were induced to differentiate towards an endothelial cell-like phenotype. We hypothesized that this approach might shorten the culture time required for endothelialization of vascular grafts using human dermal fibroblasts. Cell were seeded on scaffolds according to the following set-up: 1) endothelial cells (Fig. 5A); 2) fibroblasts and single-cell clone fibroblasts cultured in FGM (Fig. 5B); 3) fibroblasts differentiated towards an endothelial cell-like phenotype cultured in EGM (Fig. 5C); and 4) fibroblasts and single-cell clone fibroblasts seeded on scaffolds and thereafter induced to differentiate towards an endothelial cell-like phenotype (Fig. 5D).

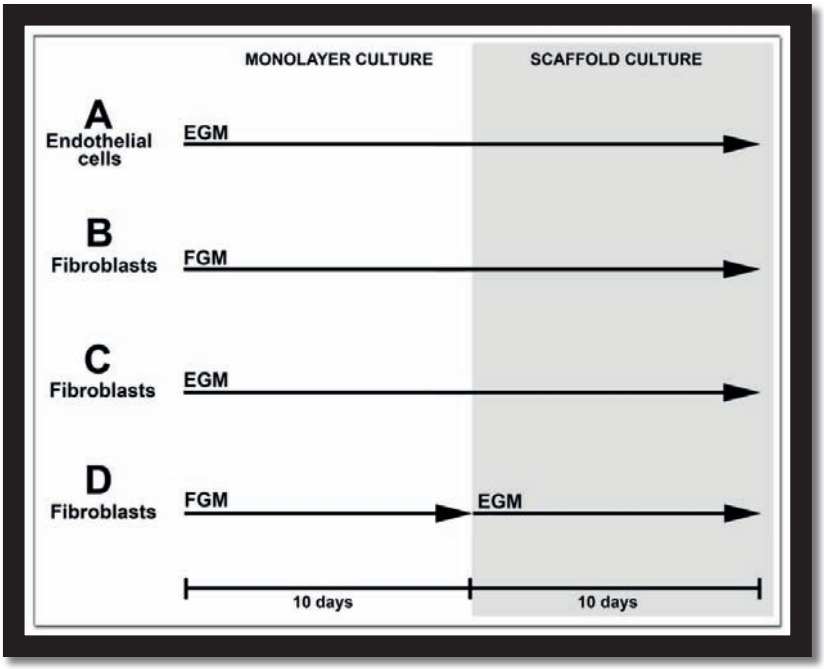


Fig. 5. *Schematic study set-up. Endothelial cells cultured in endothelial cell growth medium (EGM) for 10 days were seeded on gelatin scaffolds and cultured for another 10 days (A). Human dermal fibroblasts and single-cell clone fibroblasts cultured in fibroblast growth medium (FGM) for 10 days were seeded on gelatin scaffolds and cultured for another 10 days (B). Human dermal fibroblasts were induced to differentiate towards an endothelial cell-like phenotype by culturing the cells in EGM for 10 days prior to seeding the cells on gelatin scaffolds. The cells were cultured in EGM on the scaffold for another 10 days (C). Human dermal fibroblasts and single-cell clone fibroblast were cultured in FGM and after being seeded on to gelatin scaffolds, differentiation towards an endothelial cell-like phenotype was induced by changing the growth medium to EGM (D).*

To evaluate the organization and migration of the cells growing on scaffolds, cross-sections were stained with DAPI- and hematoxylin-eosin staining. These staining techniques revealed that the gelatin scaffold supported both migration and proliferation of all four "cell types" used in this study. All cells had the capacity to migrate into the pores of the scaffold. However, the most prominent migration was found in human dermal fibroblasts and especially the fibroblasts

that were induced to differentiate towards an endothelial cell-like phenotype after being seeded on scaffolds (Fig. 5D).

The results obtained in this study (paper II) showed that endothelial cells were organized in a confluent monolayer on the scaffold, displaying immunostaining to vWf, ve-cadherin, CD31, eNOS, and the B_2 braykinin receptor. On the other hand, fibroblasts and single-cell clone fibroblasts cultured on scaffolds grew in continuous cell layers, up to three cell layers thick. These cells did not display any immunostaining when using antibodies to vWf, ve-cadherin, CD31, or eNOS. However, the cells displayed a weak staining after incubation with antiserum to the B_2 bradykinin receptor. Human dermal fibroblasts induced to change phenotype towards an endothelial cell-like phenotype formed a confluent monolayer when cultured on gelatin scaffolds for 10 days. Moreover, these cells displayed immunostaining when using antiserum to vWf, ve-cadherin, eNOS, and the B_2 receptor. Both the organization of the cells and the staining pattern were similar to that seen in endothelial cells. These results further strengthen the hypothesis that human dermal fibroblasts can be differentiated towards an endothelial cell-like phenotype.

It is well-known that an anti-thrombogenic endothelial cell-layer is important for a functional vascular graft. Binding of bradykinin to its G-protein-coupled bradykinin B_2 receptor, results in a Ca^{2+}-dependent activation of the enzyme eNOS leading to production of NO. There is solid evidence that the production of NO is associated with anti-platelet activity and vasodilatation. The presence of the B_2 receptor and eNOS in endothelial differentiated fibroblasts is thus crucial for a production of NO, which is essential to maintain an anti-thrombotic and vascular regulatory state of these cells [30, 31]. By using western blotting and indirect immunohistochemistry with antibodies directed towards the brady-

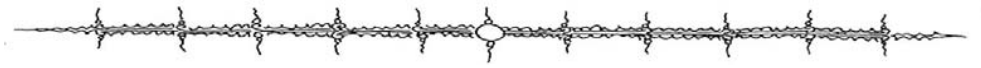
kinin B_2 receptor and eNOS, we investigated the possibilities of NO-activity in the endothelial cell-like cells. As previously mentioned, our results revealed that fibroblasts differentiated towards an endothelial cell-like phenotype (before seeding) showed immunoreactivity to both these markers. These results indicate that fibroblasts differentiated towards endothelial cell-like cells can serve as a functional cell layer, and consequently be useful as a cell source in vascular tissue engineering. However, further studies investigating the non-thrombogenic properties of the cell layer are required before these cells can be used in an *in vivo* situation, and above all before they can be used in clinical applications.

One problem with current vascular tissue engineering methods is the long culture time required. In an attempt to shorten the culture time, we investigated the possibility to seed scaffolds with human dermal fibroblasts and later commencing differentiation towards an endothelial cell-like phenotype. Our results revealed that these cells displayed vWf, ve-cadherin, eNOS, and B_2 after being cultured on a scaffold in EGM for 10 days. However, the growth pattern showed more similarities to normal dermal fibroblasts, with cells growing in multilayer, than the monolayer found in endothelial cells. As mentioned earlier, more knowledge of the precise molecular mechanisms leading to a phenotypic shift in dermal fibroblasts towards an endothelial cell-like cell type will hopingly lead to a more efficient differentiation. Worth to emphasize is that single-cell clone fibroblasts formed a monolayer similar to that seen in the endothelial cells, when cultured under the same conditions. Moreover, these cells displayed a high degree of immunostaining towards ve-cadherin.

To our knowledge this is the first study using human dermal fibroblasts in a vascular tissue engineering application. In this study a rather uncomplicated culture method where cells were cultured on flat scaffolds under static

conditions was used. Though, with the promising results obtained in the present study the work will be continued and we will investigate the possibility to seed cells on cylindrical scaffolds. Moreover, the importance of mechanical forces on cells of the vascular system have long been recognized [155, 156]. Exposing the cells to mechanical stimulation mimicking the forces of blood flow, combined with an optimized induction medium may enhance their capacity to differentiate. Hopefully this will also lead to shorter culture times. The restricted availability of human autologous cells is a great limitation in vascular tissue engineering. Thus, the results presented in this study may have an important impact cell sourcing in vascular tissue engineering. Being able to obtain cells through a simple skin biopsy would dramatically facilitate the use of autologous vascular tissue engineering methods, not only endothelialization of grafts, but also engineering of complete blood vessels and vascularization of engineered constructs.

CONCLUSIONS



With support of the results in paper I and II, we draw the following conclusions:

* Human dermal fibroblasts can alter their phenotype towards an endothelial cell-like phenotype.

* The presence of human serum is essential to induce a phenotypic shift of fibroblasts towards an endothelial cell-like phenotype *in vitro*.

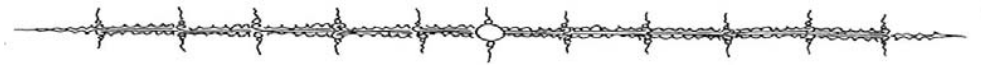
* Neither VEGF nor TGF-β1, at least not alone, is responsible for the induction of the differentiation of fibroblasts towards an endothelial cell-like phenotype.

* A shift in gene expression (transdifferentiation) and not cell-cell fusion is the underlying mechanism of the phenotypic change.

* Human dermal fibroblasts can be used to endothelialize a surface *in vitro*. Most resemblance to mature endothelial cells was obtained when using fibroblasts differentiated towards an endothelial cell-like phenotype before seeded on scaffolds.

* Human dermal fibroblasts differentiated towards an endothelial cell-like phenotype (both before and after seeding) expressed molecules essential for vessel dilatation and anti-thrombotic activity, when cultured on a gelatin scaffold *in vitro*.

FUTURE ASPECTS



The results at hand today indicate that human dermal fibroblasts are a potential cell source for vascular tissue engineering. Stem cell plasticity among autologous cells is a discussed phenomenon often met by some scepticism. Therefore, data similar to results presented in this study require rigid proofs for others to believe in its accuracy. Herein, western blot analysis and immunohistochemistry was used to confirm the presence of various molecules at a protein level. To verify these data a next step would be to study the gene expression of these molecules. Techniques, such as real-time polymerase chain reaction, would bring valuable information to the study. Moreover, the use of a quantitative technique would make it easier to visualize differences in regard to differentiation capacity.

With more knowledge of the induction factors that lead to endothelial differentiation of fibroblasts, the potential of our findings may have an impact on several vascular tissue engineering techniques and the possibility of using these methods clinically. Our studies demonstrated that human serum is necessary for this shift to occur. Human serum contains numerous factors and trying to

find one specific factor among these is like looking for a needle in a haystack. Many of the factors found in human serum also exist stored in platelets [157]. Thus, one method to reduce the amount of possible factors is to use different preparations of platelets. In an attempt to clarify the precise mechanisms that contribute to the differentiation of fibroblasts we added fractions of either activated platelets, non-activated platelets, or from platelet membranes, instead of human serum to the growth medium. However, further studies will be required to find out the precise combination of bioactive molecules that induce the phenotypic change observed in the present investigations.

With the results received in the present study we know that it is possible to induce human dermal fibroblast to express endothelial-specific molecules. However, to assess the clinical utility of these cells it is necessary to demonstrate that these cells also have the ability to function as endothelial cells. As mentioned previously, both the formation of capillary-like networks and the uptake of fluorochrome-labelled LDL are examples of assays demonstrating a function normally found in mature endothelial cell. Since our results have shown the presence of eNOS in endothelial differentiated fibroblasts, a natural continuation would be to study the activity of this enzyme in these cells. As mentioned previously, eNOS is responsible for the production of NO, and NO plays an important role in a variety of biological processes. For example, it has effects on vessel homeostasis by inhibiting vascular smooth muscle contraction, platelet aggregation and adhesion, and leukocyte adhesion to the endothelium [30, 31]. These are functions of great importance for the creation of a functional and anti-thrombotic vascular graft. Techniques commonly used to study the effects of NO are for example organ chamber methodology or aggregometry. We performed a pilot study investigating the capacity of endothelial differentiated fibroblasts to produce NO, and thereby inhibit platelet aggregation. Endothelial

cells, fibroblasts and endothelial cell-like fibroblasts were cultured on gelatin scaffolds. Subsequently, scaffolds were placed in platelet rich plasma and cells were stimulated to produce NO. Data from this early study indicates that human dermal fibroblasts differentiated towards an endothelial cell-like phenotype are capable of producing enough NO to decrease the aggregation rate of activated platelets compared to controls, upon stimulation with bradykinin.

The possibility of fibroblasts giving rise to all three cell types required for the generation of a complete blood vessel is an appealing thought. The promising results from this study combined with results from previous studies reporting a possibility to differentiate fibroblasts into smooth muscle cells, this might be achievable [74, 158, 159]. This would greatly facilitate the *in vitro* production of autologous vascular grafts and lead to improved possibilities of replacing damaged blood vessels with autologous tissue.

ACKNOWLEDGEMENT

Det är många som jag vill rikta mitt allra varmaste tack till. Människor som på olika sätt har bidragit till att det har varit möjligt för mig att genomföra det här projektet. Utan er hade jag inte stått här idag. Särskilt vill jag tacka:

Gunnar Kratz, för att du gjort det möjligt för mig att få delta i det här forskningsprojektet, för ditt engagemang och för din humor. Du har en unik förmåga att se det positiva i allt och att kunna motivera en till i princip vad som helst.

Magnus Grenegård, för att du har delat med dig utav din kunskap, dina tips och inte minst din dyrbara tid. Tack även för alla livsråd du har delat med dig av, vare sig man har bett om dem eller inte. Jag kommer alltid i fortsättningen akta mig noga för tråkiga människor!

Camilla Fredriksson och **Sofia Pettersson**, ni är bra fina ni! Tack för allt vi har delat under den här tiden. Vi har varandra i nöd och lust!

Kristina Briheim och **Anita Lönn**, utan er stannar plastiklabbet. Ni har gjort det till en sann fröjd att komma till jobbet.

Resten av gänget på ”plastiklabbet”, **Fredrik Huss, Pehr Sommar, Robert Cimermancic, Jonathan Rakar**, tack för att ni har bidragit till att göra plastiklabbet till den trevligaste forskargruppen av dem alla.

Personalen på KEF, **Pia**, **Håkan**, **Kerstin**, tack för att ni alltid hjälper till med allt mellan himmel och jord!

Alla ni härliga personer som gör att man vill stanna lite längre i fikarummet trots den outhärdliga ljudnivån: **Micke Pihl**, **Stina Axelsson**, **Micke Cheramy, Anders Carlsson, Anna Rydén** och **Anna Kivling**.

Ulrika Nilsson, min allra bästa Ulrika. Med dig känns allt så okomplicerat och det är mycket tack dig som jag har tagit mig igenom detta. Du är den absolut bästa vän man kan önska sig!

Johan Junker, du är och kommer alltid att vara min allra bästa Johan. Tusen tack för allt! (…**men** vad du än säger så heter det faktiskt ullmeRkott).

Femke Lutgendorf, Henrik Blomqvist, Henrik Åberg, Karin Skoglund, Karolin Eriksson, Patiyan Andersson, Pernilla Eliasson, Saad Salim, Sebastian Schultz, utan vänner som ni vore inte livet särskilt mycket att ha!

Åsa Elmsäter, Hanna Theis, ni är ovärderliga. Det är tack vare er som jag har orkat kämpa på trots att det har blåst motvind emellanåt.

Mamma, pappa och **Olle** för att ni är bland de viktigaste som finns. Tack för att ni alltid har litat på mig och låtit mig hitta min egen väg. Med humor och kärlek har ni stöttat mig, mer än ni förstår, trots att ni knappt vet vad det är jag gör när jag säger att jag jobbar.

Petter, mitt allra finaste. Tack för allt stöd och all hjälp. Äntligen står vi på tröskeln till vårt fortsatta liv tillsammans. Vi har stora saker på gång och jag ser fram emot att få dela dem med dig!

/Lisa Karlsson,

Linköping 2009

REFERENCES

1. Olausson M. Svensk Transplantationsförening. In: Larsson M, editor.
2. Bhishagratna K. An English translation of the Sushruta Samhita based on original Sanskrit text. 1996.
3. Langer R, Vacanti JP. Tissue engineering. *Science* 1993;**260**(5110): 920-926.
4. Rabkin E, Schoen FJ. Cardiovascular tissue engineering. *Cardiovasc Pathol* 2002;**11**(6): 305-317.
5. Kratz G. Body parts from the laboratory bench. *Br J Surg* 2005;**92**(4): 385-386.
6. Hoenig MR, Campbell GR, Rolfe BE, Campbell JH. Tissue-engineered blood vessels: alternative to autologous grafts? *Arteriosclerosis, thrombosis, and vascular biology* 2005;**25**(6): 1128-1134.
7. Kakisis JD, Liapis CD, Breuer C, Sumpio BE. Artificial blood vessel: the Holy Grail of peripheral vascular surgery. *J Vasc Surg* 2005;**41**(2): 349-354.
8. Kannan RY, Salacinski HJ, Butler PE, Hamilton G, Seifalian AM. Current status of prosthetic bypass grafts: a review. *Journal of biomedical materials research* 2005;**74**(1): 570-581.
9. Rouwkema J, Rivron NC, van Blitterswijk CA. Vascularization in tissue engineering. *Trends Biotechnol* 2008;**26**(8): 434-441.
10. Riha GM, Lin PH, Lumsden AB, Yao Q, Chen C. Review: application of stem cells for vascular tissue engineering. *Tissue Eng* 2005;**11**(9-10): 1535-1552.
11. Vats A, Bielby RC, Tolley NS, Nerem R, Polak JM. Stem cells. *Lancet* 2005;**366**(9485): 592-602.
12. Conrad C, Huss R. Adult stem cell lines in regenerative medicine and reconstructive surgery. *J Surg Res* 2005;**124**(2): 201-208.
13. Guillot PV, Cui W, Fisk NM, Polak DJ. Stem cell differentiation and expansion for clinical applications of tissue engineering. *Journal of cellular and molecular medicine* 2007;**11**(5): 935-944.
14. Hipp J, Atala A. Sources of stem cells for regenerative medicine. *Stem cell reviews* 2008;**4**(1): 3-11.
15. Kim S, von Recum H. Endothelial stem cells and precursors for tissue engineering: cell source, differentiation, selection, and application. *Tissue engineering* 2008;**14**(1): 133-147.
16. Wagner DD, Frenette PS. The vessel wall and its interactions. *Blood* 2008;**111**(11): 5271-5281.

17. Sarkar S, Sales KM, Hamilton G, Seifalian AM. Addressing thrombogenicity in vascular graft construction. *Journal of biomedical materials research* 2007;**82**(1): 100-108.
18. Michiels C. Endothelial cell functions. *Journal of cellular physiology* 2003;**196**(3): 430-443.
19. McGuigan AP, Sefton MV. The influence of biomaterials on endothelial cell thrombogenicity. *Biomaterials* 2007;**28**(16): 2547-2571.
20. Mitchell SL, Niklason LE. Requirements for growing tissue-engineered vascular grafts. *Cardiovasc Pathol* 2003;**12**(2): 59-64.
21. Hoenig MR, Campbell GR, Campbell JH. Vascular grafts and the endothelium. *Endothelium* 2006;**13**(6): 385-401.
22. Risau W. Differentiation of endothelium. *Faseb J* 1995;**9**(10): 926-933.
23. Schnittler HJ. Structural and functional aspects of intercellular junctions in vascular endothelium. *Basic Res Cardiol* 1998;**93 Suppl 3**: 30-39.
24. Weibel ER, Palade GE. New Cytoplasmic Components in Arterial Endothelia. *J Cell Biol* 1964;**23**: 101-112.
25. Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J Cell Biol* 1982;**95**(1): 355-360.
26. Denis CV. Molecular and cellular biology of von Willebrand factor. *Int J Hematol* 2002;**75**(1): 3-8.
27. Hannah MJ, Williams R, Kaur J, Hewlett LJ, Cutler DF. Biogenesis of Weibel-Palade bodies. *Semin Cell Dev Biol* 2002;**13**(4): 313-324.
28. Lampugnani MG, Resnati M, Raiteri M, Pigott R, Pisacane A, Houen G, Ruco LP, Dejana E. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J Cell Biol* 1992;**118**(6): 1511-1522.
29. DeLisser HM, Newman PJ, Albelda SM. Molecular and functional aspects of PECAM-1/CD31. *Immunology today* 1994;**15**(10): 490-495.
30. Dudzinski DM, Igarashi J, Greif D, Michel T. The regulation and pharmacology of endothelial nitric oxide synthase. *Annual review of pharmacology and toxicology* 2006;**46**: 235-276.
31. Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. *American journal of physiology* 2001;**280**(2): F193-206.
32. Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Wan W, Ratajczak J, Wojakowski W, Kucia M. Hunt for pluripotent stem cell -- regenerative medicine search for almighty cell. *Journal of autoimmunity* 2008;**30**(3): 151-162.
33. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Molecular biology of the cell* 2002;**13**(12): 4279-4295.
34. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;**7**(2): 211-228.
35. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411): 143-147.
36. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *Journal of cellular physiology* 2007;**213**(2): 341-347.
37. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981;**292**(5819): 154-156.
38. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;**282**(5391): 1145-1147.
39. Atala A. Tissue engineering and regenerative medicine: concepts for clinical application. *Rejuvenation research* 2004;**7**(1): 15-31.

40. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 2000;**408**(6808): 92-96.
41. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development (Cambridge, England)* 1998;**125**(9): 1747-1757.
42. Vittet D, Prandini MH, Berthier R, Schweitzer A, Martin-Sisteron H, Uzan G, Dejana E. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* 1996;**88**(9): 3424-3431.
43. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2002;**99**(7): 4391-4396.
44. Rippon HJ, Bishop AE. Embryonic stem cells. *Cell proliferation* 2004;**37**(1): 23-34.
45. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;**275**(5302): 964-967.
46. Friedenstein AJ, Piatetzky S, II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *Journal of embryology and experimental morphology* 1966;**16**(3): 381-390.
47. Caplan AI. Mesenchymal stem cells. *J Orthop Res* 1991;**9**(5): 641-650.
48. He Q, Wan C, Li G. Concise review: multipotent mesenchymal stromal cells in blood. *Stem cells (Dayton, Ohio)* 2007;**25**(1): 69-77.
49. Osdoby P, Caplan AI. Osteogenesis in cultures of limb mesenchymal cells. *Dev Biol* 1979;**73**(1): 84-102.
50. Grigoriadis AE, Heersche JN, Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J Cell Biol* 1988;**106**(6): 2139-2151.
51. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *Endocrinology* 1994;**134**(1): 277-286.
52. Rogers JJ, Young HE, Adkison LR, Lucas PA, Black AC, Jr. Differentiation factors induce expression of muscle, fat, cartilage, and bone in a clone of mouse pluripotent mesenchymal stem cells. *Am Surg* 1995;**61**(3): 231-236.
53. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;**238**(1): 265-272.
54. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* 1997;**64**(2): 295-312.
55. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* 1998;**4**(4): 415-428.
56. Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am* 1998;**80**(12): 1745-1757.
57. Benayahu D, Akavia UD, Shur I. Differentiation of bone marrow stroma-derived mesenchymal cells. *Current medicinal chemistry* 2007;**14**(2): 173-179.
58. Poulsom R, Alison MR, Forbes SJ, Wright NA. Adult stem cell plasticity. *The Journal of pathology* 2002;**197**(4): 441-456.
59. Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhauser M, Werner C. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem cells (Dayton, Ohio)* 2004;**22**(3): 377-384.
60. Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, Rabkin E, Moran AM, Schoen FJ, Atala A, Soker S, Bischoff J, Mayer JE, Jr. Functional small-diameter neovessels

created using endothelial progenitor cells expanded ex vivo. *Nature medicine* 2001;**7**(9): 1035-1040.
61. Hristov M, Weber C. Endothelial progenitor cells in vascular repair and remodeling. *Pharmacol Res* 2008;**58**(2): 148-151.
62. Eisenberg LM, Eisenberg CA. Stem cell plasticity, cell fusion, and transdifferentiation. *Birth Defects Res C Embryo Today* 2003;**69**(3): 209-218.
63. Li WC, Yu WY, Quinlan JM, Burke ZD, Tosh D. The molecular basis of transdifferentiation. *Journal of cellular and molecular medicine* 2005;**9**(3): 569-582.
64. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 2003;**425**(6961): 968-973.
65. Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. *Nature* 2003;**422**(6934): 901-904.
66. Camargo FD, Chambers SM, Goodell MA. Stem cell plasticity: from transdifferentiation to macrophage fusion. *Cell proliferation* 2004;**37**(1): 55-65.
67. Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J Biol Chem* 2000;**275**(13): 9645-9652.
68. Chunmeng S, Tianmin C. Skin: a promising reservoir for adult stem cell populations. *Medical hypotheses* 2004;**62**(5): 683-688.
69. Young HE, Black AC, Jr. Adult stem cells. *Anat Rec A Discov Mol Cell Evol Biol* 2004;**276**(1): 75-102.
70. Young HE, Duplaa C, Young TM, Floyd JA, Reeves ML, Davis KH, Mancini GJ, Eaton ME, Hill JD, Thomas K, Austin T, Edwards C, Cuzzourt J, Parikh A, Groom J, Hudson J, Black AC, Jr. Clonogenic analysis reveals reserve stem cells in postnatal mammals: I. Pluripotent mesenchymal stem cells. *The Anatomical record* 2001;**263**(4): 350-360.
71. Bartsch G, Yoo JJ, De Coppi P, Siddiqui MM, Schuch G, Pohl HG, Fuhr J, Perin L, Soker S, Atala A. Propagation, expansion, and multilineage differentiation of human somatic stem cells from dermal progenitors. *Stem cells and development* 2005;**14**(3): 337-348.
72. Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC, Jr. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. *The Anatomical record* 2001;**264**(1): 51-62.
73. Junker J, Sommar P, Skog M, Johnson H, Kratz G. Adipogenic, Chondrogenic and Osteogenic Differentiation of Clonally Derived Human Dermal Fibroblasts. *Cells, Tissues, Organs* 2009;**In Press**.
74. Junker JPE, Kratz C, Tollbäck A, Kratz G. Mechanical tension stimulates the transdifferentiation of fibroblasts into myofibroblasts in human burn scars. *Burns* 2008.
75. Teebken OE, Haverich A. Tissue engineering of small diameter vascular grafts. *Eur J Vasc Endovasc Surg* 2002;**23**(6): 475-485.
76. Thomas AC, Campbell GR, Campbell JH. Advances in vascular tissue engineering. *Cardiovasc Pathol* 2003;**12**(5): 271-276.
77. Kim BS, Mooney DJ. Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends Biotechnol* 1998;**16**(5): 224-230.
78. Ratcliffe A. Tissue engineering of vascular grafts. *Matrix Biol* 2000;**19**(4): 353-357.
79. Iwai S, Sawa Y, Ichikawa H, Taketani S, Uchimura E, Chen G, Hara M, Miyake J, Matsuda H. Biodegradable polymer with collagen microsponge serves as a new bioengineered cardiovascular prosthesis. *J Thorac Cardiovasc Surg* 2004;**128**(3): 472-479.
80. He W, Ma Z, Yong T, Teo WE, Ramakrishna S. Fabrication of collagen-coated biodegradable polymer nanofiber mesh and its potential for endothelial cells growth. *Biomaterials* 2005;**26**(36): 7606-7615.

81. Huss FR, Junker JP, Johnson H, Kratz G. Macroporous gelatine spheres as culture substrate, transplantation vehicle, and biodegradable scaffold for guided regeneration of soft tissues. In vivo study in nude mice. *J Plast Reconstr Aesthet Surg* 2007;**60**(5): 543-555.
82. Kumar TR, Krishnan LK. Fibrin-mediated endothelial cell adhesion to vascular biomaterials resists shear stress due to flow. *J Mater Sci Mater Med* 2002;**13**(8): 751-755.
83. Lepidi S, Abatangelo G, Vindigni V, Deriu GP, Zavan B, Tonello C, Cortivo R. In vivo regeneration of small-diameter (2 mm) arteries using a polymer scaffold. *Faseb J* 2006;**20**(1): 103-105.
84. L'Heureux N, Germain L, Labbe R, Auger FA. In vitro construction of a human blood vessel from cultured vascular cells: a morphologic study. *J Vasc Surg* 1993;**17**(3): 499-509.
85. Liu JY, Hafner J, Dragieva G, Seifert B, Burg G. Autologous cultured keratinocytes on porcine gelatin microbeads effectively heal chronic venous leg ulcers. *Wound Repair Regen* 2004;**12**(2): 148-156.
86. Meinhart JG, Deutsch M, Fischlein T, Howanietz N, Froschl A, Zilla P. Clinical autologous in vitro endothelialization of 153 infrainguinal ePTFE grafts. *The Annals of thoracic surgery* 2001;**71**(5 Suppl): S327-331.
87. Pankajakshan D, Krishnan VK, Krishnan LK. Vascular tissue generation in response to signaling molecules integrated with a novel poly(epsilon-caprolactone)-fibrin hybrid scaffold. *Journal of tissue engineering and regenerative medicine* 2007;**1**(5): 389-397.
88. Schmidt SP, Hunter TJ, Sharp WV, Malindzak GS, Evancho MM. Endothelial cell--seeded four-millimeter Dacron vascular grafts: effects of blood flow manipulation through the grafts. *J Vasc Surg* 1984;**1**(3): 434-441.
89. Weinberg CB, Bell E. A blood vessel model constructed from collagen and cultured vascular cells. *Science* 1986;**231**(4736): 397-400.
90. Atala A, Lanza RP. *Methods of tissue engineering*. Academic press: London, 2002.
91. Ratner B, Hoffman A, Schoen F, Lemons J. *Biomateriales Science An introduction to Biomaterials in Medicine*. Academic Press: San Diego, 1996.
92. Kim BS, Putnam AJ, Kulik TJ, Mooney DJ. Optimizing seeding and culture methods to engineer smooth muscle tissue on biodegradable polymer matrices. *Biotechnol Bioeng* 1998;**57**(1): 46-54.
93. Rosenberg N, Martinez A, Sawyer PN, Wesolowski SA, Postlethwait RW, Dillon ML, Jr. Tanned collagen arterial prosthesis of bovine carotid origin in man. Preliminary studies of enzyme-treated heterografts. *Ann Surg* 1966;**164**(2): 247-256.
94. Badylak SF, Lantz GC, Coffey A, Geddes LA. Small intestinal submucosa as a large diameter vascular graft in the dog. *J Surg Res* 1989;**47**(1): 74-80.
95. Wainwright DJ. Use of an acellular allograft dermal matrix (AlloDerm) in the management of full-thickness burns. *Burns* 1995;**21**(4): 243-248.
96. Silver FH, Pins G. Cell growth on collagen: a review of tissue engineering using scaffolds containing extracellular matrix. *Journal of long-term effects of medical implants* 1992;**2**(1): 67-80.
97. Stegemann JP, Nerem RM. Phenotype modulation in vascular tissue engineering using biochemical and mechanical stimulation. *Ann Biomed Eng* 2003;**31**(4): 391-402.
98. Bilodeau K, Mantovani D. Bioreactors for tissue engineering: focus on mechanical constraints. A comparative review. *Tissue Eng* 2006;**12**(8): 2367-2383.
99. Nasseri BA, Pomerantseva I, Kaazempur-Mofrad MR, Sutherland FW, Perry T, Ochoa E, Thompson CA, Mayer JE, Jr., Oesterle SN, Vacanti JP. Dynamic rotational seeding and cell culture system for vascular tube formation. *Tissue Eng* 2003;**9**(2): 291-299.
100. Niklason LE. Techview: medical technology. Replacement arteries made to order. *Science* 1999;**286**(5444): 1493-1494.
101. Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, Langer R. Functional arteries grown in vitro. *Science* 1999;**284**(5413): 489-493.

102. Nerem RM. Tissue engineering: the hope, the hype, and the future. *Tissue Eng* 2006;**12**(5): 1143-1150.
103. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev* 2005;**16**(2): 159-178.
104. Gabhann FM, Popel AS. Systems Biology of Vascular Endothelial Growth Factors. *Microcirculation* 2008;**15**(8): 715-738.
105. Ferrara N. Vascular endothelial growth factor: Basic science and clinical progress. *Endocrine Reviews* 2004;**25**(4): 581-611.
106. Massague J. How cells read TGF-beta signals. *Nature Reviews Molecular Cell Biology* 2000;**1**(3): 169-178.
107. Karamysheva AF. Mechanisms of angiogenesis. *Biochemistry-Moscow* 2008;**73**(7): 751-762.
108. Sporn MB, Roberts AB, Wakefield LM, Assoian RK. Transforming growth factor-beta: biological function and chemical structure. *Science* 1986;**233**(4763): 532-534.
109. Huang SS, Huang JS. TGF-beta control of cell proliferation. *Journal of cellular biochemistry* 2005;**96**(3): 447-462.
110. Madri JA, Pratt BM, Tucker AM. Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. *J Cell Biol* 1988;**106**(4): 1375-1384.
111. Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Munger K, Howley PM, Moses HL. TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* 1990;**61**(5): 777-785.
112. Pollman MJ, Naumovski L, Gibbons GH. Vascular cell apoptosis: cell type-specific modulation by transforming growth factor-beta1 in endothelial cells versus smooth muscle cells. *Circulation* 1999;**99**(15): 2019-2026.
113. Massague J. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990;**6**: 597-641.
114. Massague J. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 2000;**1**(3): 169-178.
115. Newton LK, Yung WK, Pettigrew LC, Steck PA. Growth regulatory activities of endothelial extracellular matrix: mediation by transforming growth factor-beta. *Exp Cell Res* 1990;**190**(1): 127-132.
116. Yang EY, Moses HL. Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. *J Cell Biol* 1990;**111**(2): 731-741.
117. Sankar S, Mahooti-Brooks N, Bensen L, McCarthy TL, Centrella M, Madri JA. Modulation of transforming growth factor beta receptor levels on microvascular endothelial cells during in vitro angiogenesis. *J Clin Invest* 1996;**97**(6): 1436-1446.
118. Pietras K, Sjoblom T, Rubin K, Heldin CH, Ostman A. PDGF receptors as cancer drug targets. *Cancer Cell* 2003;**3**(5): 439-443.
119. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 1999;**79**(4): 1283-1316.
120. Friesel RE, Maciag T. Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction. *Faseb J* 1995;**9**(10): 919-925.
121. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008;**16**(5): 585-601.
122. Tortora GaGS. *Principles of Anatomy and Physiology* (9 edn). John Wiley & Sons Inc, 2000.
123. Graham LM, Burkel WE, Ford JW, Vinter DW, Kahn RH, Stanley JC. Immediate seeding of enzymatically derived endothelium in Dacron vascular grafts. Early experimental studies with autologous canine cells. *Arch Surg* 1980;**115**(11): 1289-1294.
124. Herring MB, Dilley R, Jersild RA, Jr., Boxer L, Gardner A, Glover J. Seeding arterial prostheses with vascular endothelium. The nature of the lining. *Ann Surg* 1979;**190**(1): 84-90.

125. Herring M, Baughman S, Glover J, Kesler K, Jesseph J, Campbell J, Dilley R, Evan A, Gardner A. Endothelial seeding of Dacron and polytetrafluoroethylene grafts: the cellular events of healing. *Surgery* 1984;**96**(4): 745-755.
126. Swedenborg J, Bengtsson L, Clyne N, Dryjski M, Gillis C, Rosfors S, Haegerstrand A. In vitro endothelialisation of arteriovenous loop grafts for haemodialysis. *Eur J Vasc Endovasc Surg* 1997;**13**(3): 272-277.
127. Deutsch M, Meinhart J, Fischlein T, Preiss P, Zilla P. Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients: a 9-year experience. *Surgery* 1999;**126**(5): 847-855.
128. Deutsch M, Meinhart J, Vesely M, Fischlein T, Groscurth P, von Oppell U, Zilla P. In vitro endothelialization of expanded polytetrafluoroethylene grafts: a clinical case report after 41 months of implantation. *J Vasc Surg* 1997;**25**(4): 757-763.
129. Jarrell BE, Williams SK, Solomon L, Speicher L, Koolpe E, Radomski J, Carabasi RA, Greener D, Rosato FE. Use of an endothelial monolayer on a vascular graft prior to implantation. Temporal dynamics and compatibility with the operating room. *Ann Surg* 1986;**203**(6): 671-678.
130. Magometschnigg H, Kadletz M, Vodrazka M, Dock W, Grimm M, Grabenwoger M, Minar E, Staudacher M, Fenzl G, Wolner E. Prospective clinical study with in vitro endothelial cell lining of expanded polytetrafluoroethylene grafts in crural repeat reconstruction. *J Vasc Surg* 1992;**15**(3): 527-535.
131. Zilla P, Deutsch M, Meinhart J, Puschmann R, Eberl T, Minar E, Dudczak R, Lugmaier H, Schmidt P, Noszian I, et al. Clinical in vitro endothelialization of femoropopliteal bypass grafts: an actuarial follow-up over three years. *J Vasc Surg* 1994;**19**(3): 540-548.
132. de Mel A, Jell G, Stevens MM, Seifalian AM. Biofunctionalization of biomaterials for accelerated in situ endothelialization: a review. *Biomacromolecules* 2008;**9**(11): 2969-2979.
133. Deutsch M, Meinhart J, Zilla P, Howanietz N, Gorlitzer M, Froeschl A, Stuempflen A, Bezuidenhout D, Grabenwoeger M. Long-term experience in autologous in vitro endothelialization of infrainguinal ePTFE grafts. *J Vasc Surg* 2009;**49**(2): 352-362; discussion 362.
134. L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. *Faseb J* 1998;**12**(1): 47-56.
135. L'Heureux N, Dusserre N, Konig G, Victor B, Keire P, Wight TN, Chronos NA, Kyles AE, Gregory CR, Hoyt G, Robbins RC, McAllister TN. Human tissue-engineered blood vessels for adult arterial revascularization. *Nature medicine* 2006;**12**(3): 361-365.
136. L'Heureux N, Stoclet JC, Auger FA, Lagaud GJ, Germain L, Andriantsitohaina R. A human tissue-engineered vascular media: a new model for pharmacological studies of contractile responses. *Faseb J* 2001;**15**(2): 515-524.
137. Niklason LE, Abbott W, Gao J, Klagges B, Hirschi KK, Ulubayram K, Conroy N, Jones R, Vasanawala A, Sanzgiri S, Langer R. Morphologic and mechanical characteristics of engineered bovine arteries. *J Vasc Surg* 2001;**33**(3): 628-638.
138. Rivron NC, Liu JJ, Rouwkema J, de Boer J, van Blitterswijk CA. Engineering vascularised tissues in vitro. *Eur Cell Mater* 2008;**15**: 27-40.
139. Walgenbach KJ, Voigt M, Riabikhin AW, Andree C, Schaefer DJ, Galla TJ, Bjorn G. Tissue engineering in plastic reconstructive surgery. *The Anatomical record* 2001;**263**(4): 372-378.
140. Hollister SJ. Porous scaffold design for tissue engineering. *Nat Mater* 2005;**4**(7): 518-524.
141. Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues--state of the art and future perspectives. *J Biomater Sci Polym Ed* 2001;**12**(1): 107-124.
142. Kneser U, Polykandriotis E, Ohnolz J, Heidner K, Grabinger L, Euler S, Amann KU, Hess A, Brune K, Greil P, Sturzl M, Horch RE. Engineering of vascularized transplantable bone tissues: induction of axial vascularization in an osteoconductive matrix using an arteriovenous loop. *Tissue Eng* 2006;**12**(7): 1721-1731.

143. Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, Marini R, van Blitterswijk CA, Mulligan RC, D'Amore PA, Langer R. Engineering vascularized skeletal muscle tissue. *Nature biotechnology* 2005;**23**(7): 879-884.
144. Post MJ, Laham R, Sellke FW, Simons M. Therapeutic angiogenesis in cardiology using protein formulations. *Cardiovasc Res* 2001;**49**(3): 522-531.
145. Black AF, Berthod F, L'Heureux N, Germain L, Auger FA. In vitro reconstruction of a human capillary-like network in a tissue-engineered skin equivalent. *Faseb J* 1998;**12**(13): 1331-1340.
146. Bachetti T, Morbidelli L. Endothelial cells in culture: a model for studying vascular functions. *Pharmacol Res* 2000;**42**(1): 9-19.
147. Ichiki T. Role of cAMP response element binding protein in cardiovascular remodeling: good, bad, or both? *Arteriosclerosis, thrombosis, and vascular biology* 2006;**26**(3): 449-455.
148. Li J, Zhang YP, Kirsner RS. Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. *Microscopy research and technique* 2003;**60**(1): 107-114.
149. Stover NP, Bakay RA, Subramanian T, Raiser CD, Cornfeldt ML, Schweikert AW, Allen RC, Watts RL. Intrastriatal implantation of human retinal pigment epithelial cells attached to microcarriers in advanced Parkinson disease. *Archives of neurology* 2005;**62**(12): 1833-1837.
150. Fernandes AM, Fernandes TG, Diogo MM, da Silva CL, Henrique D, Cabral JM. Mouse embryonic stem cell expansion in a microcarrier-based stirred culture system. *Journal of biotechnology* 2007;**132**(2): 227-236.
151. Del Guerra S, Bracci C, Nilsson K, Belcourt A, Kessler L, Lupi R, Marselli L, De Vos P, Marchetti P. Entrapment of dispersed pancreatic islet cells in CultiSpher-S macroporous gelatin microcarriers: Preparation, in vitro characterization, and microencapsulation. *Biotechnol Bioeng* 2001;**75**(6): 741-744.
152. Liu JY, Hafner J, Dragieva G, Burg G. High yields of autologous living dermal equivalents using porcine gelatin microbeads as microcarriers for autologous fibroblasts. *Cell transplantation* 2006;**15**(5): 445-451.
153. Huss FR, Kratz G. 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.
154. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol* 1984;**99**(6): 2034-2040.
155. Riha GM, Lin PH, Lumsden AB, Yao Q, Chen C. Roles of hemodynamic forces in vascular cell differentiation. *Ann Biomed Eng* 2005;**33**(6): 772-779.
156. Seliktar D, Nerem RM, Galis ZS. Mechanical strain-stimulated remodeling of tissue-engineered blood vessel constructs. *Tissue Eng* 2003;**9**(4): 657-666.
157. Italiano JE, Jr., Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood* 2008;**111**(3): 1227-1233.
158. Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 2005;**13**(1): 7-12.
159. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *The Journal of pathology* 2003;**200**(4): 500-503.