Biodegradable gelatin microcarriers
in tissue engineering

In vitro studies on cartilage and bone

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ABSTRACT

Tissue engineering is a multidisciplinary field that combines cells, biomaterial scaffolds and environmental factors to achieve functional tissue repair. This thesis focuses on the use of macroporous gelatin microcarriers as scaffolds in tissue engineering applications, with a special focus on cartilage and bone formation by human adult cells in vitro.

In our first study, human articular chondrocytes were seeded on macroporous gelatin microcarriers. The microcarriers were subsequently encapsulated in coagulated blood-derived biological glues and cultured under free-swelling conditions for up to 17 weeks. Even in the absence of recombinant chondrogenic growth factors, the chondrocytes remained viable and metabolically active for the duration of the culture period, as indicated by an increased amount of cell nuclei and extracellular matrix (ECM). The ECM showed several cartilage characteristics, but lacked the cartilage specific collagen type II. Furthermore, ECM formation was seen primarily in a capsule surrounding the tissue-engineered constructs, leading to the conclusion that the used in vitro models were unable to support true cartilage formation.

The capacity of human dermal fibroblasts to produce cartilage- and bone-like tissue in the previously mentioned model was also investigated. Under the influence of chondrogenic induction factors, including TGF-β1 and insulin, the fibroblasts produced cartilage specific molecules, as confirmed by indirect immunohistochemistry, however not collagen type II. Under osteogenic induction, by dexamethasone, ascorbate-2-phosphate and β-glycerophosphate, the fibroblasts formed a calcified matrix with bone specific markers, and an alkaline phosphatase assay corroborated a shift towards an osteoblast like phenotype. The osteogenic induction was enhanced by flow-induced shear stress in a spinner flask system.

In addition, four different types of gelatin microcarriers, differing by their internal pore diameter and their degree of gelatin cross-linking, were evaluated for their ability to support chondrocyte expansion. Chondrocyte densities on the microcarriers were monitored every other day over a two-week period, and chondrocyte growth was analyzed by piecewise linear regression and analysis of variance (ANOVA). No
differences were seen between the different microcarriers during the first week. However, during the second week of culture both microcarrier pore diameter and gelatin cross-linking had significant impacts on chondrocyte density.

Lastly, a dynamic centrifugation regime (f=12.5 mHz for 16 minutes every other day) was administered to chondrocyte-seeded microcarriers, with or without encapsulation in platelet rich plasma (PRP), to study the possible effect of dynamic stimuli on cartilage formation. Presence of PRP enhanced the structural stability of the tissue-engineered constructs, but we were not able to confirm any dose-response pattern between ECM formation and the applied forces. After 12 weeks, distinct gelatin degradation had occurred independent of both dynamic stimuli and presence of PRP.

In summary, this thesis supports a plausible use for gelatin microcarriers in tissue engineering of cartilage and bone. Microcarrier characteristics, specifically gelatin cross-linking and pore diameter, have been shown to affect chondrocyte expansion. In addition, the use of human dermal fibroblasts as an alternative cell source for cartilage and bone formation in vitro was addressed.
TABLE OF CONTENTS

Abstract ........................................................................................................i
Table of contents .......................................................................................... iv
Abbreviations ............................................................................................... 6
List of papers ................................................................................................. 7
Introduction .................................................................................................. 9
  Articular cartilage ....................................................................................... 9
  Tissue Engineering ..................................................................................... 13
  Cells ........................................................................................................... 15
  Biomaterials ............................................................................................... 17
  Environmental factors ................................................................................ 21
Aims of the study .......................................................................................... 25
  Paper I ....................................................................................................... 25
  Paper II ..................................................................................................... 25
  Paper III ................................................................................................... 25
  Paper IV .................................................................................................... 25
Comments on methods .................................................................................. 27
  Cell culture ............................................................................................... 27
  Analysis methods ..................................................................................... 33
Results and discussion ................................................................................ 39
  Gelatin microcarriers support adhesion and expansion of human articular chondrocytes (HACs) and human dermal fibroblasts (HDFs) .................................................................................. 39
  Blood-derived glues can be used for microcarrier encapsulation in vitro .................. 39
  PRP encapsulation enhances pellet stability and generates pellets with more uniform morphologies ................................................................................................................................. 40
  Expanded HACs produce cartilage-like tissue components but do not generate cartilage tissue in free-swelling conditions in the absence of recombinant chondrogenic growth factors ................................................................................................................................. 41
  Dynamic centrifugation every other day does not dramatically alter ECM synthesis of microcarrier-expanded HACs but may contribute to collagen fiber organization .......... 42
  HDFs produce cartilage-like tissue components on gelatin microcarriers under chondrogenic induction ................................................................................................................................. 43
## Table of contents

HDFs produce bone-like tissue components and upregulate ALP activity on gelatin microcarriers under osteogenic induction ................................................................. 43
Flow-induced shear stress enhances ECM mineralization and affects ALP activity in HDFs ................................................................................................................................. 44
Gelatin cross-linking affects HAC proliferation during microcarrier expansion ........ 44
Pore diameter affects HAC proliferation during microcarrier expansion ............... 45
Summary ........................................................................................................................................ 46

**General discussion and future perspectives** ......................................................... 47
On the efficacy of microcarriers for tissue engineering of cartilage.................... 47
On the use of *in vitro* models ..................................................................................... 48
On the influence of microcarrier characteristics ..................................................... 48
On the use of dermal fibroblasts in tissue engineering ............................................ 49

**Conclusions** .............................................................................................................. 51
**Acknowledgements** ............................................................................................... 53
**References** .................................................................................................................. 59
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D/3D</td>
<td>two/three-dimensional</td>
</tr>
<tr>
<td>ACI/ACT</td>
<td>autologous chondrocyte implantation/transplantation</td>
</tr>
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<td>ALP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>BMSC</td>
<td>bone marrow stem cells</td>
</tr>
<tr>
<td>CS</td>
<td>CultiSpher (Paper III)</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'6-diamino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>g</td>
<td>gram (unit)</td>
</tr>
<tr>
<td>g</td>
<td>acceleration (unit)</td>
</tr>
<tr>
<td>G, GL and GLS</td>
<td>CultiSpher microcarrier types</td>
</tr>
<tr>
<td>GAG</td>
<td>glucoseaminoglycan</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HAC</td>
<td>human articular chondrocytes</td>
</tr>
<tr>
<td>HDF</td>
<td>human dermal fibroblasts</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>LM</td>
<td>light microscopy</td>
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<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cells</td>
</tr>
<tr>
<td>MTTR</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NCS</td>
<td>newborn calf serum</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>personal cell analysis (Guava PCA)</td>
</tr>
<tr>
<td>PLM</td>
<td>polarized light microscopy</td>
</tr>
<tr>
<td>PPP</td>
<td>platelet poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>PSR</td>
<td>picrosirius red</td>
</tr>
<tr>
<td>RCF</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SZP</td>
<td>superficial zone protein, also known as PRG4 or lubricin</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>WB</td>
<td>whole blood</td>
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LIST OF PAPERS

Paper I  Sofia Pettersson, Jonas Wetterö, Pentti Tengvall, and Gunnar Kratz
Human articular chondrocytes on macroporous gelatin microcarriers form structurally stable constructs with blood-derived biological glues in vitro

Paper II  Pehr Sommar, Sofia Pettersson, Charlotte Ness, Hans Johnson, Gunnar Kratz, and Johan P.E. Junker
Engineering three-dimensional cartilage- and bonelike tissues using human dermal fibroblasts and macroporous gelatine microcarriers
In press

Paper III  Sofia Pettersson, Jonas Wetterö, Pentti Tengvall, and Gunnar Kratz
Cell expansion of human articular chondrocytes on macroporous gelatine microcarriers – impact of pore diameter and degree of gelatin cross-linking on cell proliferation
Manuscript

Paper IV  Sofia Pettersson, Jonas Wetterö, and Gunnar Kratz
The role of platelet rich plasma and dynamic centrifugation on extracellular matrix formation of human articular chondrocytes on macroporous gelatin microcarriers in pellet culture
Manuscript
INTRODUCTION

A frequent problem in modern medicine involves the loss or failure of tissues and organs. The consequences can be devastating for the individual patients, and treatment options are often limited by the lack of suitable donor tissue for transplantation or of appropriate prosthetic alternatives. The field of tissue engineering aims at restoring lost tissue by combining engineering and medical sciences, to develop functional biological substitutes [Langer and Vacanti 1993].

This thesis covers the use of gelatin microcarriers in tissue engineering applications, with focus on cartilage and bone formation in vitro. The basics and issues involved with tissue engineering will be discussed using examples from articular cartilage, as this presents relevant background for all four papers included in this thesis. Hence, the general function and structure of cartilage will be outlined in the introduction. For bone tissue other issues arise, most notably the need for vascularization, but this tissue will only be discussed briefly.

Articular cartilage

Articular cartilage serves as a load bearing, shock absorbing, and lubricating tissue in diarthrodial joints, where it covers the contact ends of long bones (Figure 1a). Besides being anchored to the underlying bone, the tissue is encased with synovial fluid that is enclosed by a synovial membrane surrounding the joint. The cartilage is neither vascularized nor innervated, and relies on the movement of synovial fluid to maintain the function and metabolism of the residing cells. Despite its relatively simple composition, the tissue has remarkable material properties, withstanding contact pressures in the megapascal range in human hip joints [Hodge et al. 1986].
Biodegradable gelatin microcarriers in tissue engineering

Figure 1: Articular cartilage covers the ends of long bones in diarthrodial joints (a). Several topographical zones can be distinguished between the synovial cavity and the subchondral bone (b). These zones differ in terms of collagen fiber organization (c) and chondrocyte morphology (d).

Articular cartilage structure

Cartilage has a fairly basic structure and consists of one unique cell type, known as chondrocytes, the extracellular matrix (ECM) that these cells produce and the synovial fluid. The chondrocytes are responsible for the synthesis and turnover of the tissue, yet they are sparsely distributed and make up a mere 1% of the tissue volume. The ECM is a complex network of collagens, proteoglycans, non-collagenous proteins and glycoproteins that provide the tissue with its unique material properties. The water content is high, accounting for 75–80% of the wet tissue mass, while the remaining matrix is composed of collagens (10–30%), proteoglycans (3–10%) and non-collagenous proteins and glycoproteins [Schulz and Bader 2007]. The collagen content is dominated by collagen type II (90–95%) and these fibers provide the tissue with tensile strength. Other collagen types, including collagen type IX and VI, are found mainly in the pericellular regions surrounding the chondrocytes [Poole 1997]. The major proteoglycan in articular cartilage tissue is aggrecan. It consists of a protein backbone, to which several glycosaminoglycans (GAG), such as keratan sulfate and chondroitin sulfate, are covalently attached. The highly negative charge of these side molecules gives the biomolecule a high swelling capacity that in turn enables the tissue to absorb high compressive forces [Schulz and Bader 2007].

Articular cartilage can be categorized into different topographical zones – the superficial zone, the middle zone, the deep zone, the tidemark zone and the calcified zone (Figure 1b). In the superficial zone, chondrocyte morphology is flattened, and the ECM is densely packed with collagen fibers running parallel to the surface.
middle zone, the cell density is reduced, and the chondrocytes display a more spherical morphology. Only few direct cell–cell interactions occur between the chondrocytes as the cells are relatively isolated, either individually or in small aggregates, in small cavities within the ECM called lacunae. The organization of the collagen fibers becomes more stochastic in the middle zone, and the proteoglycan content in the ECM increases. Even further down, in the deep zone, the chondrocytes are arranged in columns perpendicular to the surface and underlying bone (Figure 1d). Collagen fibers in the deep zone are also organized in this manner. The specific molecular composition of the ECM varies between the zones, with ECM molecules residing uniquely in some zones. For example, the superficial zone protein (SZP, also known as PRG4 or lubricin) acts as a lubricant at cartilage surfaces and is not found in the deeper zones [Schumacher et al. 1994]. Among the collagens, collagen type X is coupled with chondrocyte hypertrophy and is considered unique for the calcified zone that borders on the underlying bone [Schulz and Bader 2007]. The cellular activity also differs between the topographical zones, and the metabolic potential of the cells can vary depending on their topographic origin [Lee et al. 1998; Darling and Athanasiou 2005; Stenhamre et al. 2008].

Articular cartilage homeostasis relies on biomechanical conditioning. Joint movement leads to compression of the cartilage tissue and the displacement of synovial fluid, resulting in complex patterns of direct compression, hydrostatic pressure, tensile and shear forces throughout the tissue. Chondrocytes respond to these forces and the biomechanical stimuli is converted into intracellular signals through a process known as mechanotransduction. The detailed mechanisms of the mechanotransduction pathways, or the causal relationships between the applied forces and cellular responses, are not yet fully understood, but the current view has recently been reviewed [Ramage et al. 2009].

Articular cartilage pathology
Under normal conditions, articular cartilage is subject to little or no wear. Once damaged through trauma or degenerative joint disease, for example osteoarthritis, articular cartilage has a limited capacity for self-repair. The avascular nature of the tissue inhibits progenitor cells to be introduced from the blood flow. Also, unlike other forms of hyaline cartilage, articular cartilage is not surrounded by a perichondrium from which chondroprogenitor cells can migrate and subsequently differentiate into chondrocytes that contribute to cartilage healing. Instead, chondrogenesis
Biodegradable gelatin microcarriers in tissue engineering

must be accomplished by residing cells, through interstitial growth. The intrinsic ability of chondrocytes to do so is however limited. There have been recent reports on mesenchymal progenitor cells residing in articular cartilage, but these findings also indicate a possible correlation with osteoarthritis [Alsalamh et al. 2004].

There is a vast demand for clinically applicable cartilage regeneration strategies. In Sweden alone, 9600 total knee replacements and 14000 hip replacements were performed in 2007 [Höftprotesregistret 2008; Knäprotesregistret 2008]. The clinical approaches in practice today for articular cartilage repair often rely on the introduction of progenitor cells to the wound site. Microdrilling and microfracture are both subchondral techniques that induce bleeding and clot formation in the cartilage defect [Redman et al. 2005]. Progenitor cells from the blood flow and the bone marrow are then introduced to the void where they differentiate towards a chondrogenic phenotype. Soft tissue grafts, such as perichondrium and periosteum grafts, also introduce progenitor cells.

In 1994, Britberg et al. described a procedure where a single cell suspension of expanded autologous chondrocytes is introduced underneath a peristeum flap that has been firmly sutured over the debrided cartilage defect [Britberg et al. 1994]. The autologous chondrocyte implantation (ACI) method, sometimes referred to as autologous chondrocyte transplantation (ACT), thus offers direct delivery of differentiated chondrocytes as well as the possibility of migrating progenitor cells from the periosteum to assist during cartilage regeneration. Long-term outcomes demonstrate improvement for several patients [Peterson et al. 2000; Britberg et al. 2003]. However, the original method is rather invasive, as it requires open knee surgery to attach the periosteum securely, and subsequent research has focused on finding arthroscopic methods to shorten patient recovery periods [Erggelet et al. 2003; Zheng et al. 2007]. The ACI method from 1994 is one of the most referenced methods in the field of cartilage tissue engineering.

Though several cartilage repair strategies improve joint function, most yield a cartilage tissue that is immature and fails to meet the mechanical properties of articular cartilage [Redman et al. 2005]. In addition, the repair tissue often lacks the zonal organization of articular cartilage. Combined, these shortcomings may lead to insufficient tissue integration and degradation of the repair tissue. The field of cartilage tissue engineering aims to identify materials and methods that will improve cartilage tissue repair.
Tissue Engineering

According to a recent editorial, the term tissue engineering was first described in the 1980s, and the first official definition was agreed upon in 1987 [Lysaght and Crager 2009]. There have been many subsequent attempts to redefine the term. In addition, it has been coupled with the related term regenerative medicine.

Regenerative medicine/tissue engineering is a rapidly growing multidisciplinary field involving the life, physical, and engineering sciences that seeks to develop functional cell, tissue, and organ substitutes to repair, replace, or enhance biological function that has been lost due to congenital abnormalities, injury, disease, or aging.

National Institute of Biomedical Imaging and Bioengineering, 2004

In practice, a common approach in tissue engineering is to seed cells on a biomaterial scaffold that serves as a substrate onto which anchorage dependent cells can adhere. Scaffolds can be designed and formed into practically any desired shape to suit the intended application. The cell–biomaterial construct is then cultured in vitro and/or implanted in vivo until tissue formation occurs (Figure 2). A widespread way to illustrate the field is thus to use a triangle, where each corner represent cells, biomaterials and environmental factors respectively (Figure 3). Below, environmental factors is used as a collective term for biomolecules, engineering methods, and in vitro designs that are used with the purpose of initiating, stimulating, guiding or enhancing tissue formation. The distinction between the research areas depicted in the triangle is not always obvious. Biomaterials can for example, by their design alone, stimulate cellular activities and tissue formation. This highly co-dependent pattern between parameters illustrates the multidisciplinarity of the research field.
Biodegradable gelatin microcarriers in tissue engineering

**Figure 2:** A commonly used approach in tissue engineering involves an initial harvest of donor tissue (i), from which cells are isolated and subsequently expanded (ii). Expanded cells are seeded onto a biomaterial scaffold (iii) in an *in vitro* setting (iv). The resulting cell–biomaterial construct can be cultured *in vitro* or directly implanted *in vivo* (v).

**Figure 3:** The triangle representing the tissue engineering paradigm where cells, biomaterials and environmental factors are combined to engineer tissues.

The previously mentioned approach, to seed cells on a biomaterial scaffold, has been named the *open matrix strategy* by Langer and Vacanti and is frequently used [Langer and Vacanti 1993]. However, not all regenerative strategies employ all of the three elements in the triangle. In *guided regeneration*, biomaterial scaffolds can be implanted without previous cell seeding. The biomaterial then acts as a scaffold into which cells from adjacent tissues can migrate. If instead the biomaterial is omitted, the term cell-based or *scaffold-free tissue engineering* is often seen in the literature. The environmental factors can never be fully removed, as cells are always affected by their surroundings, physiological or artificial. Current research in this area is consequently focused on identifying the environmental cues that will optimize tissue formation.
Introduction

Cells
An ideal cell type for tissue engineering must be easy to harvest, isolate and expand into great numbers in a rapid and cost-effective manner. Additionally, it should not cause harm to or elicit an immune response in the intended host. In reality, this is not always so easily accomplished. The supply of donor tissue is often limited and donor site morbidity must be considered, especially for tissues where the capacity of self-repair is limited, such as cartilage. For some cell types, the isolation of the desired cell type can also be challenging and expansion techniques complex and costly.

Cell sources in tissue engineering
The long-term clinical goal for most tissue engineering approaches involves the use of autologous cells, that is, cells that have been isolated from the intended recipient. As discussed above, the supply of these cells is often limited. For research purposes, many researchers instead opt for animal cells or commercially available cell lines. Animal cells are often easier to obtain and well-established cell lines offer the advantage of reproducibility. The relevance of such findings is however difficult to put into perspective, as the metabolic activity and phenotypic stability of the cells may vary significantly between different species [Akens and Hurtig 2005; Giannoni et al. 2005]. This makes it difficult to compare and translate results obtained with such cells to applications where primary human cells are eventually meant to be used. Even within a species, results can vary depending on donor age and biopsy location [Barbero et al. 2004; Akens and Hurtig 2005; Stenhamre et al. 2008]. The donor-to-donor variability is one of the general difficulties with experimental designs using human cells, as the reproducibility of obtained results can, and should, be questioned. Another issue concerns differences between cells from unaffected donors and the intended patient groups. For example, studies have shown discrepancies between chondrocytes from osteoarthritic patients and healthy controls [Tallheden et al. 2005a; Yang et al. 2006].

Human cell sources in tissue engineering can be divided into i) differentiated, or tissue specific, cells and ii) adult stem cells.

Differentiated cells
Differentiated cells are specialized cells that have been isolated from the tissue of interest. Intuitively, these cells are a logical choice, as they build and maintain the tissues in vivo. However, there are some problems associated with their use. Apart from the issues involving the limited supply of donor tissue and donor site morbidity that
Biodegradable gelatin microcarriers in tissue engineering

have been previously discussed, some differentiated cell types require time-consuming and costly methods, such as for example co-culture with other cells, to adhere and proliferate in vitro. The stability of the intrinsic phenotype is another issue. In the case of cartilage, chondrocytes rapidly lose their phenotype when cultured in monolayer culture in a process known as dedifferentiation [Holtzer et al. 1960; von der Mark et al. 1977]. The most crucial consequence of this process is that these cells no longer produce collagen type II, a cartilage-specific collagen that provides tensile strength. Though these cells have been shown to regain some of their phenotypic characteristics when transferred into a three-dimensional environment, a process consequently known as redifferentiation, the redifferentiation potential is related to the expansion period in vitro [Benya and Shaffer 1982; Darling and Athanasiou 2005; Kang et al. 2007]. This poses a powerful dilemma as high initial cell seeding densities significantly improves the outcome [Mauck et al. 2003; Eyrich et al. 2007; Hayes et al. 2007]. Different strategies have been evaluated to protect the chondrogenic phenotype during in vitro expansion [Malda et al. 2003a; Malda et al. 2003b; Gigout et al. 2005; Hendriks et al. 2006].

Adult stem cells
In recent years, researchers have begun to investigate alternative cell sources for regenerative medicine, such as stem cells. Stem cells are undifferentiated cells that are defined by their ability to self-renew and to differentiate along certain developmental pathways. They are classified according to their differentiation potential. Totipotent stem cells can give rise to any cell lineage, pluripotent stem cells are more limited in this capacity, and monopotent cells are considered to be tissue-committed. In vivo, these cells aid in the repair and renewal of human tissue, while maintaining the adult stem cell population by self-renewal. Embryonic stem cells are by definition totipotent, but the issues associated with the difficulty to control their differentiation in vivo, are discouraging [Blum and Benvenisty 2008].

In regenerative medicine, the controlled differentiation of adult stem cells in vitro has gained momentous attention in later years. Bone marrow stem cells, BMSCs, or mesenchymal stem cells, MSCs, are able to proliferate in vitro while maintaining their ability to differentiate towards chondrocyte, adipocyte and osteoblast phenotypes in response to biochemical factors [Jaiswal et al. 1997; Yoo et al. 1998; Pittenger et al. 1999; Jaiswal et al. 2000]. Cells isolated from various other human tissues, including adipose, muscle and dermal tissue, have also been reported to possess a certain level
of stem cell plasticity in response to environmental factors [Young et al. 1995; Warejcka et al. 1996; Young et al. 2001; Zuk et al. 2001; Jahoda et al. 2003; Bartsch et al. 2005]. The underlying mechanisms, including the identification of possible cellular sub-populations responsible for this multipotency, are subject to an ongoing debate [Eisenberg and Eisenberg 2003].

The ability of adult stem cells to differentiate into phenotypes and produce tissues distinct from their tissue of origin provides an exciting alternative to differentiated cells. For these strategies to become clinically feasible, the challenges regarding isolation, long-term expansion and classification of adult stem cells must be solved [Gardner 2007; Seeger et al. 2007]. Yet, the fact that cells isolated from several diverse tissues possess the ability to alter their phenotype, in effect, challenges the concept of adult somatic cell monopotency.

**Biomaterials**

Numerous classes of biomaterials are used in medicine today, including metals, ceramics, glasses, polymers and various composites. The field of biomaterial science is vast, and covers areas well beyond tissue engineering. In order to understand the role of biomaterials in tissue engineering, a brief introduction to the field, highlighting some of the definitions and principles that are most relevant to tissue engineering and this thesis, is given below.

In 1986, the European Society for Biomaterials agreed on the following definition [Williams 1987].

*A biomaterial is a nonviable material used in a medical device, intended to interact with biological systems.*

Williams DF, 1987

With the emergence of several biomaterial applications in later years some alterations have been suggested to the definition, especially regarding the words *non-viable* and *medical*, to include the wide range of pre-clinical, analytical and regenerative uses for these materials. The intent to interact with a biological system, specifically to a host, requires the material not to elicit an inappropriate host response. This crucial point is addressed with the term *biocompatibility*. 
Biodegradable gelatin microcarriers in tissue engineering

Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application.

Williams DF, 1987

The phrasing is deliberately vague, as both the material performance and the appropriate response may differ considerably between different applications. In fact, the definitions have recently been revisited and rephrased to mirror this more clearly [Williams 2008]. Finally, the scaffolds are often designed to act as an intermediate support for tissue regeneration and are consequently meant to gradually degrade and disappear when new tissue is formed. The term biodegradable is used with the following definition.

Biodegradation is the chemical breakdown of materials by the action of living organisms, which leads to changes in physical properties.

Williams DF, 1987

Though this is a wide definition, a host-induced deterioration of the biomaterial is implied. Needless to say, the degradation products of a biocompatible material must be biocompatible as well.

Biomaterial design in tissue engineering

The role of a biomaterial scaffold in tissue engineering can be fundamentally different from that of many biomaterials in various long-term medical devices, in that it should typically more profoundly encourage and elicit a cellular response. The chemical and architectural properties of a scaffold are utilized to trigger and optimize a response rather than to minimize it, as would be the case for any biomaterial to be used in for example coronary stents. The following definition for biocompatibility has been suggested for scaffolds to be used in tissue engineering.

The biocompatibility of a scaffold or matrix for a tissue engineering product refers to the ability to perform as a substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signaling sys-
tems, in order to optimize tissue regeneration, without eliciting any undesirable local or systemic responses in the eventual host.

Williams DF, 2008

This definition summarizes the crucial requirements for any biomaterial to be used in tissue engineering. In short, it should support and optimize the formation of new tissue without causing harm to the intended host. The challenge in biomaterial design thus lies in the identification and optimization of the scaffold that will optimize the tissue formation for each specific tissue and cell type.

A primary requirement is that the scaffold must support cellular adhesion. Not all materials allow for direct cell–material interactions, and initial protein adsorption is often a pre-requisite for such connections [Wilson et al. 2005]. Alternatively, the surfaces can be chemically modified to allow for cell adhesion [Chen et al. 2006]. In addition to surface chemistry, the topography and porosity of biomaterial surfaces can affect cellular adhesion and morphology [Lee et al. 1994; Curtis and Wilkinson 1997; Mukherjee et al. 2008]. Porous structures enhance surface areas, facilitate homogenous cell distribution and enable cell–cell interactions, provided that pores are interconnected [Spiteri et al. 2006; Chung et al. 2008; Kang et al. 2009]. The three-dimensional structure of the bulk material, such as porosity and pore diameters, can also affect cellular activities, including ECM synthesis, and should thus be identified and chosen to favor a rapid and tissue-specific metabolism [Karageorgiou and Kaplan 2005; Griffon et al. 2006; Yamane et al. 2007]. The material properties of the scaffold should be considered to promote regeneration [Kelly and Prendergast 2006]. When biodegradable scaffolds are used, the degradation rate should be adjusted to match the formation of new tissue, in order to avoid loss of integrity or inhibition of ECM synthesis during tissue regeneration. The stability of the material can be controlled by altering the chemical composition and degree of cross-linking [Zeugolis et al. 2009]. Once implanted, the newly formed tissue should preferably integrate seamlessly with the surrounding tissue, to avoid the risk of wear at the interface. This can be accomplished with a biological glue fixative, when the tissue has been developed in vitro, or by encouraging adjacent cells to partake in tissue formation in situ.
Polymer scaffolds in cartilage and bone tissue engineering
A common approach in cartilage engineering, and the most relevant for this thesis, is the use of porous polymer scaffolds. Both natural and synthetic polymers are being investigated in musculoskeletal tissue engineering. Natural polymers are derived from naturally occurring polymers. These include polymers derived from ECM proteins and polysaccharides, such as collagen, gelatin and chondroitin sulfate, as well as polymers derived from diverse parts of nature, including starch-based polymers, chitosan and alginate [Malafaya et al. 2007]. The advantages of these polymers include their intrinsic ability to support biological processes. However, it can be difficult to retain control between different batches, resulting in material variability that may affect reproducibility. In contrast, the chemistry and material properties of synthetic polymers can be well controlled. Synthetic polymers include polyesters such as poly(glycolic acid), poly[lactic acid] and their copolymers, poly(lactones), most notably polycaprolactone, as well as various polyanhydrides and polyurethanes [Gunatillake and Adhikari 2003]. These polymers offer extensive possibilities to tailor material properties and also allow longer shelf life than natural polymers. On the other hand, they lack the inherent ability to support biological processes and may therefore be more prone to elicit foreign body responses from the immune system. A common approach is to combine different polymers to optimize material properties and gain some of the advantages from each type used [Gunatillake and Adhikari 2003; Hong et al. 2005; Thissen et al. 2006; Malafaya et al. 2007; Lao et al. 2008].

The three-dimensional architecture of these materials can be varied in numerous ways. Polymers can be woven, spun, freeze-dried, solvent-cast or processed with gas foam into porous three-dimensional structures. With the ACI method from 1994 in mind, scaffold materials are often designed to allow for arthroscopic delivery. Apart from development of deformable sponges, that can also be administered arthroscopically, two strategies distinguish themselves – the use of hydrogels and microcarriers. Hydrogels are defined as water-based colloidal gels, whereas microcarriers often come in the form of sphere-shaped scaffolds, with diameters in the micrometer range. A wide range of microcarriers, manufactured from synthetic and/or natural polymers, with porous or non-porous structures, has been investigated as scaffolds for cell expansion and tissue engineering [Frondoza et al. 1996; Malda et al. 2003b; Chung et al. 2008; Lao et al. 2008]. The microcarriers that are the focus of this thesis are manufactured from gelatin, derived from collagen type I, and have a macro-porous structure (Figure 4).
Introduction

Figure 4: CultiSpher microcarriers are macroporous particles manufactured from gelatin, derived from collagen type I.

For bone, several different types of biomaterials, including ceramics, glasses and various composites, are investigated in addition to the polymer approach [Karageorgiou and Kaplan 2005]. Considering the natural link between the two tissues, some efforts towards developing biphasic scaffolds for osteochondral constructs have also been made, as has been recently reviewed elsewhere [Martin et al. 2007; Keeney and Pandit 2009].

Environmental factors
The most elusive discovery in tissue engineering concerns the identification of the environmental factors required to stimulate optimal tissue formation in vitro and in vivo. Several different strategies, all with the common goal of enhancing the quantity and quality of the engineered tissue, are currently being investigated. Combinations of two or more stimulating factors often yield synergistic effects, illustrating the need
Biodegradable gelatin microcarriers in tissue engineering

to identify the correct types, doses, combinations and sequences of these factors for each tissue and cell type. Some of the most commonly used principles in tissue engineering are discussed below.

**Biochemical factors**

A potent and commonly used approach to stimulate differentiation and tissue formation is by adding biochemical factors to the cell culture media. These include growth factors, hormones and cytokines that guide the proliferation and differentiation of cells. Their use is especially crucial for guiding adult stem cells through induction of differentiation pathways, but the strategy can also be used to preserve the intrinsic phenotype, or trigger the redifferentiation of chondrocytes after the expansion period. A large number of growth factors have been shown to have chondrogenic and osteogenic properties. For cartilage, the transforming growth factor beta (TGF-β) superfamily, including the bone morphogenetic protein (BMP) subclass, has proven especially important, but several other factors, such as fibroblast growth factor (FGF), insulin-like growth factor (IGF) as well as insulin itself have positive effects on chondrogenesis [Leboy et al. 1997; Jakob et al. 2001; Blunk et al. 2002; Awad et al. 2003; Malda et al. 2003a]. In addition, the presence and concentrations of other media additives, such as ascorbic acid and calcium, affect cellular mechanisms [Sullivan et al. 1994; Leboy et al. 1997; Gigout et al. 2005]. The chondrogenic and osteogenic differentiation pathways are closely related, and the BMPs in the TGF-β superfamily are consequently highly significant for bone formation as well [Cheng et al. 2003]. Other widely used osteogenic differentiation factors include ascorbate-2-phosphate, dexamethasone and β-glycerophosphate [Grigoriadis et al. 1988; Cheng et al. 1994; Zuk et al. 2001].

As an alternative to recombinant growth factors, autologous sources for chondrogenic growth factors have recently gained interest. For cartilage, human platelet supernatants have been used as cell culture media additives during chondrocyte expansion as well as three-dimensional culture [Gaissmaier et al. 2005; Tallheden et al. 2005b; Akeda et al. 2006]. In these cases, platelets from human plasma have been activated and the growth factors released from platelet alpha granules subsequently extracted in liquid form. Alternatively, platelet rich plasma (PRP) can be used as a scaffold or as part of the study design for cell adhesion purposes [Malicev et al. 2007; Wu et al. 2007; Haberhauer et al. 2008]. Regardless of method, the concentration of growth factors can differ as a result of donor-to-donor variability as well as differences
in the methods used during plasma coagulation and platelet activation [Weibrich et al. 2002; Tallheden et al. 2005b]. A strategy that merges with biomaterial surface modification involves recombinant growth factors to be chemically bound to or entrapped within biomaterial scaffolds to allow for controlled release during \textit{in vivo} or \textit{in vitro} culture [Fan et al. 2006].

**Biomechanical factors**

In many cases, the quantity and quality of the engineered tissue is enhanced when tissues are cultured under the influence of biomechanical conditioning. This is especially true when engineering tissues that experience biomechanical cues \textit{in vivo}, such as bone, cartilage and blood vessels.

A \textit{bioreactor} can be regarded as a well-defined \textit{in vitro} system that controls or supports a biologically active environment. As such, the definition can apply to an ordinary incubator. For tissue engineering purposes however, the optimal bioreactor system should enable a controlled environment that mimics the physiological conditions that appear \textit{in vivo} [Schulz and Bader 2007]. In reality, this task is difficult to achieve and most bioreactors mainly aim to minimize the difference between the complex, fine-tuned \textit{in vivo} environment and the comparatively crude \textit{in vitro} system. This can be achieved by maintaining conditions, for example by introducing one or more parameters, that stimulate tissue formation \textit{in vitro}. In the literature, the term is often used for any device that improves cell seeding, cell specific behavior or tissue formation \textit{in vitro}.

Several different principles for biomechanical stimulation are used in bioreactors for cartilage and bone engineering, including compression, hydrostatic pressure, perfusion, shear stress as well as combinations thereof. The rationale for using bioreactors in cartilage engineering, along with detailed descriptions on the different principles and systems used, has recently been extensively reviewed [Schulz and Bader 2007]. In addition to the underlying principle of the applied forces, the duration, frequency and amplitude of the administered regimes matter [Lee and Bader 1997; Maeda et al. 2001; Davisson et al. 2002; Waldman \textit{et al.} 2003; Waldman \textit{et al.} 2004].

**Oxygen tension**

In addition to biochemical and biomechanical factors, other environmental factors have also gained recent interest. \textit{In vivo}, articular cartilage is subjected to low oxygen levels of approximately 1–6 % [Silver 1975; Fermor \textit{et al.} 2007]. The importance of
these hypoxic conditions has been investigated recently, and reduced oxygen levels during three-dimensional in vitro culture elevate tissue formation in comparison to normoxic conditions [Domm et al. 2002; Malda et al. 2004; Wernike et al. 2008]. Hypoxia also induces chondrogenic differentiation of MSC cell lines [Robins et al. 2005].

3D environment
The three-dimensional environment experienced by the cells can also have stimulating qualities. For example, the accumulation or presence of bonelike ECM molecules enhance the osteogenic differentiation of both rat and human MSCs [Salasznyk et al. 2004; Datta et al. 2006]. This again highlights the significance of cell–material interactions.
AIMS OF THE STUDY

The overall aim of this thesis has been to investigate the use of biodegradable macroporous gelatin microcarriers in tissue engineering applications with focus on in vitro formation of cartilage and bone.

**Paper I**
To investigate the use of chondrocyte-seeded gelatin microcarriers for in vitro formation of cartilage. To evaluate the potential of blood-derived glues for encapsulation of cell-seeded microcarriers. To appraise the performance of two free-swelling culture models for in vitro formation of cartilaginous tissue in the absence of recombinant growth factors.

**Paper II**
To study in vitro formation of bone- and cartilage-like tissue by human dermal fibroblasts on gelatin microcarriers under osteogenic and chondrogenic induction. To investigate the influence of flow-induced shear stress on osteogenic differentiation of human dermal fibroblasts adhering to gelatin microcarriers in a spinner flask system.

**Paper III**
To analyze the impact of microcarrier characteristics, specifically pore diameters and degrees of gelatin cross-linking, on chondrocyte expansion in a spinner flask system.

**Paper IV**
To explore the effect of dynamic centrifugation every other day on ECM synthesis by human articular chondrocytes on gelatin microcarriers. To examine the influence of platelet rich plasma (PRP) in this model.
COMMENTS ON METHODS

This chapter features the methods used during this thesis. For further specific details regarding the materials and methods used, see the Materials and methods section of Papers I–IV.

Cell culture

Chondrocyte isolation
In Papers I, III and IV, human articular chondrocytes (HACs) were isolated from tissue obtained from total knee arthroplasties. The discarded tissue was covered with sterile saline solution during transportation, and, unless processed immediately, covered with low serum cell culture media and kept at 4 °C over night. Visibly damaged, i.e. coarse and yellowed, tissue was removed and the remaining cartilage tissue was dissected and enzymatically digested according to the details in each respective paper. Chondrocytes were isolated from both the femoral and tibial condyles, from all topographical zones. When the cell yield following chondrocyte isolation was determined, it reached approximately $8 \times 10^6$ chondrocytes per knee.

Fibroblast isolation
In Paper II, human dermal fibroblasts (HDFs) were isolated from dermal tissue following routine plastic surgery. The tissue was stored briefly in sterile saline and processed within 24 hours. The dermal layer was dissected in smaller fragments and fibroblasts were isolated by enzymatic digestion according to Paper II.

Cell culture media
Initially, all chondrocyte expansion and tissue construct cultures were conducted using a basic growth media. This cell culture media was later changed to a frequently
Biodegradable gelatin microcarriers in tissue engineering

used chondrocyte growth media [Freed et al. 1997; Malda et al. 2003a; Chung et al. 2008]. In Paper I, both cell culture media are represented in the included experiments. In Papers III–IV, only the chondrocyte growth media was used.

With the exception of Paper II, where TGF-β plays a crucial part in the study design, no recombinant chondrogenic growth factors were added to the cell culture media. Several chondrogenic growth factors, TGF-β included, are however known to be released from platelet alpha granules [Weibrich et al. 2002; Gaisser et al. 2005; Akeda et al. 2006]. This concerns all groups using PRP and whole blood encapsulation, in Papers I, II and IV respectively. Recombinant growth factors were excluded to minimize the risk of masking the effects of the included materials. For example, the relevance of any comparison between the platelet rich plasma (PRP) and platelet poor plasma (PPP) groups in Paper I would have been limited if a powerful chondrogenic growth factor had been continuously added.

The differentiation factors used in Paper II for chondrogenic and osteogenic differentiation have been described previously [Grigoriadis et al. 1988; Pittenger et al. 1999; Zuk et al. 2002]. In short, the chondrogenic induction media contained TGF-β and insulin, whereas the osteogenic induction media contained dexamethasone and β-glycerophosphate. In addition, both media contained ascorbate-2-phosphate. These induction media have previously been used for differentiation of HDFs in monolayer culture [Junker et al. 2009].

Expansion methods
In Papers I and III, HACs were expanded by traditional monolayer culture techniques using polystyrene flasks. This cell culture technique yields great expansion numbers for many cell types, including chondrocytes. The disadvantages of using this type of plastic have been mentioned previously [Holtzer et al. 1960; von der Mark et al. 1977]. In Paper I (Experiment VI) and Paper IV, HACs were seeded directly onto the gelatin microcarriers once isolated. These changes were made to enhance the chondrocyte redifferentiation potential [Malda et al. 2003a; Melero-Martin et al. 2006]. In Paper II, HDFs were expanded in monolayer using polystyrene flasks. The cells were then seeded onto microcarriers and expanded for an additional two weeks in spinner flasks before induction started.

Microcarrier selection
In Papers I, II and IV, CultiSpher GL microcarriers were used. Compared to the more commonly used CultiSpher G and CultiSpher S microcarriers, this microcar-
Comments on methods

Microcarrier has a slightly larger average internal pore diameter, 30 µm compared to 20 µm, when hydrated in PBS (for details, refer to Paper III). The microcarrier choice for these papers was made before the results of Paper III were analyzed, and was based on visual evaluation of sectioned and histologically stained material, indicating that cells did not occupy the interior of the microcarriers. The rationale for using a microcarrier with a larger pore size was thus to encourage cells to migrate into these pores and populate the entire microcarrier.

Microcarrier encapsulation
In Paper I, microcarriers were encapsulated in whole blood, re-calcified citrated PRP, re-calcified citrated PPP and diluted Tisseel [Goessl and Redl 2005]. Non-diluted Tisseel has also been evaluated, but our results indicated that it was too dense to support diffusion of nutrients in free-swelling conditions. In Papers II and IV, gelatin microcarriers were encapsulated in re-calcified citrated PRP. The basis for using PRP for encapsulation originates from previous research indicating that PRP, when used in combination with bone grafts, accelerated bone healing [Marx et al. 1998]. We hypothesized that PRP could be used to encapsulate pre-seeded microcarriers in a similar way. The whole blood and PPP groups were added to the design following a discussion regarding the role of platelets and other blood and plasma constituents in this regard. As these glues are readily available from the patient’s own blood, they present an appealing autologous alternative to the off the shelf commercial fibrin glues. It is however important to note that the PRP used in this study was not prepared from blood from the chondrocyte donors. There was no microcarrier encapsulation in Paper III.

In vitro models for tissue engineering of cartilage
In Paper I, cell-seeded microcarriers were encapsulated in PRP in two models. The first model utilizes cell culture inserts with permeable membranes. This model allows media perfusion from the top as well as from the bottom of the tissue-engineered constructs. This approach was also used in Paper II. The second experimental model involves forming of the clots as pellets in polypropylene tubes. As this strategy facilitates centrifugation of tissue-engineered constructs, the pellet model was used in Paper IV. Centrifugation of constructs in the cell culture insert model, by using microwell plate holders, has also been performed according to an acceleration curve similar to those used in Paper IV (see Biomechanical stimulation below). For both models, calcium was added to initiate clotting of the citrated blood-derived glues. The most straight-
Biodegradable gelatin microcarriers in tissue engineering

forward way of differentiating between the two models is by comparing Experiments V and VI in Paper I, where HACs from the same donor were used.

**In vitro models for tissue engineering of bone**

Two different models were used for osteogenic induced fibroblasts in Paper II. In addition to the cell culture insert model described above, cell-seeded microcarriers were kept in a spinner flask system for the duration of the experiment, to investigate if fluid flow-induced shear stress increases the level of osteogenic induction in HDFs.

**Biomechanical stimulation**

There was no biomechanical stimulation in Paper I, as the study was designed to investigate cartilage formation under free-swelling conditions.

In Paper II, cell-seeded microcarriers were kept in a spinner flask system in fibroblast and osteoinductive cell culture media. Microcarrier aggregates were allowed to form, as the primary objective in this study was to study 3D formation of bone tissue. In Paper III, using the same spinner flask system, these aggregates were disrupted to ensure a homogeneous distribution of microcarriers in the flask at each sampling point.

In Paper IV, dynamic centrifugation was administered to the microcarrier pellets according to three different acceleration curves, differing by their top relative centrifugal force $g_{\text{max}}$ (Figure 5). The centrifugation regime was administered for a total of 16 min with a frequency of 12.5 mHz every other day. This is not within physiological range, but dynamic stimuli have been proven efficacious at these frequencies previously [Sah et al. 1989; Buschmann et al. 1995; Davisson et al. 2002]. The acceleration curves also incorporated a period of total rest, to allow the constructs to regain their shape after compression [Barker and Seedhom 1997].
Figure 5: In Paper IV, dynamic centrifugation was administered with a Sigma K415C centrifuge with swing out rotors (a). Dynamic acceleration curves (f=12.5 mHz) were programmed with differing top speeds, corresponding to 500 g, 1500 g, and 3000 g respectively (b).

In an unpublished study, direct unconfined compression was tested using a custom-build compression device (Figure 6) designed for use with cell culture inserts in a 12-well culture plate. The dynamic compression regime included loaded and non-loaded periods and was run for a total of 15.5 min with a frequency of 13 mHz. Again, this frequency is outside the physiological range but inside the effective range [Sah et al. 1989; Buschmann et al. 1995; Davisson et al. 2002]. The same study included cell culture insert plates subjected to dynamic centrifugation (g\text{max}=500 g) with the same frequency as the dynamic compression. Control constructs were kept under free-swelling conditions. Due to the lack of quantitative control of the forces applied to the constructs with the compression device, these results have not been published.
Figure 6: The custom-built compression device used during supplemental experiments to this thesis.

Control groups

In Paper I, containing six separate experiments, several different types of control groups were included (see Paper I for details). In Experiment I and II, microcarrier-free clots with monolayer-expanded chondrocytes were used to assess the role of the gelatin scaffold. In Experiment V, cell-free microcarriers were embedded in PRP to investigate the stability of the microcarrier–PRP clots. In Experiment VI, pellets were formed with cell-seeded microcarriers in the absence of biological glue to study matrix formation without PRP. In Paper II, controls included cell-free microcarriers kept under identical conditions. To study the effects of the different induction media, control fibroblasts were kept in equivalent models.

In Paper III, the CultiSpher G microcarrier served as a reference in the statistical analysis. The cell counts were converted to cell densities and normalized for gram gelatin dry weight. For further details regarding the gelatin effect on cell counting, see the details for the Guava PCA analysis below. In Paper IV, unstimulated groups acted as control groups for the centrifugation regime, while PRP-free pellets served as controls for each PRP-containing equivalent.

For controls related to analysis methods, see details for each method in the following section.
**Time points**
In Paper I, time points varied between 4 hours and 17 weeks. The earlier time points were chosen to investigate when cell migration or cell death occurred at sample centers. Obtained results indicate that little or no matrix formed prior to 4 weeks. As a result, samples were only taken after 4, 8 and 12 weeks of culture in Papers II and IV. In Paper III, samples were withdrawn from the culture flasks every other day to monitor cell growth.

**Analysis methods**
This section highlights some aspects of the analysis methods that have been employed to evaluate scaffold characteristics, cell densities as well as ECM formation.

**Microcarrier characterization**
In Paper III, scanning electron microscopy (SEM) was used to visualize microcarrier structures. Dehydrated microcarriers were sputtered with a 15-µm layer of platinum prior to microscopy.

**Viability assay**
In Papers I–IV, the viability of cells seeded on the microcarriers was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [Mosmann 1983; Denizot and Lang 1986]. The method is based on the reduction of MTT and the formation of violet MTT formazan crystals in the cytoplasm of viable cells. There has been no indication of non-specific staining of MTT associated with the gelatin matrices used in this thesis. The Guava ViaCount assay can distinguish between viable and non-viable cells, but this data has been used sparsely. In Paper III, the viable cell count is used to determine the number of viable cells that were seeded onto the gelatin microcarriers; however, the viable cell count was not used when determining cell densities on the microcarriers. For details, see Cell counting below and Paper III.

**Cell counting**
In Papers I–IV, cell counts were established using a Guava Personal Cell Analysis (PCA) flow cytometer with the Guava ViaCount assay. This method uses two DNA-binding dyes with different permeabilities to distinguish between nucleated and dying cells [Phi-Wilson et al. 2001]. When seeded on microcarriers, cells were detached by dissolving the gelatin in a mixture of EDTA and trypsin. A representative plot from
Biodegradable gelatin microcarriers in tissue engineering

the ViaCount assay following microcarrier-based cell expansion can be seen in Figure 7. For well-populated microcarriers, prolonged trypsin incubations and vigorous micropipette mixing were required to ensure a homogeneous cell solution. This may well have affected the accuracy of the cell viability assay (for details, refer to Paper III). This method was validated using i) dissolved cell-free microcarriers in EDTA/trypsin, ii) EDTA/Trypsin and iii) ViaCount reagent alone. The results show that the accuracy of the measurements is acceptable up to the third significant figure.

![Figure 7: Representative plot from the Guava PCA flow cytometer ViaCount analysis used in Paper III.](image)

Sample Processing

In Papers I-IV, sample constructs were fixed in buffered formaldehyde, for up to 24 hours, and paraffin embedded prior to sectioning. Samples were uniformly positioned when possible, yet differences in pellet morphologies and the influence of the sectioning plane may have had an affect on the outcome of these analyses. In addition, artifacts such as ripping, tearing and parallel structures derived from the movement of the blade over the sample surface cannot be excluded in the sectioned material. For constructs where modest ECM formation had occurred, the risk of microtome tearing, in turn collapsing the section morphology, was evident.

Histology

Routine histology methods were used for evaluation of the composition and morphology of tissue-engineered constructs. In this thesis, the gelatin microcarriers stained intensely for Mayer’s and Weigert’s hematoxylin, obscuring individual cell
Comments on methods

morphology on the scaffolds. For Alcian Blue, we occasionally observed a partial coloration of the scaffold. As an alternative to hematoxylin, cell nuclei were counterstained with DNA-binding 4',6-diamino-2-phenylindole (DAPI) and examined under UV light.

Specialized stains were used in Papers II and IV. In Paper II, the von Kossa stain was used to visualize mineralized extracellular matrix in the osteogenic induced groups. This method is based on the binding of silver to carbonate and phosphate groups, predominantly calcium phosphate and calcium carbonate, and positive staining is indicative of mineralized matrix [Bills et al. 1971]. In Paper IV, a combination of picrosirius red and polarized light microscopy was used to investigate the occurrence of collagen fibers in centrifuged pellets [Junqueira et al. 1979]. The method uses the birefringence of collagen fibers stained with picrosirius red to distinguish between collagen fibers and randomly orientated collagen.

Immunohistochemistry

For more specific determination of the ECM components, indirect immunohistochemical analysis was performed for a number of antigens. These are described in Table 1. In Papers I and IV, identical sets of antibodies were used, including aggrecan, collagen types I and II, SOX-9 and S-100. In Paper III, SOX-9, S-100 and aggrecan were used. In Paper II, chondrogenic differentiation was evaluated with aggrecan and collagen type II, while osteogenic differentiation was evaluated immunohistochemically with anti-osteocalcin and osteonectin. The other antibodies of chondrogenic interest were taken into use after Paper II was submitted.

For most immunohistochemical applications, the signal to noise ratio was highly improved by additional antigen retrieval and autofluorescence quenching. The antigen retrieval and autofluorescence techniques used in this thesis are derived from the individual data sheets of each antibody or modified from published data after evaluating different techniques and sequences with each antibody. Primary antibody concentrations and incubation times have also been developed from the initial data sheet recommendations or through systematic experiments evaluating different techniques and sequences with each antibody. For context, cell nuclei were counterstained with a DAPI-containing mounting media.
Table 1: Primary antibodies used in this thesis.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Relevance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>Mouse monoclonal</td>
<td>Major cartilage ECM molecule</td>
<td>[Buckwalter and Mankin 1998]</td>
</tr>
<tr>
<td>Collagen Type II</td>
<td>Mouse monoclonal</td>
<td>Major cartilage ECM molecule</td>
<td>[Buckwalter and Mankin 1998]</td>
</tr>
<tr>
<td>Collagen Type I</td>
<td>Mouse monoclonal</td>
<td>Fibrocartilaginous marker</td>
<td>[Hayes et al. 2007]</td>
</tr>
<tr>
<td>SOX-9</td>
<td>Rabbit polyclonal</td>
<td>Chondrogenic transcription factor</td>
<td>[Aigner et al. 2003]</td>
</tr>
<tr>
<td>S-100</td>
<td>Rabbit</td>
<td>Chondrocyte phenotype indicator</td>
<td>[Wolff et al. 1992]</td>
</tr>
</tbody>
</table>

Negative controls were incubated with PBS instead of primary antibodies. The use of isotype control antibodies is a more precise method. The negative occurrence of positive staining for Collagen type II however acts as a control for the anti-aggrecan antibody in effect. There is no such parallel for the polyclonal rabbit antibodies. Positive controls stained for cartilage and skin respectively. When non-specific fluorescence signals have been detected, this has been stated (see SOX-9 and collagen type I in Paper I).

Enzymatic assays
In Paper II, the activity of alkaline phosphatase (ALP), a hydrolyse enzyme, was investigated as a marker of osteogenic differentiation through a modified spectrophotometric assay [Magnusson and Farley 2002]. The ALP activity was established by measuring the optical density (OD) every 5 min for a total of 30 min following the addition of cell culture supernatant to a buffer containing p-nitrophenyl phosphate, diethanolamine and magnesium chloride. The OD at the control wavelength (490
nm) was subtracted from the OD at the maximum absorbance wavelength of p-nitrophenyl phosphate (405 nm) to eliminate unspecific absorbance.

**Statistical methods**

In Paper II, the ALP activity from osteogenic induced experimental groups and corresponding control groups was analyzed with linear regression. Regression line slopes were compared to detect differences between experimental groups. In Paper III, piecewise linear regression and analysis of variance (ANOVA) was used to determine differences between growth characteristics on the four different microcarriers. A break point was introduced at 7 days to allow for time dependent changes in the growth characteristics. This time point was chosen with support from earlier studies on chondrocyte proliferation, and is discussed in Paper III [Grad et al. 2003; Melero-Martin et al. 2006; Wu et al. 2008]. Tukey pairwise comparison was used for further comparison when significant differences were detected, using the CultiSpher G microcarrier as a reference.
RESULTS AND DISCUSSION

This section highlights the major findings of this thesis. For detailed descriptions of the results, please refer to each respective paper.

**Gelatin microcarriers support adhesion and expansion of human articular chondrocytes (HACs) and human dermal fibroblasts (HDFs)**

In this thesis, four gelatin microcarriers – CultiSpher G, CultiSpher S, CultiSpher GL and CultiSpher GLS – have been verified to support adhesion and expansion of HACs (Papers I, III and IV). The CultiSpher GL microcarrier was shown to support adhesion and expansion of HDFs (Paper II). These results cohere with observations by others. The CultiSpher G microcarrier has previously been used for expansion of human nasal chondrocytes and chondroprogenitor cells, while the CultiSpher S microcarrier has been seeded with fibroblasts [Malda et al. 2003a; Melero-Martin et al. 2006; Huss et al. 2007]. Once transferred to the gelatin microcarriers, the increase in both cell number and accumulated ECM with time suggested an ongoing proliferation and synthesis for both cell types (Papers I–IV).

**Blood-derived glues can be used for microcarrier encapsulation in vitro**

Three re-calcified citrated blood-derived glues – whole blood (WB), platelet rich plasma (PRP) and platelet poor plasma (PPP) – were all able to encapsulate the microcarriers, and maintain an ongoing cell proliferation and synthesis of cartilage-like tissue components for up to 16 weeks in vitro (Paper I). These results were not dependent on the presence of platelets or other whole blood constituents, as all blood-derived glues, as well as the commercially available fibrin glue, gave relatively
similar results. When HACs were seeded in similar numbers in biological glue alone, without the microcarriers, the clots dissolved before the chondrocytes were able to produce sufficient amounts of ECM to uphold the structural integrity of the constructs. This correlates with earlier reports of rapid onset of fibrin glue disintegration in vitro [Homminga et al. 1993]. Our results are also corroborated by findings that presence of chitosan–GP polymer stabilizes blood clots from lysis [Marchand et al. 2009]. Others have evaluated PRP, blood plasma and fibrin glue as vehicles for expanded chondrocytes without supporting microcarriers, and reported that porcine chondrocytes accumulated more GAGs in blood plasma clots compared to whole blood clots [Homminga et al. 1993; Wu et al. 2007; Haberhauer et al. 2008].

Results from Paper IV show that HAC-seeded microcarriers also form cartilage-like tissue components in the absence of encapsulating glue, however at the expense of some structural stability (see below).

**PRP encapsulation enhances pellet stability and generates pellets with more uniform morphologies**

In Paper IV, the influence of PRP was examined by forming pellets of chondrocyte-seeded microcarriers with or without plasma encapsulation. When compared to PRP-free pellets, the data suggests that the engineered constructs are more stable and obtain a more uniform morphology when the microcarriers are sealed within the plasma. However, the immunohistochemical analysis did not disclose distinct differences between the two groups regarding the ECM components or the cellular markers. Nor did the plasma prove to be crucial for microcarrier degradation (Paper IV). There is however a possibility that the presence of PRP may have accelerated the chain of events, and that this could have been missed due to the choice of time points and analysis methods. It is unclear what, if any, influence the single exposure of chondrogenic growth factors from platelet alpha-granules may have had on the long-term outcome, especially considering the longevity and efficiency of its stimulating factors [Yoo et al. 1998; Landesberg et al. 2000; Jakob et al. 2001; Blunk et al. 2002; Weibrich et al. 2002; McCarrel and Fortier 2009]. When used in combination with porous collagen fleece scaffolds, both fibrin glue and blood plasma enhanced the collagen type II gene levels of HACs [Malicev et al. 2007]. The uses of PRP for cell culture media supplementation, rather than for scaffold encapsulation, is discussed in Paper I.
Expanded HACs produce cartilage-like tissue components but do not generate cartilage tissue in free-swelling conditions in the absence of recombinant chondrogenic growth factors

In each long-term culture using expanded HACs in this thesis, the amount of cells and accumulated ECM increased with time, indicating an ongoing proliferation and metabolism (Papers I and IV). Immunohistochemical analysis revealed presence of the cartilage proteoglycan aggrecan in the newly formed ECM. In addition, cells stained positive for the chondrogenic transcription factor SOX-9 and the calcium binding protein S-100 that has been associated with the chondrogenic phenotype [Wolff et al. 1992; Wehrli et al. 2003; Zheng et al. 2007]. However, the collagen content revealed a fibrocartilaginous ECM. None of the in vitro models were capable of supporting full cartilage regeneration, either in terms of homogeneity or quality, leading to the conclusion that free-swelling in vitro culture conditions do not sustain 3D cartilage formation of expanded HACs on macroporous gelatin microcarriers in the absence of recombinant chondrogenic growth factors. These results may have been affected by several factors. The chondrocytes used throughout this thesis has been isolated from patients undergoing total knee arthroplasties. HACs from OA patients have been shown to produce limited amounts of type II collagen in micromass pellet culture, even in the presence of TGF-β1, and significantly less so than chondrocytes isolated from unaffected donors [Tallheden et al. 2005a]. Though cartilage formation can be achieved in high-density micromass pellet culture under free-swelling conditions it is generally accepted that the homeostasis of articular cartilage is highly dependent on mechanical conditioning [Schulz and Bader 2007]. For tissue engineering of larger cartilage constructs, where diffusion can no longer be relied on to maintain the subsurface chondrocytes, several bioreactor approaches have been employed. These are thought not only to facilitate circulation of nutrients but also to activate the mechanotransduction pathways via integrin-mediated cell–matrix interactions [Millward-Sadler and Salter 2004; Ramage et al. 2009]. Several other parameters can have significant effects on cartilage formation in vitro. These include, but are not limited to, topographic origin of chondrocytes, cell expansion method, cell culture passage number and initial seeding density on scaffolds [Lee et al. 1998; Mauck et al. 2000; Malda et al. 2003a; Darling and Athanasiou 2005; Yang et al. 2006; Eyrich et al. 2007; Kang et al. 2007; Stenhamre et al. 2008].
Dynamic centrifugation every other day does not dramatically alter ECM synthesis of microcarrier-expanded HACs but may contribute to collagen fiber organization

In Paper IV, we evaluated dynamic centrifugation as a means of stimulating tissue formation. Acquired results demonstrate that chondrocytes seeded on gelatin microcarriers remain viable and metabolically active when subjected to relative centrifugal forces of up to 3000 g. There was no dramatic effect on matrix accumulation between the centrifuged groups and the unstimulated controls. These results are corroborated with observations from a parallel experiment, using the cell culture insert model from Paper I, where tissue engineered constructs were subjected to dynamic centrifugation with a peak relative centrifugal force of 500 g. Also, results obtained after dynamic compression via a custom-built compression device, using equivalent frequencies, closely resemble those seen with centrifuged and free-swelling constructs in the cell culture insert model. In all these experiments, histological analysis revealed clear signs of microcarrier degradation. The immunohistochemical analysis of the pellets also generated similar results for all groups, regardless of exposure to centrifugation, or presence of PRP (Paper IV). Previous studies have reported that centrifugation can stimulate proteoglycan synthesis as well as DNA content [Inoue et al. 1990; Maeda et al. 2001]. Others have used centrifugation every other day when studying different aspects of chondrocytes in pellet culture [Nakagawa et al. 1993; Oyajobi et al. 1998]. Whether the lack of detectable beneficial effect has to do with the absence of soluble factors described previously, shortages in the models themselves, or a combination of both, cannot be distinguished from the experimental data. Considering the non-quantitative nature of immunohistochemistry, potential subtle differences may have been too modest to discern. Model shortages could involve inadequate duration, frequencies or amplitudes of the administered regimes [Lee and Bader 1997; Lee et al. 1998; Maeda et al. 2001]. Furthermore, the reoccurrence of the biomechanical stimuli may affect the outcome [Maeda et al. 2001]. Centrifugal pressure may lead to alignment of collagen fibers [Maeda et al. 2005], and histological analysis of the centrifuged pellets indicated a higher level of fiber organization in the groups subjected to high relative centrifugal forces, prompting further investigation. While the picrosirius red analysis in Paper IV indeed revealed a distinct presence of collagen fibers, there is a risk that any observations on fiber organization are dependent on sectioning artifacts or discrepancies manifested during centrifugation.
HDFs produce cartilage-like tissue components on gelatin microcarriers under chondrogenic induction

In Paper II, we investigated the ability of HDFs to produce cartilage-like tissue in a 3D cell culture insert model, analogous to the one described in Paper I. In accordance with our earlier findings, the amount of cells and ECM increased with time, indicating an ongoing proliferation and metabolism. The newly formed ECM stained rich for aggrecan, indicating a cartilage proteoglycan synthesis and a differentiation towards a chondrocyte like phenotype. However, no positive staining for collagen type II could be detected. This may be attributed to the difficulty of retaining, or rather in this case inducing, the collagen type II phenotype in vitro. However, HDFs have been shown to stain positively for type II collagen when subjected to the same chondrogenic induction media in monolayer culture, a result that we were not able to reproduce here [Junker et al. 2009]. In addition, there have been reports of collagen type II formation by mesenchymal and adipose derived adult stem cells under free-swelling in vitro conditions [Awad et al. 2003; Li et al. 2005].

As has been discussed previously, the in vitro model used in Paper II proved incapable of supporting cartilage formation when used with HACs. The discussion regarding the in vitro model limitations also applies here. Furthermore, positive synergistic effects with biomechanical stimuli, such as cyclic dynamic compression and hydrostatic pressure, have been reported for chondrogenic differentiation of mesenchymal progenitor cells [Angele et al. 2003; Angele et al. 2004].

HDFs produce bone-like tissue components and upregulate ALP activity on gelatin microcarriers under osteogenic induction

In Paper II, we also investigated the ability of HDFs to produce bone-like tissue when seeded on gelatin microcarriers. Two models were used, the cell culture insert model with PRP encapsulation, and a spinner flask model. Results regarding cell proliferation and ECM formation were analogous to the chondrogenic induced fibroblasts. The total amount of matrix was greater in the spinner flask system than in the cell culture inserts, arguably due to the mechanically active environment. Presence of the bone ECM markers osteonectin and osteocalcin was confirmed by indirect immunohistochemistry in all osteogenic induced cultures. In addition, the von Kossa stain revealed presence of mineralized matrix for both osteogenic induced models. These results corroborate with earlier findings on similar induction of osteogenic phenotype by HDFs [Lorenz et al. 2008]. The differentiation towards an osteoblast phenotype
was also observed through an upregulated ALP activity of the HDFs under osteogenic induction. Importantly, fibroblast controls in the cell culture insert model showed negative results for all these analysis, demonstrating that the osteogenic induction indeed resulted in a phenotypical shift of the HDFs. These results concur with recently published data concerning single cell HDF clones in monolayer culture [Junker et al. 2009]. Results from the spinner flask model, highlighting the influence of flow-induced shear stress, are discussed below.

**Flow-induced shear stress enhances ECM mineralization and affects ALP activity in HDFs**

To investigate the influence of flow-induced shear stress on osteogenic induction, HDFs were kept in a spinner flask system as well as in cell culture inserts. Matrix mineralization was confirmed for osteogenic induced fibroblasts in both culture models, but the degree of mineralization was markedly higher in the spinner flask model for the osteogenic induced fibroblasts. A similar pattern has been reported for human MSCs on collagen scaffolds in spinner flasks [Meinel et al. 2004]. Interestingly, we detected some extent of mineralization in the matrix produced by un-induced fibroblasts in the spinner flask model as well, indicating that shear stress can induce or enhance matrix mineralization. The influence of shear stress on osteogenic differentiation in the absence of soluble osteogenic factors has been reported previously, though not with HDFs [Holtorf et al. 2005; Datta et al. 2006; Kreke et al. 2008]. We also found an increase in ALP activity for the un-induced cells in spinner flasks compared to control fibroblasts in cell culture inserts. However, there was no corresponding elevation in ALP activity between the culture models for the osteogenic induced fibroblasts. The results indicate that the key factor for increased ALP activity is the soluble factors in the osteogenic induction media rather than shear stress. Furthermore there was no formation of osteonectin or osteocalcin by control fibroblasts in the spinner flask model, illustrating that shear stress alone is not enough to cause the phenotypic shift discussed above.

**Gelatin cross-linking affects HAC proliferation during microcarrier expansion**

In Paper III, we investigated if the degree of gelatin cross-linking affects chondrocyte proliferation on gelatin microcarriers over a two-week expansion period. We found a
Results and discussion

The statistically significant impact of gelatin cross-linking on chondrocyte density during the second week of culture. The less tightly cross-linked gelatin supported higher cell densities compared to microcarriers with a stronger cross-link. In a recent study, Sisson et al. demonstrated that viability and proliferation of MG63 cells, human osteosarcoma cells with osteoblastic characteristics, on electrospun gelatin scaffolds is significantly affected by the cross-linking method [Sisson et al. 2009]. They reported that gelatin fiber morphology was influenced by the cross-linking agents, arguably due to differences in reaction times and the amount of gelatin dissolved before complete cross-linking. For the CultiSpher microcarriers investigated in Paper III, the same cross-linking agent was used during manufacturing, but it is possible that differences in the manufacturing process (see Paper III for details) may have led to a dissimilar release of gelatin during the cross-linking stage that in turn may have affected scaffold morphology, and consequently the proliferative behavior of the chondrocytes in this study.

When compared to the others, the CultiSpher G microcarrier yielded the highest chondrocyte density after two weeks. This is in contrast to earlier findings, using Chinese hamster ovary cells, where instead the CultiSpher S microcarrier gave the highest cell fold increase compared to both the CultiSpher G and the GL counterparts [Kong et al. 1999]. The CultiSpher GLS microcarrier was not part of that study. This discrepancy again highlights the importance of identifying suitable scaffolds for individual cell types. The overall cell yield in Paper III was modest, and the cell culture parameters, such as stirring speed and serum concentrations, may be optimized further to encourage faster cell growth and limit aggregate formation [Malda et al. 2003a; Gigout et al. 2005; Melero-Martin et al. 2006].

Pore diameter affects HAC proliferation during microcarrier expansion

In Paper III, we also found a statistically significant impact of microcarrier pore diameter on chondrocyte density, again manifested during the second week of culture. Gelatin matrices with an average internal pore diameter of 20 µm had higher cell densities than matrices with a 30 µm pore diameter. We have found no comparable experiments on human chondrocytes cultured on porous gelatin scaffolds with pore diameters in the 20–30 µm range. Kong et al. however reported that the CultiSpher G microcarrier yielded higher numbers of Chinese hamster ovary cells compared to the CultiSpher GL counterpart [Kong et al. 1999]. Again, no such comparison for the
Biodegradable gelatin microcarriers in tissue engineering

tighter cross-linked S-type microcarriers, CultiSpher S and CultiSpher GLS, was made by these authors. For other gelatin- or collagen-based matrices, rat chondrocyte densities were higher in scaffolds with pore diameters ranging between 50–200 µm compared to 250–500 µm pore scaffolds [Lien et al. 2008], while osteogenic MC3T3 cells adhere better to collagen–GAG based scaffolds with decreasing pore diameters [O’Brien et al. 2005]. For chitosan scaffolds, porcine chondrocytes reached higher densities in scaffolds with pore diameters of up to 120 µm [Griffon et al. 2006], but pore diameter did not significantly influence rabbit chondrocyte proliferation on chitosan hybrid materials [Yamane et al. 2007]. It has also been reported that porous PLGA microcarriers with analogous dimensions to the CultiSpher GL microcarrier supported greater DNA content with bovine chondrocytes compared to nonporous microcarriers [Chung et al. 2008].

Summary

The plausibility of using cell-seeded gelatin microcarriers in combination with PRP has been addressed in three different in vitro studies (Papers I, II and IV), each yielding results that demonstrate a capacity to support ECM formation in vitro. Still, the proposed system has yet to be proven efficacious for tissue engineering of cartilage and bone in vivo. Microcarrier characteristics, specifically internal pore diameter and degree of cross-linking, have been shown to influence HAC expansion in terms of cell density per gram dry weight gelatin over a two-week culture period in spinner flasks (Paper III). In addition, the use of HDFs for cartilage and bone formation presents a highly interesting cell source for parallel investigation (Paper II).
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The long-term clinical goal of this thesis is to enable cell delivery of human cells via gelatin microcarriers for the purpose of regenerating cartilage and bone tissue. Several experimental studies have been conducted, and the main results were discussed in the previous chapter. The efforts presented here do not give any definitive answers. Rather, it has raised additional scientific questions. This section addresses some of the topics that encourage further examination.

On the efficacy of microcarriers for tissue engineering of cartilage

Papers I, II and IV describe the plausibility of using macroporous gelatin microcarriers for tissue engineering of cartilage. Though several important cartilage molecules were detected in the synthesized ECM, true cartilage formation was not achieved. The lack of uniform tissue formation, in combination with the absence of collagen type II, may undeniably be an indication that the biomaterial scaffolds are not capable of supporting full cartilage generation, but it may also be attributed to inadequate in vitro culture systems that lack important environmental cues, according to the earlier discussion. Thus, the results from these in vitro studies are difficult to put into a clinical perspective to date. It is not until evaluated in situ that the true efficacy of the microcarrier and blood-derived glue system can be confirmed or rejected. Before such in vivo studies are launched, a number of in vitro experiments are needed, some of which will be described below.
Biodegradable gelatin microcarriers in tissue engineering

On the use of in vitro models
There is a great need for a reliable, straightforward in vitro model for initial evaluations of biomaterial characteristics, including biomaterial modifications, on chondrocyte metabolism. The dynamic centrifugation, as administered in Paper IV, did not dramatically enhance chondrogenesis in vitro. Still, centrifugation at other intervals or with other frequencies may have a positive influence on ECM synthesis in vitro. Further development of the amplitude and frequency parameters could enable an easy and affordable system for this type of screening, thus efforts towards establishing a better centrifugation regime would be beneficial. Preferably, such regimes would run with frequencies in the physiological range, 0.5–1 Hz [Lee and Bader 1997]. A fully programmable centrifuge able to withstand the humidity and temperature in an incubator would be highly advantageous for this purpose. Also, it is important not to overestimate the dynamic nature of such regimes, and consider the reoccurrence interval of such stimuli with utmost care. Dynamic compression can be administered with the custom-built compression device mentioned previously in this thesis. If this device is to become more useful for scientific purposes, further modifications are needed, such as introducing sensors above and below the tissue-engineered constructs to measure the applied forces. It is also possible that the cell culture media used during expansion and three-dimensional culture could be optimized further [Malda et al. 2003a; Tallheden et al. 2005b; Akeda et al. 2006]. Finally, the influence of hypoxic conditions on chondrocyte ECM production in a gelatin microcarrier model should be investigated.

On the influence of microcarrier characteristics
In Paper III, we showed that gelatin pore diameter and degree of cross-linking influence chondrocyte proliferation in vitro. The direct clinical relevance of this result is perhaps limited. Rather, we have identified the microcarrier that will optimize the chondrocyte expansion in vitro. Given that the CultiSpher G microcarrier yielded the highest cell count during expansion, this microcarrier is the expected frontrunner for future use. However, in vitro experiments studying ECM formation, rather than chondrocyte proliferation, on these four microcarriers are needed. In order for such an approach to be successful, quantitative analysis methods evaluating sulfate and proline incorporation, GAG and collagen accumulation, and gelatin degradation
rates in the respective microcarrier groups, should be added to the study design to allow possible differences to be statistically confirmed.

By studying the SEM images in Paper III (Figure 1A), it is evident that the surfaces of individual microcarriers differ in terms of porosity and pore diameters. Similarly, it is clear from the MTT pictures that not all microcarriers are populated during the initial days (Figure 2A, Paper III). This is in agreement with data from Kong et al., where only 14–47% of the CultiSpher microcarriers used in that study were populated by cells after 24 hours [Kong et al. 1999]. There may be a link between these two observations and further investigations into this relationship, by means of scanning electron microscopy on cell-seeded microcarriers during the first days post-seeding, may elicit a deeper understanding of cell–biomaterial interactions during adhesion, potentially enabling optimally designed scaffolds.

The experiments in Paper II were conducted on the CultiSpher GL microcarrier. As we have shown that microcarrier characteristics can influence chondrocyte behavior, an analogue screening of gelatin matrices for HDFs may be useful. Furthermore, the introduction of growth factors to the gelatin scaffold may prove beneficial to maintain or enhance the differentiated phenotype in situ. Previous non-published efforts from our lab addressed the possibility of coupling chondrogenic growth factors from the TGF-β super family to the gelatin matrices. Such strategies should be verified in vitro to ensure that the growth factors are chemically active post-binding and once released.

On the use of dermal fibroblasts in tissue engineering
The fact that HDFs, a cell type that can be isolated with minimal discomfort and expanded into great numbers, can change their phenotype offers a promising and novel alternative cell source for tissue regeneration. Yet, one crucial question remains regarding the stability of the induced phenotype – can these cells contribute to intrinsic tissue regeneration in situ? This outcome is difficult to predict. There have been reports that the prolonged exposure to osteogenic factors may suppress bone growth in vivo [Cheng et al. 2000]. Other studies have shown that HDFs are induced towards a chondrocyte phenotype in the presence of demineralized bone in vitro [Mizuno and Glowacki 1996]. Furthermore, the adhesion to bone ECM proteins such as vitronectin and collagen type I, is enough to promote osteogenic differentiation of BMSCs [Salasznyk et al. 2004]. These results could indicate that the in situ environment can, at least to some extent, help to establish and maintain a tissue specific
phenotype. For cartilage the question is even more intriguing. As most cartilage repair strategies today yield fibrocartilaginous tissues, even with all the physiological cues in place, it would be truly groundbreaking if HDFs could regenerate intrinsic cartilage formation \textit{in situ}. The influence of environmental factors on chondrogenic differentiation is interesting. The importance of these factors, including the synergistic effects between chondrogenic induction media and biomechanical stimuli and/or hypoxia, have been well established for adult stem cells [Angele \textit{et al.} 2003; Angele \textit{et al.} 2004; Robins \textit{et al.} 2005]. The incorporation of such strategies may enhance the chondrogenic differentiation of HDFs as well [Mizuno and Glowacki 2005]. Regardless of the outcome, data from such experiments may prove helpful in the understanding of the mechanisms responsible for phenotype plasticity in human adult cells.

A major challenge in tissue engineering concerns the vascularization of large tissue engineered constructs [Rouwkema \textit{et al.} 2008]. Recent efforts by members of our lab have investigated the ability of HDFs to differentiate towards an endothelial cell like phenotype [Karlsson \textit{et al.} 2009]. In addition, the differentiation of HDFs towards chondrocyte, osteoblast, and adipocyte like phenotypes has been assessed [Junker \textit{et al.} 2009]. As has been discussed earlier, the efficacy of these cells to regenerate tissue \textit{in vivo} remains to be proven. Yet, the ease with which these cells can be isolated and expanded in comparison offers a huge advantage and the use of HDFs for tissue regeneration may revolutionize cell-based therapies as we know them today.

As a final remark, the long-term clinical success of regenerative strategies does not rely solely on the identification of the cells, scaffolds and environmental factors needed to support tissue formation \textit{in vitro}. The mechanisms behind any underlying pathogenesis, such as osteoarthritis, must also be identified and addressed, in order to restore permanent tissue function for the individual patients.
CONCLUSIONS

Based on the results from Papers I–IV, we conclude the following

- Gelatin microcarriers support adhesion, expansion and matrix formation of HACs and HDFs
- Biological blood-derived glues can be used to encapsulate cell-seeded microcarriers \textit{in vitro}
- PRP encapsulation enhances structural integrity of microcarrier constructs and does not affect cartilage formation negatively
- Free-swelling conditions do not support true cartilage formation by HACs or HDFs \textit{in vitro}
- Dynamic centrifugation (for 16 min at 0.0125 Hz) every other day does not dramatically enhance or alter cartilage formation \textit{in vitro}
- PRP encapsulation or dynamic centrifugation are not crucial for microcarrier degradation \textit{in vitro}
- HDFs seeded on gelatin microcarriers can produce cartilage-like tissue components in free-swelling conditions under chondrogenic induction
- HDFs seeded on gelatin microcarriers can produce bone-like tissue components in free-swelling conditions as well as in spinner flask systems under osteogenic induction
- Flow-induced shear stress enhances matrix formation and mineralization and leads to an upregulation of ALP activity in un-induced HDFs seeded on gelatin microcarriers
- The degree of gelatin cross-linking affects microcarrier-based expansion of HACS in a spinner flask system
- The average pore diameter affects microcarrier-based expansion of HACs in a spinner flask system
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Biodegradable gelatin microcarriers in tissue engineering

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Biodegradable gelatin microcarriers in tissue engineering


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Biodegradable gelatin microcarriers in tissue engineering


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Biodegradable gelatin microcarriers in tissue engineering


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Biodegradable gelatin microcarriers in tissue engineering


