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HUMAN DERMAL FIBROBLASTS IN TISSUE ENGINEERING

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*“Jag ska göra några sista kalkyler
och studera förloppet i ett experiment.
Om förändringarna stöder teorin
så kan vatten förvandlas till vin”*
Genesarets Sjö, **Kjell Höglund**, 1984

TO MYSELF

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ABSTRACT

The loss or failure of tissues and/or organs is one of the most frequent problems in modern healthcare. The field of tissue engineering applies the principles of biology and engineering in order to develop functional substitutes for damaged tissues. Tissue engineering contains elements of medicine, material science and engineering with major components in focus being cells, biomaterials and soluble factors. All three components may be required for the development of clinical treatments.

The usage of autologous tissue specific cells for clinical treatment is often not feasible due to poor growth kinetics or unstable phenotypes of the cells. Furthermore, lack of availability of healthy tissue that can be biopsied is a major problem in many applications. One approach to overcome this problem is to use adult stem cells which have the capacity to give rise to several different cell types. Although promising, adult stem cells have major impediments for use in several tissue engineering applications. The difficulties associated with harvest, culture and storage render problems in the development of clinically relevant procedures.

During the last years, the inherent plasticity of differentiated somatic cells has been demonstrated. One of the easiest human cell types to obtain, expand and store is the dermal fibroblast. Recent reports indicate that dermal fibroblasts can be induced to differentiate towards several distinct mesenchymal lineages *in vitro*.

The main aim of this thesis was to investigate the inherent stem cell plasticity of human dermal fibroblasts and explore their possible usefulness in tissue engineering applications. The papers included in this thesis employ routine and immunohistochemical staining, enzyme activity assay, analysis of low density lipoprotein incorporation, capillary-like network formation assay and full expression micro array analysis.

Fibroblasts were shown to differentiate towards adipocyte, chondrocyte, endothelial and osteoblast-like cell types *in vitro*. The differentiation from fibroblasts to myofibroblasts in burn scar tissue upon stimulation by mechanical tension was also demonstrated. Adipogenic, chondrogenic and osteogenic induced fibroblasts display the upregulation of several genes associated with adipocytes, chondrocytes and osteoblasts.

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ABBREVIATIONS

A ₂ P	ascorbate-2-phosphate
ALP	alkaline phosphatase
ASC	adult stem cell
BGP	beta-glycerophosphate
C/EBP	CCAAT/enhancer-binding protein
cAMP	cyclic adenosine mono phosphate
CD	cluster of differentiation
COX-2	inducible cyclooxygenase
DEX	dexamethasone
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ESC	embryonal stem cell
FB	fibroblast
FCS	fetal calf serum
HSL	hormone sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IGF-1R	insulin growth factor receptor 1
IHC	immunohistochemistry
LDL	low-density lipoprotein
LDLR	LDL receptor
NO	nitric oxide
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule-1
PKA	protein kinase A
PPAR γ	peroxisome proliferator-activated receptor- γ
SC	stem cell
SCC	single cell clone
SMA	α smooth muscle actin
TGF β ₁	transforming growth factor β ₁
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
vWf	von Willebrand factor

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. **Johan PE Junker**, Pehr Sommar, Mårten Skog, Hans Johnson, Gunnar Kratz
Adipogenic, Chondrogenic and Osteogenic Differentiation of Human Dermal Fibroblasts
In press, Cells, Tissues, Organs, 2009
- II. **Johan PE Junker**, Camilla Kratz, Anna Tollbäck, Gunnar Kratz
Mechanical Tension Stimulates the Transdifferentiation of Fibroblasts into Myofibroblasts in Human Burn Scars
Burns, 2008 Nov;34(7):942-6
- III. Lisa K Karlsson, **Johan PE Junker**, Magnus Grenegård, Gunnar Kratz
Human Dermal Fibroblasts and Single-Cell Clone Fibroblasts Have the Capacity to Alter Their Phenotype Towards an Endothelial-Like Cell type
Under revision, European Cells and Materials Journal, June 2009
- IV. Lisa K Karlsson, **Johan PE Junker**, Magnus Grenegård, Gunnar Kratz
Human Dermal Fibroblasts: a Potential Cell Source for Endothelialization of Vascular Grafts
In press, Annuals of Vascular Surgery May 2009
- V. **Johan PE Junker**, Jonathan Rakar, Hans Johnson, Gunnar Kratz
Gene Expression Analysis of Adipogenic, Chondrogenic and Osteogenic Induced Human Dermal Fibroblasts
Manuscript

INTRODUCTION

The loss or failure of tissues and/or organs is one of the most frequent problems in modern healthcare. Reasons for this loss or failure include trauma, congenital anomalies and conditions related to clinical treatment (i.e. tissue loss after tumor resection). Current regimes for treatment of these conditions often rely on transplantation of healthy tissue to the site of injury. If possible, autologous tissue is the preferred source for transplantation, the reason being the absence of immune system modulated rejection. In many cases autologous transplantation is not a plausible option due to the fact that no functional tissue can be obtained from the patient. When using allogeneous donor tissue several difficulties arise. First and foremost there are currently more than 100,000 candidates awaiting donor organs and tissues in the U.S. alone (<http://www.unos.org>, May 2009). Furthermore, the required immunological matching of donor and recipient limits transplantation options even more.

TISSUE ENGINEERING

Tissue engineering, which is one of the fastest growing areas in medicine today, applies the principles of biology and engineering to the development of functional substitutes for damaged tissues. The term tissue engineering was coined at a National Science Foundation workshop in 1988. Later, Langer and Vacanti published a paper in the journal *Science* defining the field:

“Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function”

(LANGER and VACANTI 1993)

During the last decades the area has gained a lot of interest and a number of clinically relevant therapies have been developed. Cultured autologous autografts has been used extensively when treating severe burns (GALLICO, O'CONNOR *et al.* 1984; GUSTAFSON and KRATZ 1999). Autologous melanocyte transplantation has been employed in the treatment of vitiligo (OLSSON and JUHLIN 1992). *In vitro* expanded chondrocytes transplanted to damaged knee joints has been shown to lead to substantial long-term improvements for patients (BRITTBERG, LINDAHL *et al.* 1994; PETERSON, MINAS *et al.* 2000). A tissue engineered airway has been employed in clinical treatment with successful results (MACCHIARINI, JUNGEBLUTH *et al.* 2008). Artificial grafts seeded with autologous cells that promote regeneration of a functional cornea have been used in clinical

treatments (MCLAUGHLIN, TSAI *et al.* 2009). Several ongoing trials regarding the engineering of urinary tissue have reported promising results (GUSTAFSON, ELDH *et al.* 1998; FOSSUM, NORDENSKJOLD *et al.* 2004; ATALA 2008; LIU, BHARADWAJ *et al.* 2009).

Tissue engineering contains elements of medicine, material science and engineering, thus requiring interdisciplinary collaborations. The major components in focus of tissue engineering research include cells, biomaterials and soluble factors (fig 1). In many cases all three components are required for the development of clinical treatments.

This thesis will focus on possible cell sources for use in tissue engineering applications, and the means of *in vitro* modification of cell cultures that can render viable alternatives to allogenic transplantation. Paper IV also incorporates the use of a biomaterial in order study the possible generation of vascular tissue constructs.

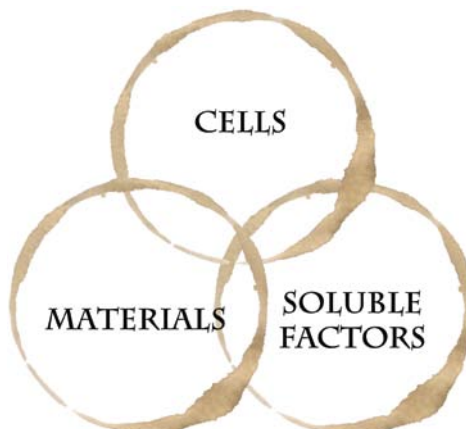


Fig 1. The three main components in tissue engineering

CELL SOURCES IN TISSUE ENGINEERING

Donor cells

The availability of donor cells is a limiting factor in the treatment of many patients (SHIMAZONO 2007). Furthermore, patients receiving grafted cells require treatment with immunosuppressive drugs, which leads to a reduced quality of life and imbues heavy costs on healthcare systems (GUTIERREZ-DALMAU and CAMPISTOL 2007).

Autologous cells

The problem of immunosuppression can largely be avoided by using autologous tissue as a source for cell transplantation. Harvested cells can often be expanded *in vitro* prior to engraftment, generating a higher number of cells than what was obtained originally. Several procedures widely used in clinical settings incorporate the use of autologous cells, for example cultured epidermal autografts in the treatment of large burns (GUSTAFSON and KRATZ 1999).

In many cases, this approach may not be feasible due to poor growth kinetics or unstable phenotypes of the autologous cells. Furthermore, lack of

availability of healthy tissue that can be biopsied is a major problem in many applications.

STEM CELLS

The term *stem cell* (SC) was first used 1908 by Alexander Maximov at a congress in Berlin. Since then, the term has come to include several different cell types with different properties. SCs are characterised by their ability to undergo asymmetric division, thus renewing themselves and giving rise to differentiated daughter cells.

The term *potency* specifies the differentiation potential of a certain SC. **Totipotent** stem cells can differentiate into embryonic as well as extraembryonic cell types, and are therefore capable of generating a complete organism. **Pluripotent** SCs are the descendants of totipotent cells and can differentiate into nearly all cells derived from all three germ layers. **Multipotent** SCs can differentiate into several different cells, but are considered to be less potent than pluripotent SCs. **Oligopotent** SCs can differentiate into only a few cell types, and are often limited to cells of a certain tissue. **Unipotent** cells can produce only one cell type, but have the property of self-renewal which distinguishes them from non-SCs.

SCs can be divided into two groups based on their origin. Embryonic SCs (ESCs), which are present in the developing embryo, and adult SCs (ASCs), which are present in the adult organism.

Embryonic stem cells

The earliest ESCs are derived from the *morula* and are totipotent, thus able to form all cells and tissues of an organism. ESCs derived at a later developmental stage, from the inner cell mass of the blastocyst are pluripotent, giving rise to all cells required for the generation of a complete organism except the placenta.

With the extensive potency of ESCs in mind, the development of cell-based therapies using ESCs is appealing at first, but many considerations must be taken into account. The problem with rejection of grafted cells and/or tissues has to be overcome, as there is no plausible scheme for acquiring autologous ESCs in most cases. There is also an evident risk of teratoma formation, i.e. transplanted cells leading to uncontrollable growth and tumor formation. Furthermore, many ethical issues exist when harvesting cells from human embryos, including the moral status of the embryo, the sanctity of life and the possible use of savior siblings as a source of ESCs (SPRIGGS and SAVULESCU 2002; TOWNS and JONES 2004).

Adult stem cells

The existence of SCs in the adult organism was reported in the 1950s, and their potential for use in tissue engineering applications have been debated extensively (CAPLAN 1991; CAPLAN 2007; TOGEL and WESTENFELDER 2007). Several studies have reported the successful differentiation of human ASC populations from a wide array of tissues towards mesenchymal lineages *in vitro* (PITTENGER, MACKAY *et al.* 1999; JAISWAL, JAISWAL *et al.* 2000; ASAKURA, KOMAKI *et al.* 2001; DEASY, JANKOWSKI *et al.* 2001; ALESSANDRI, PAGANO *et al.* 2004; SCHULTZ and LUCAS 2006; GAO, YAO *et al.* 2007; PASSIER, VAN LAAKE *et al.* 2008). The bone marrow derived stem cell was one of the first ASCs shown to have multilineage potential as shown by experiments performed *in vitro* (JAISWAL, HAYNESWORTH *et al.* 1997; YOO, BARTHEL *et al.* 1998). Reports of several ASC populations derived from other human tissues were published during the following years (YOUNG, STEELE *et al.* 2001; ZUK, ZHU *et al.* 2002).

During the last decade several reports indicating that SCs transplanted into a new environment resulted in detectable contribution in several lineages distinct from their tissue of origin have been published (BRUDER, KURTH *et al.* 1998; ARINZEH, PETER *et al.* 2003; YOUNG, DUPLAA *et al.* 2004; YOUNG, DUPLAA *et al.* 2005; CANCEDDA, GIANNONI *et al.* 2007; DRAGOO, CARLSON *et al.* 2007; BAJADA, MAZAKOVA *et al.* 2008; HEYDARKHAN-HAGVALL, SCHENKE-LAYLAND *et al.* 2008; SVENDSEN 2008).

Although promising, ASCs have major impediments for use in several tissue engineering applications. The difficulties associated with harvest, culture and storage render problems in the development of clinically relevant procedures (GARDNER 2007; FU and LI 2009). Currently, there is a lack of standardisation procedures for SC preparations. It has been demonstrated that procedures need to be carefully monitored in order to obtain comparable stem cell populations (SEEGER, TONN *et al.* 2007). One major concern about cell therapy in general is the potential consequences associated with a treatment that result in long-term or permanent presence of foreign cells in the recipient. Administered cells remain in the body and although many studies have shown only limited or transient engraftment (LANGE, TOGEL *et al.* 2005; VACANTI 2006), it cannot be excluded that there is long term engraftment. ASCs are not as prone to form tumors as compared to reports on implantation of ESCs (BLUM and BENVENISTY 2008), but there is a risk present.

There has been an intense discussion about the mechanisms underlying differentiation of ASCs and there are a variety of different hypothesis on the subject (EISENBERG and EISENBERG 2003; JIN and GREENBERG 2003). For instance, the possible fusion of different cell types giving rise to new phenotypes has been proposed (CAMARGO, CHAMBERS *et al.* 2004; O'MALLEY and SCOTT

2004; SCOTT 2004). Several studies performed on clonal populations of ASCs imply that fusion is not required for the phenotypical switch associated with the differentiation process (YOUNG, DUPLAA *et al.* 2001; GUILAK, LOTT *et al.* 2006).

STEM CELL PLASTICITY IN "NORMAL" ADULT CELLS

During the last years, the concept of adult cells being restricted in their ability to generate only the differentiated cell phenotypes of their own tissue has been challenged (fig 2). Several reports have shown the plasticity inherent in cells considered terminally differentiated, and their phenotypical change towards other cell types, both *in vitro* and *in vivo* (CHUNMENG and TIANMIN 2004; THOWFEEQU, MYATT *et al.* 2007).

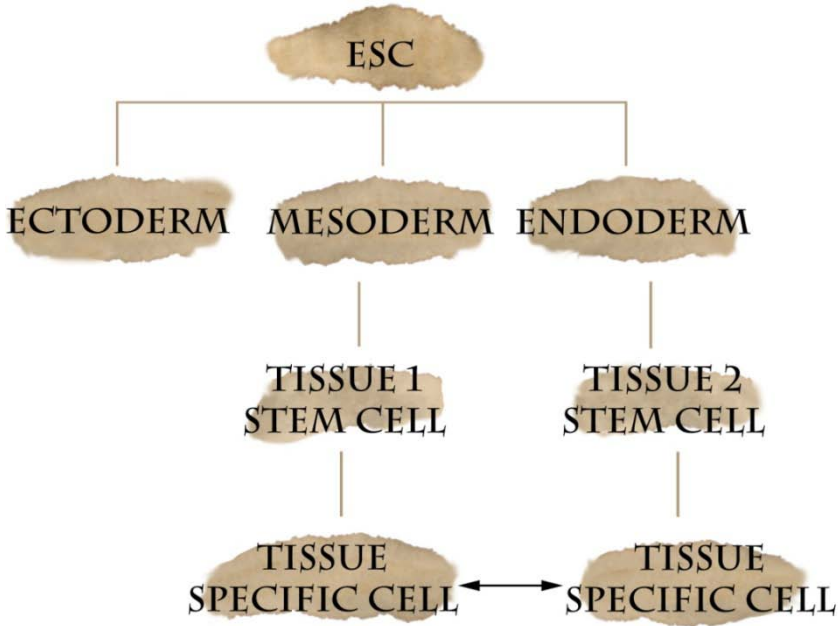


Fig 2. Schematic visualisation of embryonal development of tissue specific cells. The proposed phenotype switch form one differentiated cell type in to another cell type marked with black arrow. Adapted from Slack (SLACK 2007).

The regeneration of liver and pancreatic tissues by the means of transdifferentiation has been proposed, and shown in model organisms as reviewed by Zaret and Grompe (ZARET and GROMPE 2008). Although this phenomenon of transdifferentiation might be rarely occurring in nature, the possibility of using reprogrammed cells for therapeutic purposes has been suggested (SLACK 2007).

DERMAL FIBROBLASTS IN TISSUE ENGINEERING

Several publications describe the presence of cells exhibiting SC plasticity in the dermis. Experiments performed *in vitro* have shown the possible osteogenic differentiation of these so called skin-derived precursor cells (TOMA, AKHAVAN *et al.* 2001; BURANASINSUP, SILA-ASNA *et al.* 2006). Reports indicate that these cells may reside in close proximity to the dermal hair follicles (JAHODA, WHITEHOUSE *et al.* 2003; FERNANDES, MCKENZIE *et al.* 2004; RICHARDSON, ARNOTT *et al.* 2005). Fibroblasts (FBs) derived from adult dermis display stem cell properties when genetically modified both *in vitro* (PHILLIPS, GULDBERG *et al.* 2007; TAKAHASHI, TANABE *et al.* 2007) and *in vivo* (RUTHERFORD, MOALLI *et al.* 2002). Furthermore, adult dermal FBs cultured in demineralised bone powder have shown promising results in regard to chondrogenic induction (MIZUNO and GLOWACKI 1996; MIZUNO and GLOWACKI 2005).

The inherent plasticity of FBs has been proposed, suggesting that they are able to differentiate towards several mesenchymal lineages (FRENCH, ROSE *et al.* 2004; BARTSCH, YOO *et al.* 2005; CHEN, ZHANG *et al.* 2007; HIRATA, MIZUNO *et al.* 2007; LORENZ, SICKER *et al.* 2008; LOWRY, RICHTER *et al.* 2008; SOMMAR, PETTERSSON *et al.* 2009). The dermal FB exists in large numbers throughout the connective tissue in the adult human and primary cultures can be obtained through minimally invasive biopsies. The dermal FB is one of the easiest human cell types to process and expand *in vitro* (WONG, MCGRATH *et al.* 2007). The usage of this cell in cell-based therapies may facilitate the *in vitro* production of autologous tissues in a most dramatic way and thus open up for new regimes in the reconstruction of damaged tissues and organs.

Surface markers in stem cell populations

The cluster of differentiation (CD) is a protocol that can be used for the identification and investigation of cell surface molecules present on various SC populations (GRONTHOS, FRANKLIN *et al.* 2001; WOGNUM, EAVES *et al.* 2003; YOUNG and BLACK 2004). CD molecules have numerous cellular functions, and are often acting as receptors or ligands. The number of CD markers is constantly increasing and there are currently more than 350 unique markers identified

(www.hcdm.or, May 2009). The analysis of CD markers present in a cell population is most often performed using flow cytometry. This method relies on the labeling of CD marker specific antibodies with fluorochromes. In the flow cytometer cells are interrogated using laser beams directed onto a hydrodynamically focused stream of fluid. A number of detectors are aimed at the interrogation point and each cell passing through is measured in regard to size, granularity and fluorescent signal, indicating the expression of labelled surface markers.

SINGLE CELL CLONING

The method of single cell cloning relies on the production of separate cultures which originate from one single cell. There are several methods available for producing single cell clones (SCCs). Serial dilution is a process where a cell suspension is diluted to the point where the expected number of cells in a certain volume is below one. The solution is then aliquoted in cell culture wells. After giving the cells time to attach to the bottom of the wells, visual examination is used to locate wells containing one single cell. This method is very time consuming and an evident risk is the presence of more than one cell in a culture well. If the observer does not realise this, the single cell origin of the population is compromised.

Another possible method for creating SCCs is by employing flow cytometric cell sorting. The main problem with this method is the low survival rates of cells being trypsinised and passed through the cytometer. Furthermore, proper setup of the instrument is needed to ensure that only one cell is dispersed in every well.

In the present study (paper I) single FBs were transferred to cell culture wells by using a micro manipulator fitted with a micro pipette. The single cells were then allowed to divide during several weeks, rendering cell populations with identical clones. This method is very robust, although time consuming.

DIFFERENTIATION OF CELLS *IN VITRO*

Commonly, differentiation can be induced by modifying the medium in which cells are cultured. A plethora of soluble factors are known to modulate the transcription of target genes, thus leading to a phenotypical change of the cultured cells. ASC populations have been subjected to these media modifications, and the effect on differentiation has been extensively studied (JAISWAL, HAYNESWORTH *et al.* 1997; PITTENGER, MACKAY *et al.* 1999; ZUK, ZHU *et al.* 2001; ZUK, ZHU *et al.* 2002).

Soluble factors used for induction of differentiation

Adipogenic induction

To induce adipogenic differentiation cells have been subjected to treatment with 3-isobutyl-1-methylxanthine (IBMX), insulin, indomethacin and dexamethasone (DEX) (STUDENT, HSU *et al.* 1980; HAUNER and LOFFLER 1987; HAUNER, SCHMID *et al.* 1987; KAWAI, NAMBA *et al.* 2007).

IBMX is a phosphodiesterase (PDE) antagonist mediating an increase in intracellular cyclic adenosine mono phosphate (cAMP) levels. Increased cAMP levels leads to protein kinase A (PKA) activation which causes phosphorylation of hormone sensitive lipase (HSL), which increases lipolysis. Insulin, however, inhibits HSL by both increasing PDE activity, thus decreasing cAMP levels, and by non-cAMP dependent mechanisms involving phosphatase activation leading to deactivation of HSL (STRALFORS and HONNOR 1989). These effects may not be important as such for the differentiation of adipocytes, but in concert with DEX and insulin treatment, the increased cAMP level seems to play an important role. DEX and IBMX are have been shown crucial for the differentiation of the 3T3 fibroblast cell line to adipocytes (GREGOIRE, SMAS *et al.* 1998). Insulin has been shown to increase peroxisome proliferator-activated receptor- γ (PPAR γ) transcriptional activity (KERSTEN, SEYDOUX *et al.* 1999). PPAR γ is a central adipogenic transcription factor (LING, NURCOMBE *et al.* 2009) and is required for adipogenic differentiation, as determined by murine chimeric knock-out models (ROSEN, SARRAF *et al.* 1999). DEX is a potent synthetic glucocorticoid that stimulates a range of transcription factors, including some from the CCAAT/enhancer-binding protein (C/EBP) family, members of which are important for the transcription of PPAR γ (ROSEN, SARRAF *et al.* 1999; KAWAI, NAMBA *et al.* 2007). C/EBP α is also vital for adipocyte differentiation as it transactivates promoters for genes important for adipocyte function and differentiation such as leptin, adipocyte fatty-acid binding protein and insulin receptors (GREGOIRE, SMAS *et al.* 1998). PPAR γ and C/EBP are necessary for the establishment of insulin-sensitive glucose uptake (DESVERGNE and WAHLI 1999).

Indomethacin is a non-steroid anti-inflammatory drug that inhibits the function of inducible cyclooxygenase (COX-2). While acting to modulate the presence of neutral lipids, indomethacin is primarily used at higher concentrations as an agonist to PPAR γ (DESVERGNE and WAHLI 1999; RIVAL, STENNEVIN *et al.* 2004). Indomethacin binds directly to the PPAR γ ligand binding domain (LEHMANN, LENHARD *et al.* 1997). After transcription of PPAR γ during adipogenic differentiation, its activity is boosted by the presence of indomethacin, leading to increased expression of PPAR γ gene targets.

Chondrogenic induction

To induce chondrogenic differentiation cells have been cultured in medium containing low amounts of fetal calf serum (FCS), supplemented with insulin, ascorbate-2-phosphate (A₂P) and transforming growth factor β_1 (TGF β_1) (IWASAKI, NAKATA *et al.* 1993; MACKAY, BECK *et al.* 1998; AWAD, HALVORSEN *et al.* 2003; GAO, YAO *et al.* 2007). The low concentration of FCS is thought to mimic the low perfusion of nutrients in cartilage tissue *in vivo*.

TGF β promotes chondrocyte differentiation and causes expression of collagen. It has been shown that TGF β leads to the formation of precartilaginous mesenchyme (MOSES and SERRA 1996). TGF β signaling is thought to co-operate with insulin signaling to promote chondrocyte differentiation (MCMAHON, PRENDERGAST *et al.* 2008).

Insulin binds to insulin growth factor receptor 1 (IGF-1R). Intracellular mediators coupled to IGF-R₁ include PI₃K-Akt/PKB, ERK_{1/2}-MAPK and p38-MAPK pathways which all has been proposed to be important for chondrogenesis (MCMAHON, PRENDERGAST *et al.* 2008).

A₂P has been shown to increase collagen content in the extracellular matrix (ECM) of cultured FBs (HATA and SENOO 1989). This increase reflects the upregulation of pro-alpha 1 and 2 collagen gene transcriptions in chondrocytes (KURATA, SENOO *et al.* 1993). The ECM-cytoskeleton interactions involve many intracellular mediators of chondrogenesis and provide both positive and negative feed-back. Dense culture with high amounts of cells and matrix is required for the maintenance of a chondrocyte phenotype *in vitro* (TACCHETTI, TAVELLA *et al.* 1992; WOODS, WANG *et al.* 2007). Therefore, high density micro mass culture is commonly used when culturing cartilage tissue (DENKER, NICOLL *et al.* 1995; HANDSCHEL, DEPPRICH *et al.* 2007).

Endothelial induction

Human serum contains numerous growth factors and bioactive molecules including vascular endothelial growth factor (VEGF), TGF β_1 , and platelet-derived growth factor (PDGF) amongst others. There are several proangiogenetic and proendothelial mechanisms attributed to these factors. Previous studies have shown the endothelial differentiation of cells derived from adipose tissue upon subjection to serum (BALWIERZ, CZECH *et al.* 2008).

VEGF acts by binding to tyrosine kinase receptors (the VEGFR family) on cell surfaces, causing them to dimerize and become activated through transphosphorylation. The VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a split tyrosine-kinase domain. VEGFR-2 appears to mediate most known cellular responses to VEGF. Several

studies show the generation of endothelial-like cells from adult stem cells after VEGF treatment *in vitro* (REYES, DUDEK *et al.* 2002; OSWALD, BOXBERGER *et al.* 2004). VEGF and its receptors have important functions in the differentiation of endothelial progenitor cells, angiogenesis and endothelial cell proliferation (KUBO and ALITALO 2003; THURSTON and GALE 2004).

TGF β_1 has been shown to promote capillary formation related apoptosis in endothelial cells (FERRARI, COOK *et al.* 2009). Furthermore, TGF β_1 regulates endothelial cell attachment, migration and differentiation *in vitro* (ZHU, YING *et al.* 2005).

PDGF is a dimeric glycoprotein that binds to tyrosine kinase receptors (the PDGFR family). PDGF has been shown to play a significant role in embryonic development of the vascular system as well as postnatal angiogenesis, as reviewed by Andrae *et al* (ANDRAE, GALLINI *et al.* 2008).

Osteogenic induction

To induce osteogenic differentiation cells have been subjected to treatment with DEX, A2P and beta-glycerophosphate (BGP) (CHENG, YANG *et al.* 1994; ZUK, ZHU *et al.* 2002).

A2P increases the collagen production of the fibroblasts. It has been extensively used in differentiation of osteogenic precursors, and is even said to be essential for osteoblast differentiation (TAKAMIZAWA, MAEHATA *et al.* 2004). A2P has also been shown to stimulate ALP activity, an early marker for osteoblast differentiation (TAKAMIZAWA, MAEHATA *et al.* 2004).

BGP increases collagen deposition and calcification of the ECM of FB cultures (KWIATKOWSKI, MELCHIOR *et al.* 2008). Type I collagen, stimulated by both BGP and A2P, acts as a ligand to osteopontin in bone, which promotes osteogenesis (SHIN, KIM *et al.* 2008). Osteopontin is postulated to be presented on the progenitor cell surface during osteogenesis, but the mechanisms regulating the presence of osteopontin, or indeed the determination of cell fate, has yet to be elucidated (SHIN, KIM *et al.* 2008).

DEX has been shown to promote osteogenesis of mesenchymal stem cells, specifically by regulating the expression of osteocalcin, osteopontin and alkaline phosphatase (ALP) (BERESFORD, GALLAGHER *et al.* 1984; GRIGORIADIS, HEERSCHKE *et al.* 1988; SUBRAMANIAM, COLVARD *et al.* 1992; CHENG, YANG *et al.* 1994). DEX upregulates the parathyroid hormone receptor, and increases the basal level of cAMP by activation of adenylate cyclase (OGSTON, HARRISON *et al.* 2002). The mechanisms that promote osteogenesis on behalf of DEX are poorly understood. The DEX-mediated inhibition of collagenases has been shown to be a potent differentiation stimulus (HAYAMI, ZHANG *et al.* 2007).

Mechanical tension

Myogenic induction

Several reports describe the induction of FB to myofibroblast transdifferentiation by mechanical tension (DARBY, SKALLI *et al.* 1990; HINZ, MASTRANGELO *et al.* 2001; DESMOULIERE, CHAPONNIER *et al.* 2005; HINZ 2007).

Upon mechanical tension the first phenotypic change occurs in response to changes in the composition, organisation, and mechanical property of the extracellular matrix (HINZ and GABBIANI 2003). With increasing stress in the ECM resulting from their own remodeling activity, the myofibroblasts further differentiate and express α smooth muscle actin (SMA), the most widely used myofibroblast marker. Expression of SMA is precisely controlled by the joint action of growth factors like TGF β_1 , specialised ECM proteins like fibronectin and of the mechanical microenvironment (TOMASEK, GABBIANI *et al.* 2002). Incorporation of SMA into stress fibers significantly augments the contractile activity of fibroblastic cells and hallmarks the contraction phase of connective tissue remodeling (HINZ, MASTRANGELO *et al.* 2001).

CONFIRMATION OF DIFFERENTIATION

Several different methods can be used to detect the genotypic and phenotypic changes associated with the differentiation process of a cell population. The papers included in this thesis employ routine and immunohistochemical (IHC) stainings, enzyme activity assay, analysis of low density lipoprotein incorporation, capillary-like network formation assay and full expression micro array analysis.

Adipogenesis

Oil red O

Oil red O is a non-polar dye that stains triglycerides, lipids and some lipoproteins. Although not specific for adipocytes, it is frequently used to visualise intracellular lipid accumulation (KUTT and TSALTAS 1959; HAUSMAN 1981; KOOPMAN, SCHAART *et al.* 2001).

Perilipin

Perilipin is a phosphorylated protein localised on the surface of intracellular lipid droplets in adipocytes and acts as protective coating against endogenous lipases (BLANCHETTE-MACKIE, DWYER *et al.* 1995). The expression of perilipin is strongly associated with mature adipocytes (GREENBERG, EGAN *et al.* 1991).

There is also evidence that perilipin is a vital component in the regulation of fat metabolism (WANG, SONI *et al.* 2008).

Chondrogenesis

Alcian blue

Alcian blue is a copper containing phthalocyanine based dye that binds to negatively charged macromolecules. It is widely used to stain glucose-aminoglycans in cartilage tissue (LEV and SPICER 1964; GOLDSTEIN and HOROBIN 1974; JOHNSTONE, HERING *et al.* 1998; WOODS, KHAN *et al.* 2007).

Aggrecan

Aggrecan is a chondroitin sulphate proteoglycan and one of the two major components of cartilage. Aggrecan is highly specific for cartilage produced by chondrocytes (WILSON, BELLUOCIO *et al.* 2008). Aggrecan binds to hyaluronan and Link protein, forming aggregates. These aggregates lead to a hydrated gel-like structure of cartilage and resistibility to compression and deformation in joints (WATANABE, YAMADA *et al.* 1998).

Collagen II

Collagen exists in several different forms in the human body. Collagen II is the other major component of hyaline cartilage (together with aggrecan) and provides strength to the cartilage matrix. The expression of collagen II is specific for chondrocytes *in vivo*.

Endotheliogenesis

B₂ bradykinin receptor

The B₂ bradykinin receptor is a G protein-coupled receptor that is expressed in various tissues. When activated it leads to an increased intracellular calcium concentration mediated by phospholipase C. It is highly expressed in endothelial cells, and causes vasodilatation when activated by bradykinin.

Endothelial nitric oxide synthase

Endothelial nitric oxide synthase (eNOS) is an enzyme expressed in endothelial cells that generates nitric oxide by catalyzing an oxidation of nitrogen on L-arginine. NO diffuses into adjacent smooth muscle cells and activates guanylate cyclase, thus leading to blood vessel dilation via smooth muscle cell relaxation.

Platelet endothelial cell adhesion molecule-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1) (also referred to as CD31) is most abundant in endothelial cells, but also expressed in leukocytes, lymphocytes and osteoclasts. It has been demonstrated to be involved in the initial formation and stabilisation of cell-cell contacts at lateral junctions of endothelial cells, the maintenance of a vascular permeability barrier, modulation of cell migration, transendothelial migration of monocytes and neutrophils and formation of new blood vessels in angiogenesis (JACKSON 2003).

Vascular endothelial cadherin

Vascular endothelial (VE) cadherin is a calcium-dependent cell-cell adhesion glycoprotein comprised of five extracellular cadherin repeats, a transmembrane region and a cytoplasmic tail. It plays an important role in endothelial cell biology through control of the cohesion and organisation of the intercellular junctions.

VE cadherin is crucial for vascular development (CARMELIET, LAMPUGNANI *et al.* 1999; GORY-FAURE, PRANDINI *et al.* 1999). VE cadherin has important functions in maintaining newly formed vessels (CROSBY, FLEMING *et al.* 2005).

Von Willebrand factor

Von Willebrand factor (vWf) is a multimeric glycoprotein produced in endothelial cells. It is stored in granules in the Weibel-Palade bodies. vWf binds to proteins and is a vital component in the blood coagulation process (SADLER 1998).

Low-density lipoprotein uptake

Low-density lipoprotein (LDL) is a lipoprotein that transports cholesterol and triglycerides from the liver to the peripheral tissues. Endothelial cells have the LDL receptor (LDLR), which enables endocytosis of LDL (VOYTA, VIA *et al.* 1984). LDLR is a chimeric protein and has also been proposed to be involved in intracellular signaling (MAY, BOCK *et al.* 2003).

Capillary-like network formation

The process of angiogenesis can be studied by using *in vitro* culture models where endothelial cells are seeded on gel substrates (i. e. ECMatrix™, Matrigel™ etc). The gel substrates contain a number of proteins and resemble the extracellular matrix associated with endothelial cells. One major limitation to these culture systems includes the lack of other cell types.

Myogenesis

α -smooth muscle actin

The expression of SMA is considered to be a marker for the transdifferentiation of FBs into myofibroblasts in contractile tissues (SKALLI, ROPRAZ *et al.* 1986; DARBY, SKALLI *et al.* 1990; SERINI and GABBIANI 1999; HINZ 2007). The expression of α -smooth muscle actin *in vivo* is regulated by various growth factors, including the TGF β family (WANG, SONI *et al.* 2008).

Osteogenesis

Von Kossa

Von Kossa staining uses a silver nitrate solution to detect calcified tissues like bone. Silver ions replace calcium ions present in a sample and are visible as a dark brown/black precipitate. Positive von Kossa staining is generally considered to be indicative of mineralised bone-like matrix (BILLS, EISENBERG *et al.* 1974).

Osteocalcin

Osteocalcin is a noncollagenous protein secreted by osteoblasts in bone tissue. It is the most abundant non-collagenous protein in bone. Osteocalcin can be used as a biochemical marker for the bone formation process (PAGANI, FRANCUCCI *et al.* 2005).

Osteonectin

Osteonectin is a calcium binding glycoprotein present in bone tissue. It is a high affinity calcium binding protein secreted by osteoblasts during bone formation, initiating mineralisation and mineral crystal formation (YAN and SAGE 1999).

Alkaline phosphatase assay

ALP is a hydrolase enzyme with three closely spaced metal ions present at the active center. It is most effective in an alkaline environment. ALP is expressed in several human tissues, but is most prominent in bone, liver and kidney (COLEMAN 1992).

Full expression micro array

The development of micro array methods has lead to the possibility of analyzing the expression of the complete genome in a sample of interest. Arrays can be purchased from commercial vendors and are highly standardised.

A microarray is a nano scale probe matrix, where each probe is present multiple times at different positions on the array surface. Each probe is

complementary to a specific sequence of a gene transcript. Multiple probes are used for different parts of each gene to increase the accuracy of the hybridisation assay.

The Affymetrix Full Expression Micro Array relies on the hybridisation of cDNA to short (~25 mer) oligonucleotides probes attached to a hydrophobic surface. The sequences are derived from sequence databases, and each gene or mRNA is represented by multiple different anti-sense probes.

The sense-strand cDNA sample is fragmented at predictable sites which are taken into account when constructing the probes. The fragments of sense cDNA are fluorescently labelled and the signal at each probe-spot on the array is measured by a micro array scanner. The resulting intensity data is transformed to a quantification of the hybridisation at specific spots, which are grouped and translated to the level of expression of each gene in the sample. Numerous control spots are present to enable comparison of two or more arrays by utilizing quality control analyses.

There are three layers of replicates in the microarray experiment: biological replicates, technical replicates and duplicate spots (KIM, RHA *et al.* 2004). Biological replicates are similar samples that are grouped and display biological variance. Technical replicates display variance inherent to the method protocol and include multiple array controls. Duplicate spots serve to average out stochastic differences in the hybridisation kinetics.

Statistical analyses used to interpret micro array data are numerous, and there is no consensus on which analytical method that is the most suitable (ALLISON, CUI *et al.* 2006). The collected data is first pre-processed in order to normalise the data to minimise systematic variation. Following normalisation, the data is filtered and transformed to exclude non-relevant differences in between the samples in the experiment. This filtration is highly subjective and depends on the criteria defined when designing the experiment. The obtained data is then be classified and differences between samples can be interpreted. Methods to classify data include clustering according to gene ontologies (i.e. grouping genes according to recognised functions or pathways), which requires access to bioinformatic databases. Clustering can also be performed using similarity-matching algorithms for gene expression in hierarchical fashions or using theoretical “expression spaces” to visualise differential expression (MOCELLIN and ROSSI 2007).

AIMS

The main aim of this thesis was to investigate the inherent stem cell plasticity of human dermal FBs and explore their possible usefulness in tissue engineering applications. This was achieved by studying differentiation induced by media supplements and mechanical tension *in vitro*.

The specific aims for this thesis were:

- To investigate the possibility of adipogenic, chondrogenic and osteogenic differentiation of dermal FBs mediated by culture in media supplemented with soluble factors previously used for the differentiation of stem cell populations.
- To attempt to identify whether subpopulations are present in the dermal FB culture, and assess their possible contribution to multilineage differentiation of the culture.
- To study the differentiation from FB to myofibroblast upon mechanical tension in burn scars by using a tissue culture model.
- To explore possible methods for achieving differentiation towards an endothelial cell-like phenotype of dermal FBs and their possible use for the endothelialisation of a biomaterial surface.
- To study the full genome expression of adipogenic, chondrogenic and osteogenic induced human dermal FBs, and identify differentially expressed genes as compared to normal uninduced FBs.

MATERIALS AND METHODS

All *in vitro* culture of cells used for experiments described in this thesis were performed on cell culture grade polystyrene culture flasks unless stated otherwise. Cells were incubated at 37° C, 95 % humidity, 21 % oxygen and 5 % carbon dioxide. When reaching subconfluence cells were enzymatically detached using Trypsin/EDTA and reseeded in new culture flasks at a 1:3 ratio.

FIBROBLASTS

Primary cultures of FBs were obtained from skin taken from healthy patients undergoing routine reduction mammoplasty or abdominoplasty procedures at the university hospital in Linköping, Sweden. The dermal component of the skin was dissected into 1 mm³ fragments and enzymatically digested using collagenase and dispase. After centrifugation and resuspension, cells were seeded in FB growth medium. Several identical or highly similar procedures have been widely used for preparing dermal FBs, and are generally considered to give primary cultures with high purity and proliferative capacity. FBs were cultured in Dulbecco's minimum essential medium (DMEM) with 10 % fetal calf serum (FCS), 50 U/mL penicillin and 50 µg/mL streptomycin.

PREADIPOCYTES

Primary cultures of preadipocytes were obtained from adipose tissue taken from patients undergoing routine reduction abdominoplasty procedures at the university hospital in Linköping, Sweden using an adapted protocol described by Entenmann et al (ENTENMANN and HAUNER 1996). Visible blood vessels were removed and the remaining tissue was dissected into 1 mm³ pieces before enzymatic digestion. After centrifugation and resuspension, cells were seeded in culture flasks and cultured in Ham's F-12/DMEM with 10 % FCS, 50 U/mL penicillin and 50 µg/mL streptomycin.

ENDOTHELIAL CELLS

Primary culture of endothelial cells were obtained from umbilical cords taken from newborns at the university hospital in Linköping, Sweden using previously described protocol (JAFFE, NACHMAN *et al.* 1973). The veins of the umbilical cords were cannulated and injected with PBS containing collagenase. After 20 minutes, vessels were massaged gently and perfused. The cell solution

obtained was seeded in gelatin coated culture flasks and cultured in DMEM containing 30 % human serum, 50 U/mL penicillin and 50 µg/mL streptomycin.

HYPERTROPHIC BURN SCAR TISSUE

Hypertrophic burn scar tissue was obtained from burn patients with deep partial or full thickness injuries. At the time of biopsy the scars were at least 12 months old. The tissue was incubated in DMEM containing 10 % human serum, 50 U/mL penicillin and 50 µg/mL streptomycin during the experiments.

SINGLE CELL CLONING

SCCFBs were prepared by using a TransferMan micromanipulator fitted with a CellTramAir micropipette (Eppendorff, Germany) fitted to an IX51 inverse light/fluorescence microscope (Olympus, Sweden). Capillary tips were custom ordered and had an inner diameter of 30 µm. Precautions were taken to ensure that only one cell was transferred from the primary culture to each of the culture wells used. SCCFBs were cultured in DMEM with 10 % fetal calf serum (FCS), 50 U/mL penicillin and 50 µg/mL streptomycin.

FLOW CYTOMETRY

Primary culture FBs and SCCFBs were analysed by flow cytometry using antibodies towards several CD markers previously used in studies of populations exhibiting stem cell plasticity (table 1) (PITTENGER, MACKAY *et al.* 1999; ZUK, ZHU *et al.* 2002; LIU, AKIYAMA *et al.* 2008; LORENZ, SICKER *et al.* 2008). 5×10^5 to 10^6 cells from FB or SCCFB cultures were used in each antibody labeling. Cells were detached from the culture flasks using trypsin/EDTA, filtered to remove clusters and kept on ice throughout the staining procedure. 10 µl of FITC-conjugated antibodies were added to each reaction. Antibodies directed towards CD4, CD9, CD14, CD19, CD34, CD45 and CD106 did not stain the FBs or SCCFBs and were pooled in one sample. Controls included omission of antibodies and matched isotype controls. Analyses were performed using a BD LSR flow cytometer (Becton Dickinson, Brøndby, Denmark). The mean fluorescence intensity was compared between CD-marker and the corresponding isotype control and the fold increase was calculated. Furthermore, the percentages of positively labelled FBs and SCCFBs were compared. Normal FBs and three SCCFB populations from separate donors were analysed at 8 different occasions. Each cell population was divided into several samples and each reaction only contained one fluorescently labelled antibody. All experiments were performed in duplicates. Statistical analysis was performed using a non-parametric Mann-Whitney test. A p value of less than 0.05 was considered significant.

Table I. Antigens used for flow cytometric analyses.

Antigen	Protein	Function	Cell type	Supplier
CD4	Co-receptor for T-cell receptor	Binds MHC II	T _h cells	Immunotools
CD8	Co-receptor for T-cell receptor	Binds MHC I	T _c cells	Immunotools
CD9	Motility related protein-1	Adhesion	?	Immunotools
CD13	Alanine aminopeptidase	Trimming of peptides	Early granulocyte and monocyte progenitors	Immunotools
CD14	Pattern recognition receptor	Recognition of lipopolysaccharide	Macrophages	Immunotools
CD19	Lymphocyte surface antigen	Co-receptor together with CD21 and CD81	B cells	Immunotools
CD29	Integrin β_1	Migration	Most cells	Immunotools
CD34	Adhesion factor	Attachment and migration of stem cells	Haematopoietic stem cells, endothelial precursor cells	BD Biosciences
CD44	Hyaluronic acid receptor	Homing and migration of stem cells	Mesenchymal stem cells	Immunotools
CD45	Leucocyte common antigen	Involved in TCR signaling	Haematopoietic cells	Immunotools
CD90	Thy-1	Cell-cell and cell-matrix interactions	Stem cells	Serotec
CD105	SH2, Endoglin	Component in TGF- β receptor system	Stem cells	Immunotools
CD106	VCAM-1	Endothelial adhesion molecule	Mesenchymal stem cells, Endothelial cells	BD Biosciences

INDUCTION OF DIFFERENTIATION

In order to induce the phenotypic shift of FBs, PAs and SCCFBs towards adipocyte, chondrocyte, endothelial and osteoblast-like cell types specific induction media were used (paper I, III-V). The specific induction factors and concentrations used can be reviewed in respective papers. To induce myogenic differentiation in burn scar tissue, mechanical tension was applied (paper II).

CONFIRMATION OF DIFFERENTIATION

Uninduced FBs were used as control samples in experiments included in paper I, III, IV and V. In paper I, uninduced PAs were also included as controls. Unstretched burn scar tissue was used as control samples in paper II.

When staining 3D cultures and tissue (paper I, II and IV) samples were dehydrated through ethanol-xylene series, embedded in paraffin, sectioned using a microtome (Leica, Stockholm, Sweden), mounted on microscopy slides and rehydrated before analysis.

Routine staining

All routine stainings were performed at room temperature. Cells were fixated using 4 % neutral buffered paraformaldehyde (Apoteket, Stockholm, Sweden). All routine stainings were performed using previously described protocols (KUTT and TSALTAS 1959; LEV and SPICER 1964; MASON 1971; BILLS, EISENBERG *et al.* 1974; HAUSMAN 1981; WOODS, KHAN *et al.* 2007). Oil red O staining in paper I was quantified by examining randomly selected high power fields and counting stained and unstained cells. Alcian blue and von Kossa stainings in paper I were scored by independent blinded observers judging the staining intensity on a four degree scale. All quantifications were statistically compared using a non-parametric Kruskal-Wallis test with a Dunn's post test.

Immunohistochemistry

IHC were performed using primary antibodies towards a number of antigens. Primary antibodies were localised using peroxidase or fluorochrome labelled secondary antibodies. For a list of all antibodies used see table II. To exclude unspecific binding of secondary antibodies control samples where primary antibodies were omitted were included in all experiments. Matched isotype non-specific immunoglobulins were employed in paper II to control for unspecific binding of the primary antibodies. When staining intracellular antigens in paper III and IV 0.01 % Triton-X was added to the blocking buffer to permeabilise cell membranes.

In paper II, immunostaining was scored by independent observers. The scoring was compared using a non-parametric Kruskal-Wallis test with a Dunn's post test. A two-tailed Spearman correlation analysis was performed to examine if the observers' scoring of the groups were similar.

Uptake of Fluorochrome-labelled LDL

Acetylated LDL labelled with 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethyl-indocarbocyanine perchlorate (Ac-DiI-LDL) was used to label FBs differentiated towards endothelial cell-like phenotype. Cells were visualised using a fluorescence microscope with an excitation wave length maximum of 488 nm.

Capillary-like network formation

The ability of endothelial induced FBs to form capillary-like networks was evaluated by light microscopy after 6 hours of culture on an *in vitro* angiogenesis assay kit, ECMatrix™. The gel used in this kit consists of various extracellular matrix proteins (e.g. laminin, collagen type IV, heparin sulphate, and proteoglycans) and several growth factors and proteolytic enzymes.

Alkaline phosphatase assay

Alkaline phosphatase activity was measured by adding *para*-nitrophenylphosphate (pNPP) to cell cultures of osteogenic induced cells. pNPP is a substrate for alkaline phosphatase and is cleaved into the yellow colored *para*-nitrophenol (MAGNUSSON and FARLEY 2002). The reaction can be quantified by using a spectrophotometer measuring absorbance at a wavelength of 405 nm. The results obtained were statistically compared using linear regression analysis.

Table II. Primary and secondary antibodies used in this thesis.

Antigen	Supplier	Dilution	Secondary antibody	Supplier	Dilution	Used in paper
Aggrecan	Abcam	1:50	ALEXA488	Molecular Probes	1:500	I, V
B₂ receptor	Sigma-Aldrich	5 µg/mL	Vectastain ABC	Immunkemi	Ready to use	IV
Collagen II	Abcam	1:100	ALEXA488	Molecular Probes	1:500	I, V
eNOS	Santa Cruz Biotech	1:50	Vectastain ABC	Immunkemi	Ready to use	IV
Osteocalcin	Chemicon	1:500	ALEXA546	Molecular Probes	1:500	I, V
Osteonectin	Chemicon	1:1000	ALEXA546	Molecular Probes	1:500	I, V
PECAM-1	Abcam	1:50	Vectastain ABC	Immunkemi	Ready to use	III, IV
PECAM-1	Abcam	1:50	ALEXA546	Molecular Probes	1:500	III, IV
Perilipin	Research Diag Inc	1:200	ALEXA488	Molecular Probes	1:500	I, V
SMA	Dako Cytomation	1:50	Vectastain ABC	Immunkemi	Ready to use	II
VE Cadherin	Santa Cruz Biotech	1:50	Vectastain ABC	Immunkemi	Ready to use	IV
vWF	Dako Cytomation	1:200	ALEXA546	Molecular Probes	1:500	III
vWF	Dako Cytomation	1:20	Vectastain ABC	Immunkemi	Ready to use	IV

Full expression micro array

RNA was extracted from cultured cells using the NucleoSpin RNA II kit (Macherey-Nagel, Germany). In accordance with the provided protocol, a lysis buffer (RA1) and β -mercaptoethanol were added to each sample. After lysis of cells each sample was passed through a clean-up filter column by centrifugation. Ethanol (70 %) was added to the filtrate to precipitate the nucleic acids which, after centrifugation were bound to a filter-column. The populated filter-column was treated with a membrane desalting buffer to minimise the risk of salts interfering with subsequent DNase treatment. The DNase degrades the DNA which is then removed from the filter, leaving only the RNA in the filter-column. The RNA was eluted in RNase-free water.

1 μ l of the RNA suspension was used to spectrophotometrically analyse the purity of the RNA using a NanoDrop instrument (Thermo Scientific, Wilmington, USA). Nucleic acid content was measured at set wavelengths of 260 nm for nucleic acids, 230 nm for phenols and other contaminants, and 280 nm for proteins. Purity was indicated by 260/280 and 260/230 ratios, and a measure of yield was also given.

Furthermore, 1 μ l of the RNA suspension was used to analyse the purity and degree of degradation in the Agilent Bioanalyzer 2100 (Agilent Technologies Inc, Santa Clara, USA). Samples were transferred to separate wells in a microfabricated 12-well chip (RNA 6000 LabChip) and analysed using a gel-electrophoretic technique. The banding is scanned and then analysed by the accompanying software to calculate a RIN value (RNA integrity number). The RIN value is set between 1 and 10, where 1 is very degraded and 10 is very intact.

For micro array analysis, the Affymetrix Human Gene 1.0ST full expression array was used. The method was performed according to the supplied protocol for 300 ng RNA starting material (Affymetrix GeneChip WT ST Labeling Assay Manual rev 4, P/N 701880, with addendum rev 1, P/N 702577).

Poly-A RNA controls and primers with N-terminal T7-promoters were added to the 300 ng of total RNA. Samples were run in a first-cycle first-strand cDNA synthesis with a reverse transcription master-mix containing RNase inhibitors, dNTPs and the Superscript II enzyme. The product was mixed with a second master-mix containing MgCl₂, dNTPs, RNase H and DNA polymerase I for a first-cycle second-strand cDNA synthesis step. In the first-cycle cRNA synthesis and clean-up a master-mix containing IVT 10X Buffer, IVT NTP mix and IVT enzyme mix was used. During the following cRNA clean-up steps the samples were diluted with RNase-free water, cRNA Binding Buffer and EtOH (99.5 %). Subsequently, samples were transferred to an IVT cRNA Clean-up Spin Column and centrifuged. Flow-through was discarded and the filter column was moved into a new collection tube.

Each filter column was washed in cRNA Wash Buffer and centrifuged, again discarding the flow-through. Another wash was performed with EtOH (80 %) and samples were dried by centrifugation. cRNA was eluted in RNase-free water and the yield measured in the NanoDrop.

The second cycle first-strand cDNA synthesis was commenced by the addition of random primers to samples. Reverse transcription components were added to each sample (5X 1st strand buffer, 0.1 M DTT, 10 mM dNTP+dUTP, Superscript II enzyme). The following hydrolysis of cRNA and cleanup of ssDNA, were performed by adding RNase H to each sample to remove all RNA. RNase-free water and cDNA Binding Buffer was added and each sample was transferred to a cDNA Spin column. The samples were centrifuged and the flow-through discarded. cDNA Wash Buffer was added to the filters, samples centrifuged and flow-through discarded again. The filter columns were dried by centrifugation, samples eluted using cDNA elution buffer and yield determined using the NanoDrop.

The obtained ssDNA was fragmented by adding 10X fragmentation buffer, UDG, APE1 and RNase-free water. The fragmented ssDNA was labelled by adding 5X TdT buffer, TdT and DNA labeling reagent.

A hybridisation cocktail was prepared containing Control oligonucleotide B2, 20X Eukaryotic Hybridisation Controls (1.5 pM bioB, 5 pM bioC, 25 pM bioD and 100 pM cre), 2X Hybridisation Mix, 7 % DMSO and RNase-free water. The arrays were allowed to reach room temperature before use and marked with sample designations. Each sample was pipetted into the designated array through the septa on the back of the array. The arrays were then placed in a hybridisation oven at 45° C for 17 ± 1 hours.

The Fluidics Station 450 was prepared (with bottles of dH₂O, Wash Buffer A and Wash Buffer B), initiated and primed in preparation for the array washing and staining steps. The experiment information was entered in the computer and the sample in each array was removed and replaced with Wash Buffer A. Three vials were prepared with Stain Cocktail 1, Stain Cocktail 2 and Array Holding Buffer for each array. The vials and the arrays were placed in the Fluidics Station and the run started with four arrays at a time, while the other four were still in the oven.

The fluidics run included a number of steps which were performed and monitored by the Fluidics Station itself. The arrays were washed, stained with SAPE solution at 35° C, and washed again. Second stain was performed in antibody solution and third stain with SAPE solution again. After the final wash the array was filled with array holding buffer. After completion the fluidics station wash shut down according to the protocol. All arrays were inspected visually to ensure that the wash and stain had not left any bubbles or other

obstructions. The arrays were scanned, generating .CEL-files, containing raw scanning-data. The eight .CEL-files were imported into the GeneSpring GX 10.0 (Agilent) microarray data analysis program and grouped according to induction or growth media. GeneSpring GX10 by default uses the RMA16 (Robust Multichip Averaging) algorithm to perform normalisation, background correction and probe summarisation of each array and also performs a median transform to baseline on all samples.

Entity lists were created based on further filtering and analysis selected by the user. Filtering included probe sets that gave signals between 20th to 100th percentile, and avoiding ambiguous signals below the recommended 20th percentile threshold to eliminate background noise. Approximately 28810 entities out of 28869 were retained by the procedure recommended by Agilent.

The filtered entity list was analysed for fold change between the induced and uninduced FBs, in three separate steps to avoid multi-group correction stringencies. Fold change cut-off was set $t \geq 2$ and statistical significance was calculated using an unpaired t-test with $p < 0.01$. Benjamin-Hochberg corrections were performed on the probe set p-values to lower the number of expected false positives. The fold-change entity lists were separated into upregulated and downregulated for induced group relative to uninduced control FBs. Venn diagrams were constructed to visualise and select the distribution of genes that were differentially regulated for each cell type. Regulated genes were checked manually for phenotype relevance.

RESULTS

Detailed descriptions of all results obtained can be reviewed in respective paper. The most significant findings are presented below.

PAPER I

FBs can be induced to differentiate towards several mesenchymal lineages *in vitro*. Indications of adipogenic differentiation include intracellular lipid accumulation and the expression of perilipin localised to droplet surfaces. Chondrogenic differentiation causes FBs to produce cartilage-resembling extracellular matrix and produce proteins specific for mature chondrocytes. Osteogenic differentiation induced a phenotypical change regarding matrix composition, alkaline phosphatase activity and protein expression.

The experiments using clonal populations presented in paper I also exclude the presence of contaminant cells in primary FB cultures giving rise to differentiated phenotypes.

PAPER II

The *in vitro* transdifferentiation from FB to myoFB mediated by mechanical tension in burn scars were shown in paper II. The FBs in the scar tissue expressed SMA in a time dependent manner. This has a profound effect on the healing process of burn wounds, and may prove to be a significant factor when treating large burn wounds.

PAPER III AND IV

When subjected to high amounts of human serum, FBs alter their phenotype into an endothelial cell-like state. FBs start to produce several proteins relevant for endothelial functions and form capillary-like networks *in vitro*. The acquired ability of LDL uptake also reflects an important function in endothelial induced FBs. Furthermore, FBs can be seeded onto a gelatin scaffold and be induced to differentiate towards an endothelial phenotype on site.

PAPER V

Adipogenic, chondrogenic and osteogenic induced FBs display the upregulation of several genes associated with adipocytes, chondrocytes and osteoblasts. Genes related to the differentiation process of progenitor cells into the above mentioned cell types were also identified (BENAYAHU, KLETTER *et al.* 1989; HUNG, CHANG *et al.* 2004; MEHLHORN, NIEMEYER *et al.* 2006; DJOUAD, DELORME *et al.* 2007; PERRIEN, AKEL *et al.* 2007).

DISCUSSION

In recent years, autologous ASCs have been proposed as a cell source for tissue engineering and reconstructive surgery. Several difficulties associated with the use of ASCs still remain, and the search for alternative cell sources is ongoing. The results presented in this thesis demonstrate that human dermal FBs possess stem cell plasticity, and can be induced to differentiate into cells with phenotypes resembling adipocytes, chondrocytes, endothelial cells, smooth muscle cells and osteoblasts.

Several reports published during the last years have supported the identification of differentiation of ASCs towards adipocyte-, chondrocyte- and osteoblast-like cells by using a variety of histological routine staining and immunohistochemical methods similar or identical to protocols used in paper I and V in this thesis (ZUK, ZHU *et al.* 2002; BARTSCH, YOO *et al.* 2005; YOUNG, DUPLAA *et al.* 2005; MASUOKA, ASAZUMA *et al.* 2006; CHEN, ZHANG *et al.* 2007; SOMMAR, PETTERSSON *et al.* 2009).

The results presented in paper I confirm the differentiation of FBs towards adipocyte-, chondrocyte- and osteoblast-like cell types. The results obtained from this study combined with present knowledge renders the FB a possible source for multiple autologous cell types for use in tissue engineering and reconstructive surgery.

Scar contracture is a common complication of deep and partial thickness burn wounds which can lead to severe disability for affected patients. Thus, it is of great importance to limit this pathological process in the scar during the rehabilitation of a burn wound. The contraction of the scar is largely dependent on cells generating mechanical forces in the granulation tissue. Current regimes for treating burn scar contraction involve active stretching of the burn area. Paper II demonstrates the effects of mechanical tension applied on burn scars *in vitro*, and that a continuous stretching of a burn scar in human skin increases the transdifferentiation of fibroblasts into myofibroblasts. This implies that current protocols used for the treatment of burn scars are counterproductive in regard to contraction of the tissue. The *in vitro* model used in paper II is limited by the exclusion of systemic inflammatory responses. However, all local components including extracellular matrix and viable cells are present in the tissue, thus the culture model represents a standardised way to investigate the responses to mechanical tension.

In paper III and IV, the phenotypical shift of FBs towards an endothelial-like cell type was studied. The results presented showed that FBs cultured in human serum expressed bradykinin B₂ receptor, eNOS, VE-cadherin and vWf,

incorporated fluorochrome labelled LDL, and formed capillary-like structures in an endothelial cell-like fashion. These findings are, to our knowledge, the first that describe a phenotypic change of human dermal FBs towards an endothelial-like cell type. The methods used for confirmation of differentiation have been employed to characterise endothelial cells in previous studies (VOYTA, VIA *et al.* 1984; RISAU 1995; BACHETTI and MORBIDELLI 2000; HANNAH, WILLIAMS *et al.* 2002). The results showed that the presence of human serum has a decisive role in the phenotypic change of FBs towards an endothelial cell-like phenotype. Human serum contains numerous growth factors that are crucial for the differentiation and proliferation of endothelial cells, some of which may be essential for inducing the phenotypic shift of FBs. VEGF plays an important part in angiogenesis, and TGF β ₁ is an important regulator of differentiation of endothelial cells and a major modulator of angiogenesis (YANG and MOSES 1990; SANKAR, MAHOOTI-BROOKS *et al.* 1996). It has also been postulated that VEGF is necessary for the *in vitro* differentiation of both endothelial precursor cells and hematopoietic stem cells into mature endothelial cells (ASAHARA, MUROHARA *et al.* 1997). However, neutralizing VEGF or TGF β ₁ did not affect the phenotypic shift. We therefore concluded that neither VEGF nor TGF- β ₁, at least not alone, is responsible for the induction of differentiation of FBs towards an endothelial-like cell type.

Endothelial differentiated FBs formed a confluent monolayer similar to that of endothelial cells, when cultured on a gelatin scaffold *in vitro*. This further strengthens the claim that FBs can be induced towards an endothelial cell phenotype. A confluent, anti-coagulative, and anti-thrombogenic cell layer is crucial for the function of a vascular graft. Previous studies have shown a correlation between the production of nitric oxide (NO) and the patency rates of vascular grafts used in bypass surgery (AMIEL, KOMURA *et al.* 2006). eNOS and bradykinin B₂ receptor are both vital for NO production, hence the presence of these proteins an important feature of FBs differentiated towards an endothelial cell-like state (GOVERS and RABELINK 2001; DUDZINSKI, IGARASHI *et al.* 2006). Results presented in paper IV indicate that FBs differentiated towards endothelial-like cells can serve as a functional cell layer substitute to endothelial cells. The results of these studies may have an impact on future cell sourcing in vascular tissue engineering. Endothelial differentiated FBs might function as a cell source for the formation of vessel networks in engineered three-dimensional tissues *in vitro* and facilitate the creation of tissue engineered grafts. Results obtained in paper II as well as previous published reports have suggested that fibroblasts can differentiate into smooth muscle cells (GABBIANI 2003; DESMOULIERE, CHAPONNIER *et al.* 2005). This implies the possibility of FBs giving rise to all three cell types required for the generation of a complete blood

vessel, which could facilitate the *in vitro* production of autologous vascular grafts and lead to improved possibilities of replacing damaged blood vessels with autologous tissue.

With the advancement of gene expression analysis and by employing micro array technology a greater understanding of the genetic basis for cellular differentiation can be achieved. In paper V adipogenic, chondrogenic and osteogenic FBs were studied in regard to gene expression using micro array analysis. Several genes shown up-regulated have important function in regard to differentiation of adult stem cells and normal function of terminally differentiated adipocytes, chondrocytes or osteoblasts. The three induction media used in paper I and V modified the cultures to different degrees, and while an overall shift of histology and morphology was seen, the heterogeneity of the induced cultures was apparent. Taking this into account, the gene expression analysis was performed on RNA obtained from heterogeneous cells along the entire spectrum of differentiation. This consideration becomes relevant in the choice of fold-change cut-off, which greatly affects the results of the micro array analysis. In paper V, a fold-change cut-off of 1.5 instead of 2.0 would have increased the number of identified genes 5-fold in all different groups. Chances are that more relevant genes are found in such extended lists than using a 2-fold cut-off, but at the same time the chances of random differences supporting false assumptions may overshadow the increased data output.

The presence of contaminant stem or precursor cells in the primary culture of FBs could possibly render false positive results in regard to differentiation capacity. This issue was addressed in several ways. Differentiation experiments in paper I, III and IV were performed using SCCFBs, which minimises the risk of contamination. Furthermore, flow cytometric analysis of SCCFBs in paper I confirmed them to not to be circulating bone marrow derived stem cells. The flow cytometric analysis of the FBs and SCCFBs revealed a set of CD markers upregulated on the SCCFBs. These markers have been employed in other studies focusing on adult stem cells from various tissues. The CD marker profile can be used in order to further characterise and purify the SCCFB populations.

The results obtained from our studies on SCCFBs also indicate that fusion between different cell types does not play a significant role in the phenotypical shift of FBs, as suggested by previous publications (CAMARGO, CHAMBERS *et al.* 2004). This is in line with publications exploring the inherent plasticity of FBs derived from dermis (BARTSCH, YOO *et al.* 2005). The results presented in this thesis suggest that the multilineage potential of dermal FBs is not necessarily dependant on the presence of subpopulations in the dermis.

FUTURE PERSPECTIVES

Further studies investigating additional features of adipogenic, chondrogenic, endothelial, myogenic and osteogenic differentiated FBs are needed before the employment of these cells in clinical applications. Studies which focus on the stability of the phenotypic shift after induction, both *in vitro* and *in vivo*, are of great importance in order to develop beneficial treatment regimes.

Future studies focusing on other lineages of differentiation might show how extensive the differentiation potential of FBs is. Current ongoing work is exploring differentiation spanning across germ layers. If possible, this feature may alter the current perception of the differentiation process inherent in adult human cells.

The *in vivo* differentiation of fibroblasts is an appealing scenario, which could lead to new regimens of treating diseases without the need of harvest and *in vitro* modification of cells.

CONCLUSIONS

In summary, the findings presented in this thesis suggest the use of human dermal FBs in tissue engineering applications. Given the apparent possibility of a phenotypic shift of FBs into cells with the characteristic of adipocytes, chondrocytes, endothelial cells, osteoblasts and smooth muscle cells, the generation of autologous cells for transplantation purposes is evident. This might render a plethora of clinically relevant treatment procedures using dermal FBs.

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