Cartilage Tissue Engineering For Auricular Reconstruction

Cartilage Constructs in Complex Shape

&

Differentiation Processes

Dissertation to obtain the Degree of Doctor of Natural Sciences

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presented by

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To my parents

‘Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.’

Thomas A. Edison

This work was carried out from August 1st, 2006 until June 30th, 2010 at the Department of Pharmaceutical Technology of the University of Regensburg.

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Chapter 1

Introduction

Loss of cartilage due to trauma, tumour resection or congenital defects is a major challenge in craniofacial surgery [1]. Established methods for auricular reconstruction such as costal grafts are often accompanied by chest wall deformities or application of alloplastic implants causing foreign body sensation as well as bearing aesthetic deficits [2]. Thus, there is an increasing demand for satisfying alternatives. Tissue engineering holds the promise to enable the generation of autologous implants that likely meet the clinical need. The overall goal is to establish a procedure to obtain autologous cells by a method with minimal burden on the patient, after expansion seed the cells on custom-designed scaffold, differentiate or redifferentiate the cells applying growth factors and subsequently transplant the engineered cartilage construct.

Cartilage Biology

Understanding cartilage biology is inevitable for cartilage tissue engineering. Chondrocytes are embedded in extracellular matrix (ECM). Cartilage is not rich in cells, in human hyaline cartilage chondrocytes only represent 1% of the volume of hyaline cartilage thus cartilage ECM is of particular interest [3]. The interterritorial matrix is composed of a collagen network formed by collagen fibrils, which provides tensile strength and retains proteoglycans. Type II collagen represents the principle component of the macrofibrils. Type VI collagen forms the macrofibrils in the pericellular area and type IX collagen is crosslinked to the surface of the fibrils. Type X collagen is only synthesized by hypertrophic cells and is usually present only in calcified areas. Type XI collagen is another type of collagen, which can be found within the macrofibrils [4, 5]. Besides collagen proteoglycans are also responsible for cartilage characteristics. Their core is associated with one or more varieties of glycosaminoglycan chains. Glycosaminoglycans represent unbranched polysaccharides built of disaccharides. At least one disaccharide always bears a negative charge, which allows

interaction with water. The tissue fluid is another essential part of hyaline cartilage, because in exchange with the synovial fluid it provides nutrients and oxygen [3].

Hyaline cartilage which is present in the joints and in the nose shows no fibres and has a glassy appearance. Besides hyaline cartilage, which is the most predominantly investigated type of cartilage, there are two other types. In comparison to hyaline cartilage fibrocartilage, which is localized at the end of the tendons and ligaments, has a higher content of collagen in the extracellular matrix, and in contrast to the other cartilage types, whose predominant collagen is type II, it also contains considerable amounts of type I collagen in addition to type II. Auricular cartilage is an elastic cartilage, which can also be found in the epiglottis. The ECM of this third type of cartilage also contains elastin in contrast to articular cartilage and fibrocartilage [3, 6–9]. Cartilage tissue engineering aims at a cartilaginous tissue mainly composed of the ECM described above, as the ECM determines the characteristics of the engineered cartilaginous tissue. Expansion of chondrocytes in 2-dimensional (2D) environment, which is often necessary due to the limited number of harvestable cells, often is accompanied by dedifferentiation. Dedifferentiation causes changes in gene expression of type I and II collagen as well as of aggrecan. Consequently, often fibrocartilaginous tissue is formed, which is biochemically and biomechanically inferior compared to native cartilage [10, 11].

Chondrocytes are metabolically relatively inactive. Cartilage lacks innervation and vascular supply, nevertheless chondrocytes respond to mechanical stimuli, growth factors and cytokines influencing cartilage homeostasis [5]. The function of chondrocytes depends on their localization. Chondrocytes located in supporting tissue, as articular cartilage or nasal cartilage, synthesize and maintain ECM and therefore the tissue’s function. Chondrocytes are able to cope with conditions of low oxygen tension (ranging from 10% at the surface and 1% in deeper zones) as the majority of the energy required is obtained by glycolysis [8].

After damage cartilage has a very low capacity of self-repair, as by being avascular, thus progenitor cells from blood or bone marrow have no access to the tissue [12]. Inherited, traumatic or degenerative cartilage defects demand engineered cartilage for repairing joint defects as well as for plastic reconstruction of the nose and the ear.

Scaffolds for Cartilage Tissue Engineering

Scaffolds are one of the key components of cartilage tissue engineering. A scaffold serves as cell carrier and may act simultaneously also as delivery system for proteins. It provides stability as well as the desired shape for the new tissue. The scaffold can not be reduced to being merely a mechanical support structure. It interacts with the cells, bioactive molecules and mechanical stimuli, which contribute to tissue generation and regeneration after implantation [13]. The desired, ideal scaffold is a 3-dimensional (3D) highly porous and interconnective construct, allowing for cell growth, nutrient supply and transport of metabolic waste. Moreover, it is supposed to be biocompatible and bioresorbable following controlled degradation. The chemistry of its surface should favour cell attachment, proliferation and differentiation. Another requirement is the mechanical stability which should fit the mechanical properties at the site of implantation [14].

A great variety of scaffold materials has been investigated for cartilage tissue engineering purposes [15]. The focus lay on polymeric scaffolds in form of hydrogels, sponges or meshes. Alginate, agarose, fibrin, collagen, chitosan, chondroitin sulphate, and gelatine are prominent examples for applied natural polymers. Natural polymers are often able to interact with cells, but are also capable of prompting an immune response. Inferior mechanical properties and variance in enzymatic degradation are other disadvantages of natural materials. In contrast to natural polymers, one advantage of synthetic materials is that they allow a design close to the mechanical requirements by physical and chemical modifications during synthesis [9]. Two

groups can be distinguished: degradable and non-degradable scaffolds. As the permanence of non-degradable materials is a great concern with regard to their long-term effects, a lot of efforts have been undertaken towards the development of degradable synthetic polymers for tissue engineering applications. For example, poly (lactic acid) (PLA), poly (glycolic acid) (PGA), as well as their copolymers poly (lactic-co-glycolic acid) (PLGA) are such materials approved by the Food and Drug Administration (FDA) [15].

Cell Sources for Cartilage Tissue Engineering

To date several cell sources have been investigated for their application in regenerative medicine. Beside chondrocytes which represent the most obvious choice, mesenchymal stem cells (MSC) are in focus for cartilage tissue engineering purposes [13]. Chondrocytes have been isolated from articular, auricular, nasoseptal or costal cartilage, which are all capable of producing cartilaginous ECM [9]. All sources have in common that they do not render the necessary number of cells, thus making expansion necessary. Expansion of chondrocytes in 2D environment is accompanied by rapid dedifferentiation [11] thus requiring redifferentiation, e.g., by the application of growth factors [13]. Undifferentiated progenitor cells possessing a multilineage potential would be a good alternative as they are easy to obtain and expand in vitro without loosing their ability to differentiate into various mesenchymal lineages [16]. MSC can be isolated from bone marrow (BMSC), adipose tissue (ASC), the synovial membrane or trabecular bone. Stem cells from bone marrow are the best characterized type of mesenchymal stem cells, although due to the risks and pain associated with their sampling procedure bone marrow may not be the ideal cell source for MSC. Adipose tissue would be an attractive alternative, as the access is easy, ASC are available in larger quantities and generally display a proliferation and differentiation potential comparable to that of BMSC [17]. However, with regard to cartilage engineering, it is still under

investigation of ASC can be stimulated to match the chondrogenic potential proven for BMSC [18–22].

Growth Factors for Cartilage Tissue Engineering

Growth factors play a key role in cartilage tissue engineering, either with regard to improve tissue quality by triggering matrix production, increasing proliferation or in (re-) differentiation processes. They are inevitable for the differentiation of progenitors towards a chondrogenic phenotype. Redifferentiation of dedifferentiated chondrocytes after expansion also demands the application of growth factors. As proteins from the transforming growth factor-β (TGF-β) superfamily are key players during cartilage development they are also in the focus of the investigations concerning growth factor application in cartilage tissue engineering. TGF-β, BMPs, bFGF, and IGF-I have been investigated independently and simultaneously. Beside inducing differentiation and redifferentiation or improving construct quality by increasing matrix production growth factors can also be used to support proliferation of the cells [9].

Goals of the Thesis

The goals of this work were defined in major parts defined by the project ‘Regenerative Implants’ which is a cooperation of several research groups from industry, hospitals and universities supported by a grant of the Bavarian Research Foundation (‘Bayerische Forschungsstiftung’) in the years 2006 to 2009. It comprised cartilage and bone tissue engineering as well as the generation of osteochondral constructs. A subgroup of the research consortium including our department aimed at the generation of an ear-shaped cartilage construct and also participated in the generation of osteochondral constructs.

In order to obtain cartilage in the complex shape of the human external ear, a scaffold providing shape and stability for the growing tissue is required. In the preceding project 'FORTEPRO' (also supported by grants of the Bavarian Research Foundation), a system combining the beneficial effects of soft and hard scaffolds had been established and the great benefits combining long-term stable fibrin gel [23] and polycaprolactone-based polyurethane (PU) scaffolds had been shown [24]. Following these results, this system was to be transferred to polyurethane scaffolds in the complex shape of the external ear (Chapter 3 and 4). As an alternative system for auricular cartilage engineering oligo (poly (ethylene glycol) fumarate (OPF) scaffolds, produced in the department, were either alone or combined with long-term stable fibrin gel, investigated with regard to cartilage development (Chapter 5). Furthermore, the combination of long-term stable fibrin gel and polyurethane scaffolds was also applied aiming at osteochondral constructs (Chapter 6).

The goal of attaining autologous cartilage constructs in clinically relevant size is associated with the challenge to provide a sufficient number of cells. As mentioned above (see 'Cell Sources for Cartilage Tissue Engineering'), the most obvious cell source is cartilage from a non load-bearing area, from which autologous chondrocytes can be isolated. Due to the fact, however, that cartilage tissue is not rich in cells, the obtained chondrocytes have to be expanded in vitro. In turn, the rapid proliferation in 2-dimensional (2D) environment, e.g. culture flasks, is accompanied by rapid dedifferentiation [11], thus rendering a tissue rich in type I collagen, which is not desired for cartilage tissue engineering purposes. Thus, options for redifferentiation of dedifferentiated chondrocytes have to be established to obtain cartilage tissue employing expanded chondrocytes. Redifferentiation can be reached by application of growth factors. One part of this work dealt with the effect of the application of growth and differentiation factor-5 (GDF-5), a member of the BMP subfamily, either alone or in combination with insulin on cartilage construct quality using expanded chondrocytes (Chapter 7).

A second possibility to yield a sufficient cell number is the use of mesenchymal stem cells. Mesenchymal stem cells from bone marrow are already well described and their multilineage potential has been proven, however, their application is associated with several disadvantages (see above). Another emerging cell source for mesenchymal stem cells is adipose tissue. It implies the advantage of a very good accessibility, is in most cases available in sufficient quantities and the multilineage potential of adipose tissue-derived stem cells (ASC) has also been shown [25, 26]. Thus, here it was investigated if the combination of GDF-5 and insulin similarly exerts similarly advantageous chondrogenic effects on ASC as seen for expanded chondrocytes (Chapter 7). Finally, in order to contribute to the clarification of the ongoing debate on the utility of the different stem cells, those publications in the literature were reviewed that directly compared BMSC and ASC within the same study with regard to their differentiation potential (Chapter 8).

Chapter 2

Materials and Methods

Chondrocyte Isolation

Bovine Chondrocytes

Knee joints from 8-12 weeks old calves were obtained from a local abattoir (VION EGN, Vilshofen, Germany). Articular cartilage was attained from the surface of the femoral patellar groove. The isolated cartilage was cut into small pieces and kept in complete chondrocyte medium (CCM) in petri-dishes (Corning, Schiphol-Rijk, Netherlands) until the isolation of chondrocytes by enzymatic digestion with collagenase type II (Worthington, via Cell Systems, St. Katharinen Germany). Complete chondrocyte medium comprised the following constituents: Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose, 584 mg/l glutamine, 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin, 10mM HEPES, 0.1 mM non-essential amino acids (all from Gibco-Invitrogen, Karlsruhe, Germany), 0.4 mM proline (Sigma-Aldrich, Taufkirchen, Germany) and 50 µg/ml ascorbic acid (Sigma-Aldrich, Taufkirchen, Germany). The enzymatic digestion was conducted over night (17 hours) with collagenase type II followed by filtration through a 149 µm filter (Spectra Medical Industries via Novodirect, Kehl/Rhein, Germany). The filtrate was centrifuged (1200 rpm, 5 min) and washed three times with phosphate buffered saline (PBS) containing 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco-Invitrogen, Karlsruhe, Germany) [27]. For determination of the cell number the obtained cell pellet was resuspended in CCM and counted with a hemocytometer and an inverted phase contrast microscope.

Human Chondrocytes

Primary human chondrocytes were isolated using sterile technique. Cartilage explants were obtained from patients (age of 20 – 55 years) undergoing nasal or auricular surgery after having given their consent (according to the approval of the local Ethics Committee, votum #05/169). Explants were cut into small pieces. After pre-digestion with pronase E (Sigma-

Aldrich, Taufkirchen, Germany) digestion of the small pieces was conducted over night (17 hours) with collagenase type II (Worthington, CellSystems, St.Katharinen, Germany). The digest was filtered through a 70 µm cell strainer (BD Falcon™ via Schubert & Weiss OMNILAB GmbH & Co KG, München, Germany). After centrifugation at 1200 rpm for 5 minutes the chondrocytes were washed three times with PBS containing containing 50 U/ml penicillin and 50 µg/ml streptomycin. Cell number was determined using a hemocytometer and an inverted phase microscope.

Chondrocyte Expansion

Bovine Chondrocytes (Chapter 5, 6)

Primary chondrocytes were seeded in T150 cm^2 culture flasks (7 500 cells/ cm^2) (Corning, Schiphol-Rijk, Netherlands) and in complete chondrocyte medium over 7 days in an incubator at 37° C, 95% humidity and 5% CO_2. After reaching confluency the cells were washed with PBS and detached from the flask with trypsin 0.25% (Gibco-Invitrogen, Karlsruhe, Germany) (P1). After determination of the obtained cell number in passage 1 (P1), cells were again seeded in T150 cm^2 culture flasks (7 500 cells/ cm^2). After 4 days cells reached second confluency and were harvested by trypsinization as described above.

Human Chondrocytes (Chapter 6)

For the experiments with human passage 2 (P2) chondrocytes primary cells were seeded in T75 culture flasks at a density of 7500 cells per cm^2 for expansion in 2D. After 12 days and reaching first confluency they were washed with PBS and trypsinized (trypsin 0.25% EDTA; Gibco-Invitrogen, Karlsruhe, Germany). In order to obtain passage 2 (P2) cells P1 chondrocytes were seeded again in T75 culture flasks ($5*10^5$ cells per flask). After further 7 days cells were confluent and harvested for pellet culture.

In Vitro Cell Culture in 3D Environment

Chondrocyte Culture on Polyurethane (PU) Scaffolds (Chapter 3)

a) PU Discs

Polycaprolactone-based polyurethane (PU) scaffolds were manufactured as previously described using a gas foaming process [28] and provided by polyMaterials AG (Kaufbeuren, Germany). If not stated otherwise, discs of 2 mm thickness with a diameter of 5 mm were punched out of the polyurethane scaffold (= routine discs). In order to increase the wettability of the PU scaffolds they were treated for 5 min with ethanol 70%. Afterwards ethanol was removed by rinsing the scaffolds five times with PBS. Subsequently the scaffolds were sterilized in PBS by autoclave sterilization. Under sterile conditions scaffolds were blotted to remove the fluid and placed in a 6-well plate for subsequent seeding with chondrocytes in long-term stable fibrin gel [23]. After first experiments, in which chondrocytes were suspended in the fibrinogen (Fibrinogen from bovine plasma, Sigma Aldrich, Taufkirchen, Germany) solution (100 mg/ml dissolved in Trasylol® (aprotinin solution, Bayer, Leverkusen, Germany), a separate study showed that concerning cartilage development there was no significant difference between starting with suspending the cells in the fibrinogen solution compared to suspending the chondrocytes in the thrombin solution. As the latter had distinct advantages during the seeding procedure, in the following, the chondrocytes were suspended in the thrombin solution (5 U/ml) (kind gift from Baxter, Germany) by adding 20 µl of thrombin solution per $5*10^6$ cells. Due to the volume of the chondrocytes, which was determined in a separate experiment, the resulting cell suspension had a concentration of $1.25*10^5$ chondrocytes per 1 µl suspension. 20 µl of this mixture were mixed with 20 µl of fibrinogen solution. The mixture was subsequently inserted into the scaffold disc in several portions using a 1ml syringe and a 20G needle (Braun, Melsungen, Germany). The resulting cell number per scaffold was $2.5*10^6$ cells. After gel insertion the constructs were kept at 37° C for 45 min in the incubator to allow gelation of the fibrin gel.

In order to evaluate the impact of the fibrin gel on cartilage development and on cell distribution, scaffold discs were also seeded with chondrocytes suspended in CCM. The expected initial number of cells per scaffold was $2.5*10^6$ cells. The distribution of the cells in the PU scaffold disc was investigated at several time points (day 3, day 7, day 14 and day 21). Besides, the effect of gentle agitation (50 rpm) on the cell distribution was also analysed at several time points (d3, d7, d14 and d21).

Then the scaffold discs were covered with 4 ml of CCM and cultured in an incubator at 37° C, 95% humidity and 5% CO_2 for 3 or 4 weeks respectively. Medium was replaced thrice a week.

b) Ear-shaped PU Scaffolds

The ear-shaped scaffold was treated before seeding as described above for the routine PU discs. Then the ear-shaped scaffold was placed in a petri-dish and seeded with a nominal cell number of $250*10^6$ chondrocytes in 2 ml of fibrin gel. Due to the rapid gelation of the fibrin gel, the gel was mixed and inserted in portions. After 45 min of gelation in the incubator at 37° C the construct was transferred into a spinner-flask. The flask was filled with 120 ml CCM supplemented with 2.5 µg/ml insulin and stirred at a speed of 50 rpm during the culture period of 4 weeks in an incubator at 37° C, 95% humidity and 5% CO_2. Medium was replaced thrice a week. After 28 days the ear-shaped cartilage construct was harvested. Prior to biochemical analysis respective samples were freeze-dried.

Chondrocyte Culture on Oligo (poly) ethylene glycol) fumarate) (OPF) Scaffolds) (Chapter 5)

a) *OPF Discs*

OPF hydrogels, which are based on poly (ethylene glycol), were prepared as scaffolds [29] in our group using a gas foaming technique (M. Henke, manuscript in preparation). Three differently composed OPF hydrogel types were investigated. Foam 1 and foam 2 contained

$NaHCO_3$ as foaming agent, whereas for foam 3 $KHCO_3$ was chosen. Foam 1 contained only 2.5% bisacrylamide, whereas foam 2 comprised 5% bisacrylamide as stabilizing agent, inhibiting rapid degradation. Foam 3 was stabilized by addition of poly (ethylene glycol) diacrylate. After γ-irradiation sterile disc-shaped OPF scaffolds with a diameter of approximately 9 mm and a height of approximately 2 mm were placed in a 6-well plate. 200µl of a suspension containing $12.5*10^6$ in CCM were gently dropped onto the scaffold disc. After 5 min of reconstitution of the OPF scaffold-hydrogels 7 ml of CCM were added and constructs were cultured in an incubator at 37° C, 95% humidity and 5% CO_2 for 4 weeks respectively. In order to possibly enhance seeding efficiency as well as cell retention and mechanical stability during the culture period OPF hydrogels were additionally seeded with chondrocytes suspended in fibrin gel. In brief chondrocytes were suspended in thrombin solution (5 U/ml), 100 µl of the suspension containing $12.5*10^6$ cells were mixed with 100 µl fibrinogen solution (100 mg/ml) and inserted into the scaffold. After leaving the constructs for 45 min in the incubator (37° C, 95% humidity and 5% CO_2) for gelation of the fibrin gel, discs were covered with 7 ml CCM. Constructs were cultured in an incubator at 37° C, 95% humidity and 5% CO_2 for 4 weeks.

b) Ear-shaped OPF scaffolds

Ear-shaped OPF-scaffolds were prepared in our group according to the preparation of the OPF-discs but foamed in a custom-made silicone mold with the shape of the human external ear (Henke, M., manuscript in preparation). After γ-irradiation, ear-shaped OPF scaffolds were seeded with primary chondrocytes suspended in fibrin gel. 1 ml thrombin solution (5 U/ml) containing $250*10^6$ chondrocytes was mixed in portions with fibrinogen solution (100 mg/ml). The construct was then placed in the incubator (37° C, 95% humidity and 5% CO_2) for gelation of the fibrin gel for 45 min. The ear-shaped construct seeded with primary cells

was pre-cultured in a petri-dish (60 ml CCM) for 1 week and for further 3 weeks in a flask (120 ml CCM) under gentle agitation. Medium was replaced thrice a week.

Pellet Culture of Human Chondrocytes (Chapter 7)

Pellets of human chondrocytes were prepared in V-bottomed 96-well plates (Nunc via Fisher Scientific GmbH, Schwerte, Germany) by pipetting 200 µl suspension of $2.5*10^5$ chondrocytes in culture medium into each well and subsequent centrifugation at 1300 rpm for 5 min. After 24 hours for pellet consolidation growth factors were added to the complete chondrocyte culture medium (composition described above, Chondrocyte Isolation, Bovine Chondrocytes).

Characterization of Polyurethane Scaffolds (Chapter 4)

If not stated otherwise, the polyurethane scaffolds produced by polyMaterials AG (Kaufbeuren, Germany) is based on polycaprolactone-diol (molecular weight of 2000) and –triol (molecular weight of 900) in equal parts. Furthermore, scaffolds derived from polymerisation of polycaprolactone-diol (MCI) (molecular weight of 1250) or polyvalerolactone (MVI) on mannitol and transformed with isophorondiisocyanate or polycaprolactone-diol polymerized on mannitol and transformed with hexamethylendiisocyanate (MCH) were characterized. Disc-shaped routine scaffolds as well as scaffolds in the shape of the external ear were investigated. Seeding efficiency tests were conducted to gain further insight in the characteristics of the scaffolds. Interconnectivity and pore structure were analyzed by scanning electron microscopy (SEM).

Seeding Efficiency Test

For determination of seeding efficiency of routine scaffolds discs with a diameter of 5 mm and a height of 2 mm were applied. In case of the scaffolds in the shape of the external ear samples from different representative areas #1 - #6 (Fig. 1) were punched out to get an overview of the characteristics in the different areas of the ear-shaped scaffold. The thickness was determined and the required fibrin gel volume and the respective cell number, corresponding to the amount used for the routine scaffold discs were calculated.

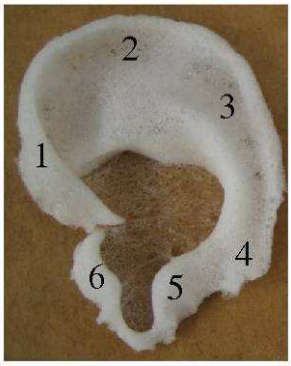


Figure 1: Areas # 1 - # 6 from which discs were punched out for seeding efficiency test.

For the seeding efficiency test scaffolds were pre-treated and seeded with chondrocytes suspended in long-term stable fibrin gel as described above (Chondrocyte Culture on PU Discs). The calculated initial cell number seeded on the routine discs (5 mm diameter, 2 mm thickness) was $2.5*10^6$ cells (in 40 µl fibrin gel). The seeded scaffold samples were incubated at 37° C for 45 min to allow gelation of the fibrin gel. Then the scaffold-fibrin gel combinations were covered with 4 ml of CCM and incubated over night in an incubator at 37° C, 95% humidity and 5% CO_2. After 24 hours constructs were harvested. After removal of the medium constructs were washed with PBS and subjected to a digestion with papainase solution (papain suspension, Worthington via Cell Systems, St. Katharinen, Germany; 3.2 U/ml in buffer) overnight and determination of the DNA content, i.e. the cell number.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy providing a deeper insight into the structure of the scaffold was applied for further characterization. For this purpose scaffold discs with a diameter of 5 mm were punched out of the ear-shaped scaffold (#1-6, see Fig. 1). Similarly discs from round scaffolds were prepared with a thickness of 2 mm and a diameter of 5 mm. Then discs were mounted on aluminium stubs applying a conductive carbon tape (Leitabs, Plannet GmbH, Germany) and coated with gold by sputtering four times for 20 seconds (SEM autocoating Unit E5200, Polaron Equipment LTD, UK). Investigation of pore structure and interconnectivity was conducted using a JSM 840 scanning electron microscope (Jeol, Japan).

Towards Osteochondral Constructs (Chapter 6)

Selective Seeding Procedure

For osteochondral constructs bilayered scaffolds with a diameter of 9 mm comprising a PU part (4 mm thickness) supposed for cartilage engineering and a hydroxyapatite-based composite part (2 mm thickness, 9 mm diameter) for bone culture were provided by polyMaterials (Kaufbeuren, Germany) in cooperation with the Friedrich-Baur-Institute (Bayreuth, Germany). Two types of bilayered scaffolds were distinguished. For one type of bilayered scaffolds, the composite part was plotted on a membrane, which covered the surface of the PU scaffold and which was introduced during the scaffold production process ("with membrane"). The second type was fabricated by plotting the composite-part directly on the open pores of the PU-part ("open").

First fibrin gel [23] with bromophenolblue (Serva, Heidelberg, Germany) was injected radially and selectively into the PU part of the bilayered scaffold combination (Figure 2) with a syringe to establish the insertion technique. The established procedure was then applied to

seed the PU part of the bilayered scaffold combination with $16.5*10^6$ chondrocytes suspended in fibrin gel. Constructs were held in cell culture for 3 weeks.

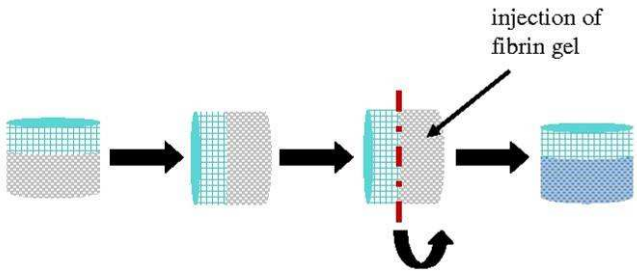


Figure 2: Selective gel insertion in PU part of scaffold combination

According to the results (c.f. Chapter 6), in subsequent experiment, the PU part was reduced to 2 mm thickness. Consequently, the cell number was also distinctly reduced, as compared to the preceding experiment. Moreover, to optimize selectivity of the seeding procedure and to avoid insertion of fibrin gel with chondrocytes in the composite part, only 80% of the fibrin gel containing the cells (i.e. $3.7*10^6$ chondrocytes per construct) was selectively injected into the PU part of the scaffold combination.

In the following experiments the composite part was blocked during the seeding procedure with a thermo-reversible gelatine gel to increase selectivity in seeding, i.e. to ensure that exclusively the PU part was seeded with chondrocytes. The thermo-reversible gelatine gel described by Joly-Duhamel [30] was modified for this purpose. In brief, 1.12 g gelatine (from bovine skin, Sigma-Aldrich, Taufkirchen, Germany) were dissolved in 25 ml of a saccharose solution (64 g saccharose dissolved in 36 g Millipore water, 30 g of this syrup was diluted with 70 g Millipore water) at 50° C. Then the tempered (50° C) gelatine solution was subjected to sterile-filtration. 100 µl of the gelatine solution were inserted in the composite part of the combination scaffolds. The bilayered scaffolds were pre-treated with ethanol 70% for 5 min, then rinsed 5 times with PBS and sterilized by autoclave sterilization beforehand.

The constructs were kept at 4°C over night in 6-well plates. The PU part was seeded with $3.7*10^6$ cells suspended in 102 µl fibrin gel. During gelation of the fibrin gel in the incubator at 37° C for 45 min, the gelatine simultaneously became fluid again and could be removed by washing with PBS. Then the constructs were covered with 7 ml CCM. After 24 h the cell number was determined in the PU as well as in the composite part to analyze seeding selectivity. Cartilage development and cell distribution were also evaluated after 1 week in culture.

Cartilage-Bone Co-Culture

For the first actual co-culture combination scaffolds with a PU part of 4 mm thickness and a diameter of 9 mm were applied. They were seeded with chondrocytes in fibrin gel ($16.5*10^6$ cells per construct) and after 1 week pre-cultivation in CCM the composite part of the scaffolds was seeded with GFP-labelled bone-marrow derived stem cells (BMSC). Constructs seeded with chondrocytes only served as control. After 3 days, constructs were harvested for determination of cell viability and cell distribution (Cooperation with research group Prof. Matthias Schieker, LMU Munich, Germany, BMSC seeding and viability testing was performed at the LMU).

The second attempt towards cartilage-bone co-culture was conducted applying the modified system. Combination scaffolds with a PU part with the reduced thickness of 2 mm were seeded with fibrin gel with a distinctly reduced cell concentration, i.e. $3.7*10^6$ cells per construct while the composite part was blocked with the thermo-reversible gelatine gel as described above. After 1 week of pre-culture, constructs were seeded with GFP-labelled BMSC. After 3 days constructs were harvested for determination of cell viability and cell distribution.

Effect of Growth and Differentiation Factor-5 (GDF-5) and Insulin on Expanded Chondrocytes (Chapter 7)

Bovine Chondrocytes in Fibrin Gel

For the preparation of fibrin gels seeded with expanded (P2) bovine chondrocytes, the cells were isolated, expanded and harvested as described above with some modifications. In order to investigate the influence of the application of growth factors during the expansion process on the capability of redifferentiation in subsequent 3D culture in fibrin gel [23] three experimental groups were created. The control group (Exp: CTR) was expanded without protein supplementation of CCM, the other two groups were expanded either in CCM supplemented with HuGDF-5 (kindly supplied by Biopharm, Heidelberg, Germany) at a concentration of 0.1 µg/ml (Exp: G 0.1) or in CCM supplemented with HuGDF-5 at a concentration of 0.1 µg/ml in combination with 2.5 µg/ml insulin (Sanofi-Aventis, Frankfurt/ Main, Germany) (Exp: G 0.1+I). After reaching second confluency (passage 2) cells were harvested for fibrin gel culture.

$5*10^6$ passage 2 (P2) chondrocytes were suspended in 20 µl fibrinogen solution (100 mg/ml). 20 µl of this suspension (containing $2.5*10^6$ chondrocytes, due to the volume of the cells) were pipetted into a silanized glass ring place at the bottom of a 6-well culture plate. Then 20µl of thrombin (5 U/ml) solution were added and gently mixed with the fibrinogen component. After 45 min for gelation at 37° C the glass ring was removed and the fibrin gels were covered with 4 ml of CCM. Each of the three groups was sub-divided into three groups (Fig. 3). The fibrin gels were cultured either in complete chondrocyte medium (CTR) or complete chondrocyte medium supplemented with 0.1 µg/ml HuGDF-5 (G 0.1) or the combination of HuGDF-5 0.1 µg/ml and insulin 2.5 µg/ml (G 0.1+I) for 21 days. Medium was changed thrice a week. After 21 days in culture constructs were weighed, cut into two

halves, one for histological examination one for biochemical analysis. The samples for biochemical analysis were freeze-dried prior to digestion.

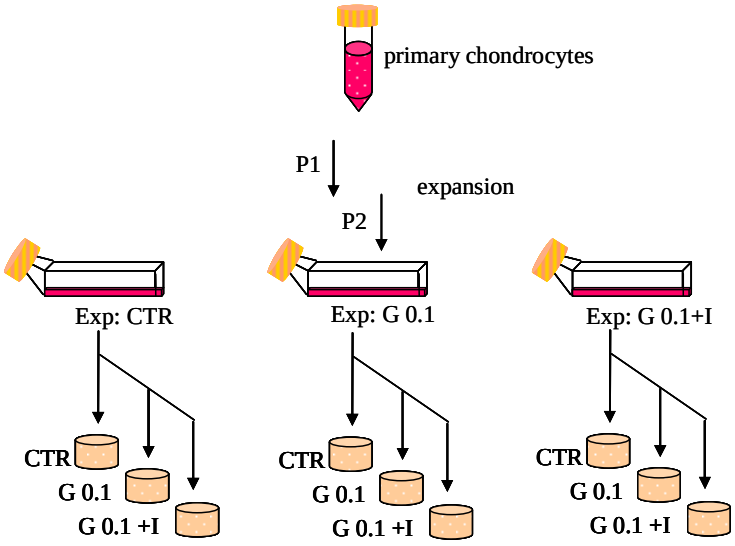


Figure 3: Experimental design of the investigation of the effect of GDF-5 application in combination with insulin during expansion on the subsequent redifferentiation in fibrin gel

Human Chondrocytes in Pellet Culture

Pellets of human chondrocytes were prepared in V-bottomed 96-well plates (Nunc via Fisher Scientific GmbH, Schwerte, Germany) by pipetting 200 µl suspension of $2.5*10^5$ chondrocytes in culture medium into each well and subsequent centrifugation at 1300 rpm for 5 min. After 24 hours for pellet consolidation growth factors were added to the culture medium (CCM) (composition described above) containing 10% FBS and from then on the pellets received growth factor supplemented culture medium with each change of medium. Due to the results with bovine chondrocytes insulin was applied at 2.5 µg/ml (I 2.5), HuGDF-5 at 0.1 µg/ml (G 0.1) and HuGDF-5 at 0.1µg/ml combined with insulin at 2.5 µg/ml (G 0.1 +I). Control constructs received medium without growth factor supplementation, but with addition of the equal volume of the dilution buffer used as solvent for HuGDF-5 and insulin.

Effect of GDF-5 and Insulin on Chondrogenic Differentiation of Adipose - Derived Stem Cells (ASC) (Chapter 7)

Human adipose - derived stem cells (ASC) (Promo Cell, Heidelberg, Germany) were expanded up to passage 3 in MSC growth medium (Promo Cell, Heidelberg, Germany). Then they were further expanded up to passage 6 in CCM.

After reaching confluency for the 6^{th} time (P6), cells were harvested for pellet culture. After detachment with trypsin cells were counted and 250 µl of the cell suspension containing $2.5*10^5$ cells were pipetted into wells of V-bottomed 96-well plates. Pellets were formed by centrifugation at 1300 rpm for 10 min. After 8 hours incubation for pellet consolidation 75 µl of the medium were removed, followed by addition of 175 µl growth factor containing CCM with the following final concentrations in the well: 0 (CTR), 2.5 µg/ml insulin (I 2.5), 0.1µg/ml HuGDF-5 (G 0.1), 2.5 µg/ml insulin and 0.1 µg/ml HuGDF-5 (G 0.1 +I). Pellets were cultured for 21 days in an incubator at 37° C, 95% humidity and 5% CO_2. Medium was changed thrice a week. Constructs were harvested for biochemical and histological assessment.

Biochemical Analysis of Engineered Tissue

Biochemical analysis of engineered constructs was conducted as previously described [24]. With the exception of pellets, constructs were cut into half after determination of wet weight (WW). In the case of pellet culture three pellets of each group were pooled for biochemical analysis. One half of the constructs or three pooled pellets respectively were digested with papainase solution (3.2 U/ml in buffer) over night (16 hours) at 60°C for subsequent determination of cell number, sulphated glycosaminoglycan (GAG) content and collagen content. The cell number was calculated from the DNA content which was determined by a

fluorometrical assay using Hoechst 33258 dye (Polysciences, Warrington, PA, USA) [31]. The content of GAG was assessed spectrophotometrically after reaction with dimethylmethylenblue (Sigma Aldrich, Taufkirchen, Germany) at 525 nm as chondroitin sulphate (ICN, Aurora, Ohio, USA) [32, 33]. The hydroxyproline content was determined after acid hydrolysis and reaction with chloramine-T (Merck, Darmstadt, Germany) and p-dimethylaminobenzaldehyde spectrophotometrically at 557nm [34]. In order to obtain the total amount of collagen a hydroxyproline to collagen ratio of 1:10 was applied [35].

Histochemical and Immunohistochemical Analysis of Engineered Tissue

Histological samples were fixed with 2% glutaraldehyde (15-30 min, dependent on construct characteristics) and stored in 10% formalin (both from Merck, Darmstadt, Germany). Formalin-fixed samples were then embedded into paraffin and cross-sectioned into 5µm slices. The cross-sections were then stained with safranin-O, fast green and haematoxylin [36].

For immunohistochemistry the cross-sections were deparaffinized before staining. The slides were incubated overnight with primary antibody solution dissolved at a ratio of 1:500 for type I collagen antibody (col-1) (Sigma-Aldrich, Taufkirchen, Germany) and 1:25 for type II collagen antibody (CIIC1) (DSHB, University of Iowa, USA) followed by incubation with secondary biotinylated antibody and subsequently with streptavidin-HRP (both Dako, Hamburg, Germany). Staining was performed with 3,3'-Diaminobenzidine Enhanced Liquid Substrate System (Sigma-Aldrich, Taufkirchen, Germany). Nuclei were counterstained with Weigert's haematoxylin.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

Pellets of expanded human chondrocytes were subjected to RT-PCR. RNA extraction was conducted using RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance to the manufacturer's protocol. Superscript II (Invitrogen, Karlsruhe, Germany) was applied for synthesis of cDNA in the presence of oligo-dt primers, nucleotides and ribonuclease inhibitor (Invitrogen, Karlsruhe, Germany).

Quantitative real-time RT PCR was performed with Platinum Sybr Green qPCR Supermix (Invitrogen, Karlsruhe, Germany) with ABI Prism 7000 (Applied Biosciences, Darmstadt, Germany). Glyceraldehyde phosphate dehydrogenase (GAPDH) served as housekeeping gene. Expression levels of the target genes were normalized to GAPDH. Expression levels were also normalized to levels of the control samples (set as 100%). Sequences of the applied primers are given in Table 1.

Primer	Sequence
GAPDH	fw: 5'- gaa ggt gaa ggt cgg agt c –3' rv: 5'- gaa gat ggt gat ggg att tc – 3'
Col1a1	fw: 5'- agg gcc aag acg aag aca tc -3' rv: 5' - aga tca cgt cat cgc aca aca – 3'
Col2a1	fw: 5'- ttc agc tat gga gat gac aat c -3' rv: 5' - aga gtc cta gag tga ctg ag – 3'

Table 1: Sequences of the primers applied for RT-PCR

Statistical Evaluation

The obtained data were analyzed using ANOVA (one way analysis of Variance) with subsequent Tukey test to determine statistical significances using SigmaStat 3.5 for Windows. In case of failure of the normality test analysis of variance on ranks was conducted (Kruskal Wallis analysis of variance on ranks indicated by $_1$with subsequent Tukey test).

Chapter 3

Chondrocyte Cell Culture Combining Polyurethane Scaffolds and Fibrin Gel for Auricular Reconstruction

(Manuscript in preparation, c.f. Appendices, Publications to be submitted)

Introduction

The external ear is one of the most complex 3-dimensional (3D) structures of the external body [37] and consists of three major components, the helix-antihelical complex, the conchal and the lobule. The 3D shape of the external ear is maintained by auricular cartilage, which is constituted by elastic cartilage [38]. Loss of auricular cartilage due to trauma, tumour resection or congenital defect is a major challenge in craniofacial surgery [1, 2]. The incidence of microtia is 100 to 150 cases per year in Germany [39]. There is no universally accepted classification of the severity of microtia. A commonly applied system was introduced by Tanzer and modified by Aguilar and distinguishes three grades of severity. Grade I is characterized by a slightly smaller than normal ear with basically normal features. A rudimentary and malformed auricle, which however displays some noticeable components, is classified as grade II. Grade III describes a severely reduced ear with a small clot of malformed tissue and anotia [37]. The most prevalently applied therapeutical method is costal cartilage graft reconstruction, which was established and described by Nagata and Brent [40]. Temporoparietal fascia flap, alloplastic implants and the combination thereof as well as prosthetic aids are further options for the treatment of microtia. All of the mentioned methods, however, are associated with certain disadvantages. Reconstruction by costal grafts on the one hand is reproducible if conducted by an experienced, skilled surgeon, but on the other hand requires multiple surgical procedures and causes donor site morbidity, as visible chest wall deformities [37]. Using the temporoparietal fascia is a procedure requiring special precaution. Alloplastic implants made of silicone or polypropylene are easily available, inherently stable, but also bear the risk of infection, extrusion, biocompatibility and uncertain long-term stability [41]. Prosthetic aids have not been tolerated and accepted very well in the past due to problems like skin irritation, change of the colour of the prosthesis over time. Consequently, established surgical methods for the reconstruction of the external ear applying autogenous

tissue represent the state of the art. Cosmetic results, however, are often unsatisfactory. Thus, tissue engineering is considered to be a promising alternative. For less complex shapes, engineering neocartilage applying scaffolds and chondrocytes has already been established using various scaffold materials. Here, the goal is to create an individual, aesthetic and autologous ear-shaped cartilage construct for the individual patient. This implicates the creation of a custom-made scaffold using the patient's healthy ear as a model, which is seeded with autologous chondrocytes, pre-cultured in vitro and then transplanted.

Rapid prototyping offers the opportunity to generate even complex, individual scaffolds for tissue engineering purposes [42–44]. Data needed for the fabrication of a custom-made mold in the shape of the human external ear can be acquired by imaging techniques like computer tomography (CT). Further data processing and conversion permits the production of the silicone mold through computer aided design (CAD) and computer aided manufacturing (CAM). In the presented study, a silicone mold was manufactured in a two-step process starting with the generation of a positive using stereolithography and subsequent generation of the silicone mold as negative. Subsequently, the silicone mould was used for the production of the scaffolds.

Porous scaffolds are a key-component in cartilage tissue engineering. Scaffolds have to comply with various requirements. They serve as a space-filling material and provide the three-dimensional shape for the desired engineered tissue as well as retention of the newly synthesized extracellular matrix. For tissue engineering approaches, soft hydrogels as well as sponge-like porous scaffolds are applied. Hydrogels are directly injectable into the defect and are easy to prepare, but often lack the mechanical stability and are not forming. The forming characteristic is one of the great advantages of porous scaffold materials, besides their mechanical stability.

Polymers widely used for the preparation of porous scaffolds are, for example polhydroxyacids, polylactides or polyglycolides. These materials support cell attachment as

well as proliferation, but are on the other hand characterized by limited elasticity and deformability. The latter characteristics, however, are considered to be of great importance for scaffolds applied for cartilage tissue engineering. Elastomeric polyurethanes have gained increasing interest as an alternative material due to their molecular stability in vivo and in case of the biodegradable polyurethanes due to the non-toxic degradation products [45]. Moreover, they have been used in many implantable devices in clinical application [24].

Previously, the benefit of combining a hydrogel and a porous scaffold thus exploiting the inherent advantages of each scaffold type was demonstrated in the group [24]. Fibrin gel was chosen as hydrogel and polycaprolactone based polyurethane scaffolds [28] as mechanically stable component. Being physiological fibrin gel does not raise questions concerning biocompatibility. In combination with the porous scaffold, it provides a good retention of the cells as well as of the produced new extracellular matrix. It has also been reported that chondrocytes retain their round morphology and do not dedifferentiate when embedded in fibrin gel. It also allows cell migration within the scaffold [46].

In this study, the PU-fibrin system was utilized for the generation of engineered complex cartilage constructs. Before generation of an ear-shaped cartilage construct, the impact on cartilage development of suspending the cells in the thrombin or in the fibrinogen component when preparing the fibrin gel was investigated. A second experiment was conducted to enlighten the role of the fibrin gel on cell distribution and cartilage development in the polyurethane scaffold by directly seeding PU scaffolds without fibrin gel. Ear-shaped scaffolds (polyMaterials AG, Kaufbeuren, Germany) were manufactured in silicone molds produced by rapid prototyping techniques (KL Technik, Krailing, Germany).

Then the established concept of combining fibrin gel and PU scaffolds for cartilage tissue engineering was transferred to the complex ear-shaped PU scaffolds using bovine chondrocytes and a first prototype of ear-shaped cartilage was analyzed.

Results

Comparison of Two Seeding Procedures

The fibrinogen solution used in forming the fibrin gels is comparatively highly viscous. By suspending the chondrocytes in the fibrinogen solution, the viscosity is even increased making its handling difficult. Thus, the seeding procedure may be improved by suspending the chondrocytes in the thrombin solution, the other component in fibrin gel formation. In order to guarantee reproducibility of the results a comparison between the two methods was conducted. Comparing wet weight and cell number of both groups after 21 days showed only a slightly higher wet weight and cell number for the group seeded with chondrocytes suspended in the thrombin solution before mixture with the fibrinogen component and insertion into the scaffold discs (Fig. 4A-C). Considering the content of extracellular matrix (ECM) (Fig. 4D,E,H,I) and the activity in synthesizing extracellular matrix components, i.e. glycosaminoglycans (Fig. 4F) and collagen (Fig. 4G) there was no detectable difference between the two methods.

Glycosaminoglycan (GAG) distribution was determined by staining cross-sections of both groups with safranin-O. After 21 days in culture both groups exhibited substantial amounts of GAG homogenously distributed in the construct, indicated by an intense and evenly distributed red staining without a subtle distinction between the two experimental groups (Fig. 5).

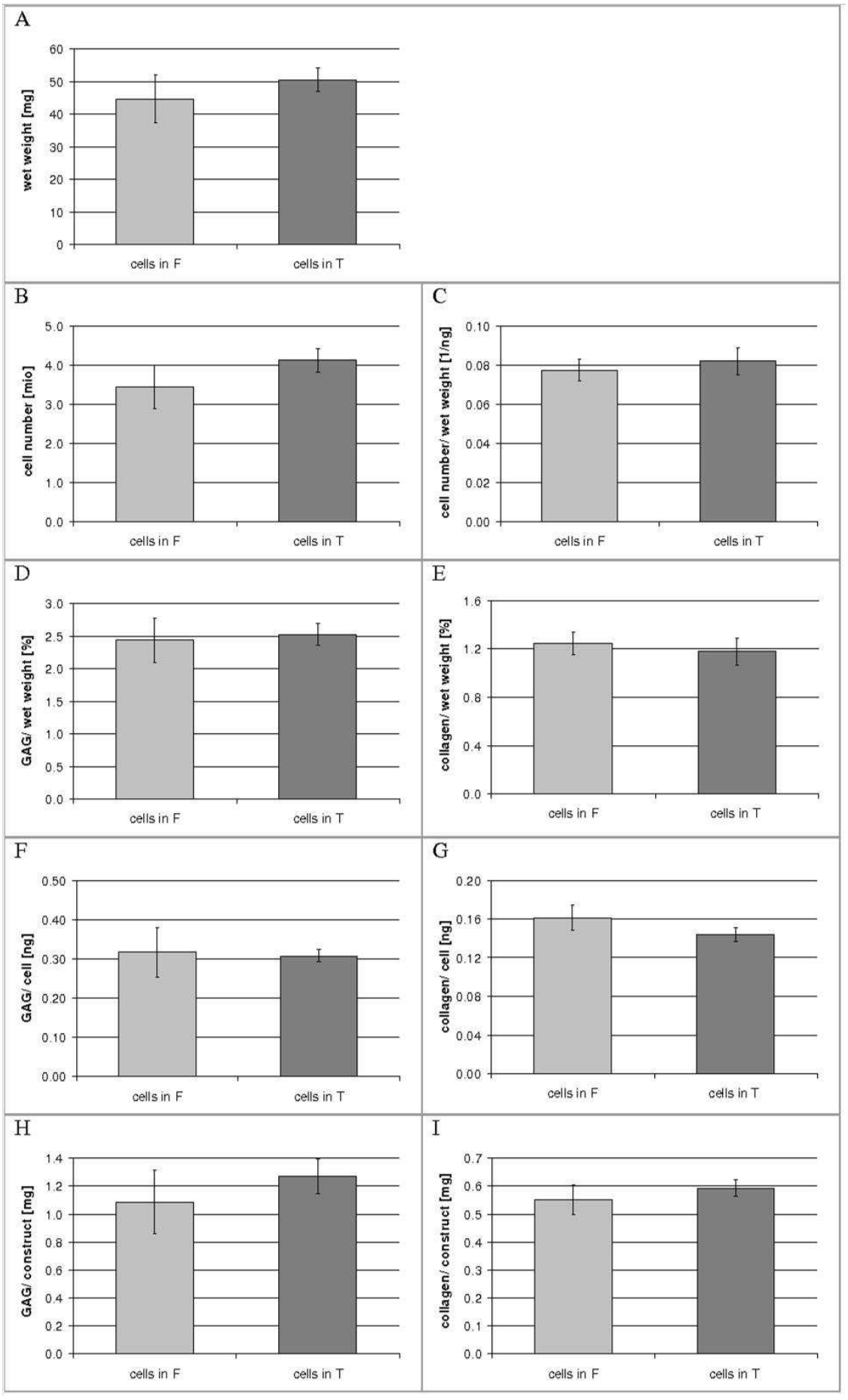

A
wet weight [mg]
60
50
40
30
20
10
0
cells in F
cells in T
B
cell number [mio]
5.0
4.0
3.0
2.0
1.0
0.0
cells in F
cells in T
C
cell number/ wet weight [1/ng]
0.10
0.08
0.06
0.04
0.02
0.00
cells in F
cells in T
D
GAG/ wet weight [%]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cells in F
cells in T
E
collagen/ wet weight [%]
1.6
1.2
0.8
0.4
0.0
cells in F
cells in T
F
GAG/ cell [ng]
0.50
0.40
0.30
0.20
0.10
0.00
cells in F
cells in T
G
collagen/ cell [ng]
0.20
0.16
0.12
0.08
0.04
0.00
cells in F
cells in T
H
GAG/ construct [mg]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
cells in F
cells in T
I
collagen/ construct [mg]
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
cells in F
cells in T

Figure 4 (p. 40): Wet weight (A), cell number (B-C), GAG-content (D,F,H), and collagen content (E,G,I) of cartilage constructs after 21 days of culture. Cells in F: chondrocytes were suspended in the fibrinogen component before mixture with thrombin solution and subsequent insertion into the polyurethane scaffold disc; Cells in T: chondrocytes were suspended in thrombin solution before mixture with fibrinogen component and following insertion into the polyurethane scaffold disc. Data represents the average ± SD of four independent constructs.

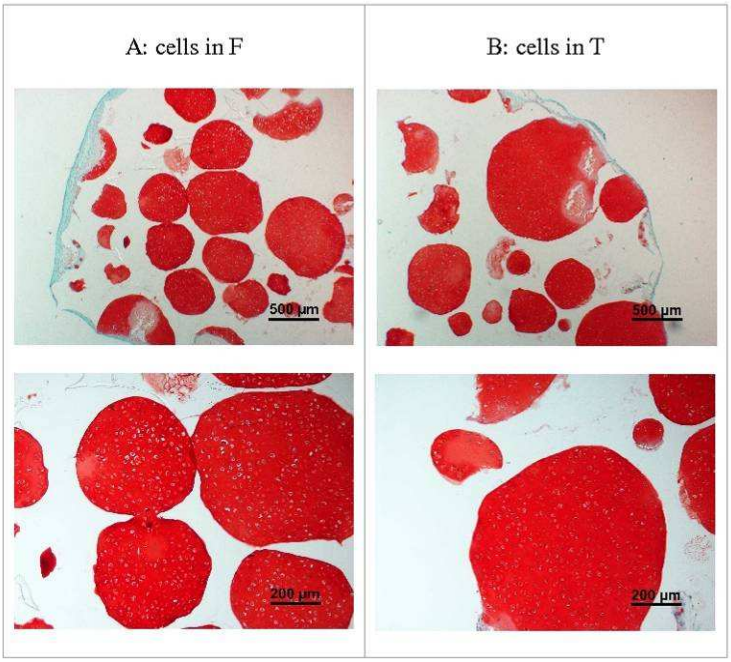


Figure 5: Glycosaminoglycan (GAG) distribution in cross-sections of cell-fibrin-PU scaffold constructs after 21 days in culture. GAG was stained red with safranin-O. Cells in F: chondrocytes were suspended in fibrinogen component before mixture with thrombin solution and subsequent insertion into the polyurethane scaffold disc; cells in T: chondrocytes were suspended in thrombin solution before mixture with fibrinogen component and following insertion into the polyurethane scaffold disc.

Consequently, for further experiments the procedure was adjusted and the chondrocytes were suspended in the thrombin solution before mixture with the fibrinogen solution and subsequent insertion into the scaffold, as the thrombin solution displayed a comparatively lower viscosity. Thus, the suspension of chondrocytes in the thrombin solution was much easier to process in the seeding procedure.

Influence of the Fibrin Gel on Cell Distribution and Development of Cartilage Tissue within PU Scaffolds

In a second experiment, the role of the fibrin gel particularly on cell distribution within the PU scaffold discs was investigated by seeding the constructs without fibrin gel and subsequent comparison with the results obtained applying the combination of fibrin gel and PU scaffolds (see Figs. 4 & 5). The wet weight was increased over the 21 days in culture (Fig. 6A). For seeding without fibrin gel, the extraordinarily high standard deviation on day 3, regarding the mean of the wet weight as well as of the cell number, indicated a low reproducibility of the seeding procedure without the fixation of the cells in the scaffold with the fibrin gel (Fig. 6A-C). Cell number on day 3 lay far behind the expected cell number, as initially $2.5*10^6$ chondrocytes were applied for each scaffold. From day 7 onwards cell number increased (Fig. 6B). Matrix content, i.e. glycosaminoglycans (GAG) per wet weight (Fig. 6D) and collagen per wet weight (Fig. 6E), also increased during the culture period. GAG production (GAG/ cell [ng]) reached already a plateau on day 7, collagen production (collagen/ cell [ng]) on day 14, hence further increase in matrix was due to proliferation of the cells (Fig. 6F,G). Total GAG and collagen, however, ascended over the entire culture period (Fig. 6H,I).

For the purpose of visualization of cell distribution within the pores cross-sections were stained with haematoxylin/ eosin (H&E). In order to analyze the development and location of GAG in the constructs, cross-sections were subjected to safranin-O staining. H&E staining of the cross-sections showed a relatively high cell number at the walls of the pores from the beginning of the culture period onwards. This phenomenon was ascribable to the adherence of the cells to the walls during the seeding process. Over the culture period the pores were filled with cells and matrix, with a lower cell density located towards the centre of the scaffold. Remarkably, the structure resembled the zonal organization observed in native cartilage.

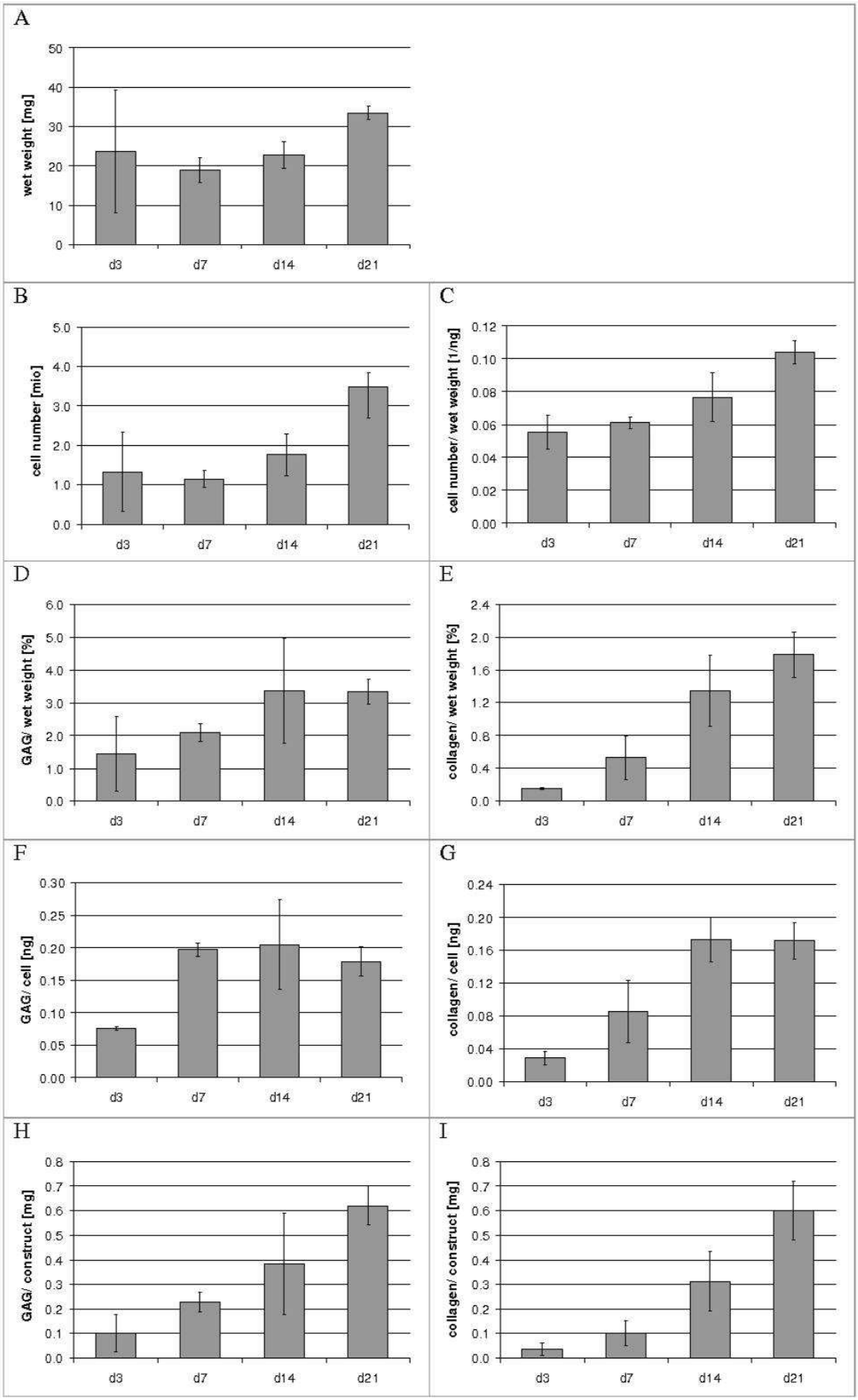

A
wet weight [mg]
50
40
30
20
10
0
d3
d7
d14
d21
B
cell number [mio]
5.0
4.0
3.0
2.0
1.0
0.0
C
cell number/ wet weight [1/ng]
0.12
0.10
0.08
0.06
0.04
0.02
0.00
D
GAG/ wet weight [%]
6.0
5.0
4.0
3.0
2.0
1.0
0.0
E
collagen/ wet weight [%]
2.4
2.0
1.6
1.2
0.8
0.4
0.0
F
GAG/ cell [ng]
0.30
0.25
0.20
0.15
0.10
0.05
0.00
G
collagen/ cell [ng]
0.24
0.20
0.16
0.12
0.08
0.04
0.00
H
GAG/ construct [mg]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
I
collagen/ construct [mg]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

Figure 6 (p. 43): Wet weight (A), cell number (B,C), GAG content (D,F,H), and collagen content (E,G,I) of cell-PU scaffolds on d3, d7, d14 and d21 of culture. Data represents the average ± SD of three independent constructs

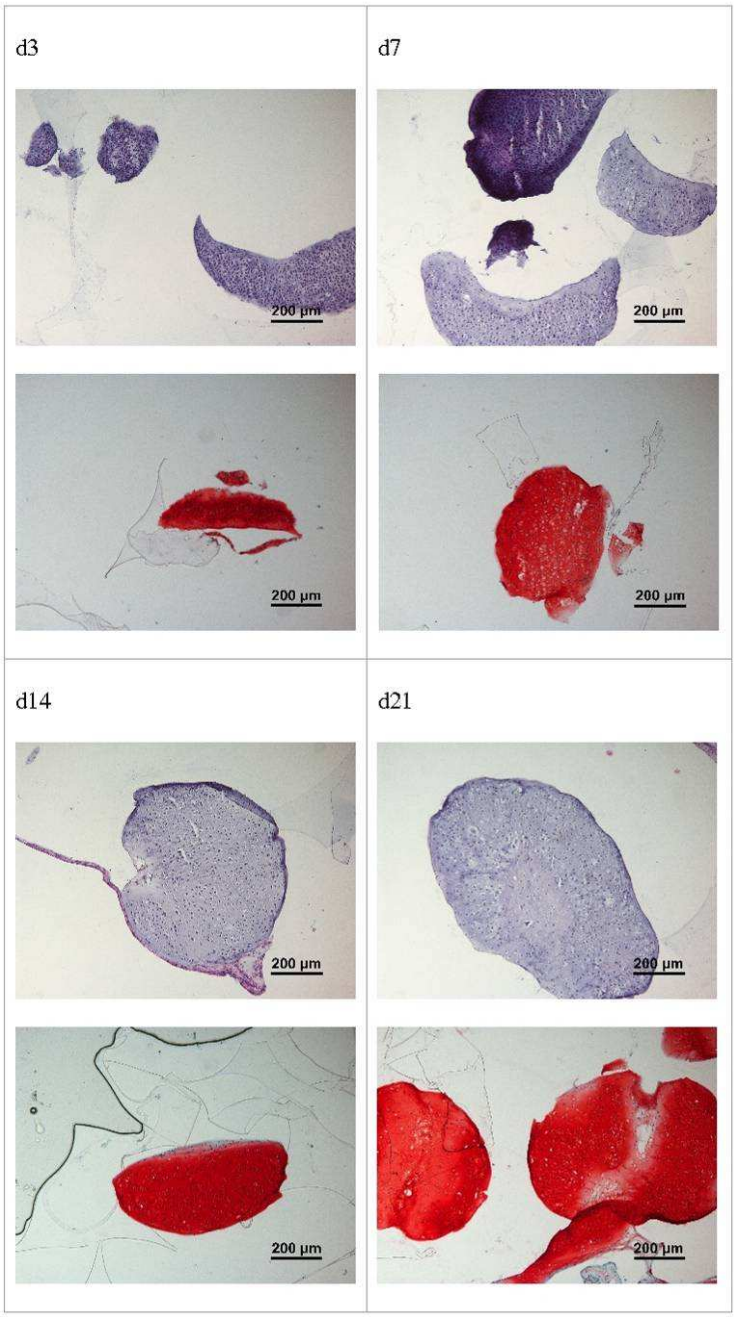


Figure 7: Upper rows: Haematoxylin/Eosin staining of the nuclei counterstained with eosin (blue coloured) of the cross-sections of the polyurethane scaffold discs directly seeded with chondrocytes on day 3 (d3), day 7 (d7), day 14 (d14) and day 21 (d21). Lower rows: Glycosaminoglycan (GAG) distribution in cross-sections of polyurethane scaffold discs seeded with chondrocytes. GAG was stained with safranin-O.

Safranin-O stained cross-sections displayed a very homogenous and intense red staining of GAG already on day 3, indicating an early start of cartilaginous matrix production, and development of a homogenous cartilaginous construct over the culture period of 21 days (Fig. 7). Comparing the stained cross-sections of discs seeded with chondrocytes suspended in CCM (Fig. 7), and those seeded with chondrocytes suspended in fibrin gel (Fig. 5), cell distribution was significantly more homogenous when using fibrin gel.

Ear-Shaped Cartilage Constructs Combining Fibrin Gel and PU Scaffolds

Due to the easier processing using the thrombin solution to suspend the cells with given comparability of the results to those of the original method (see above), the modified method was applied for seeding the ear-shaped PU scaffold.

Figure 8 shows the ear-shaped construct obtained after 28 days in culture. Macroscopic inspection of the yielded cartilage construct suggested that it contained a relatively high amount of culture medium, indicated by the red colour.

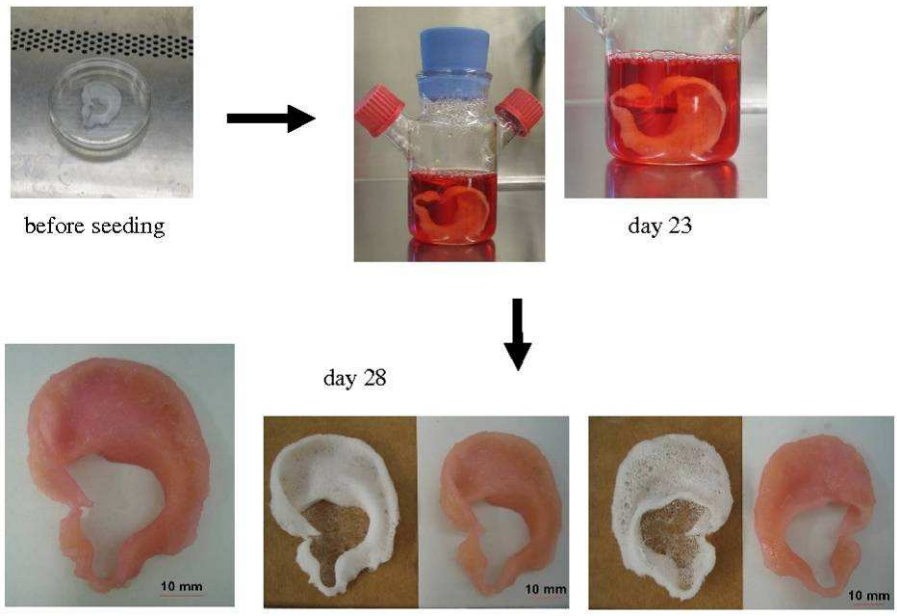


Figure 8: Development of an ear-shaped cartilage construct. After seeding it was cultured in a custom-made bioreactor and harvested after 28 days in culture.

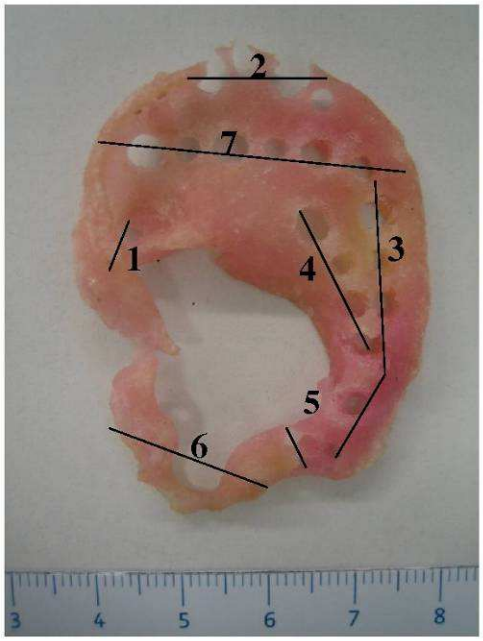


Figure 9: Section planes and localization of the samples taken for biochemical and histological analysis to obtain a comprehensive analysis of the development of cartilage tissue covering the whole construct after 28 days in culture.

In order to facilitate a comprehensive analysis of the whole construct with high spatial resolution, samples (# 1 – 7) were punched out from various section planes of the ear-shaped construct (Fig. 9) for biochemical and histological analysis.

Due to the fact that the discs obtained from the ear-shaped cartilage for biochemical analysis had a slightly smaller diameter (3 mm) than the routine discs, which served as a control (CTR), and furthermore differed in thickness depending on their location in the ear-shaped construct, wet weight could not really be compared (Fig. 10A). The cell number per wet weight was much smaller in the samples derived from the ear than in the control group (CTR). The same applied to the content of glycosaminoglycans and collagen per wet weight (Fig. 10C, E). This was plausible considering the comparatively low cell number in the ear-shaped construct. Regarding GAG production per cell, however, the performance of the chondrocytes in the ear-shaped construct reached a level comparable to the chondrocytes in

the control discs (Fig. 10D). The collagen production, however, expressed as collagen [ng] per cell was also remarkably lower in the samples of the ear-shaped construct in comparison with the control group (CTR) (Fig. 10F).

In accordance with the results of the biochemical analysis, glycosaminoglycan distribution in the cross-sections was neither coherent nor homogenous. Single cells or conglomerates of some chondrocytes embedded in cartilaginous matrix could be detected in every section plane, but without forming a coherent cartilaginous construct, whereas in the control discs an intense and homogenous safranin-O staining throughout the pores of the routine disc was detected indicating production of a coherent cartilage tissue (Fig. 11).

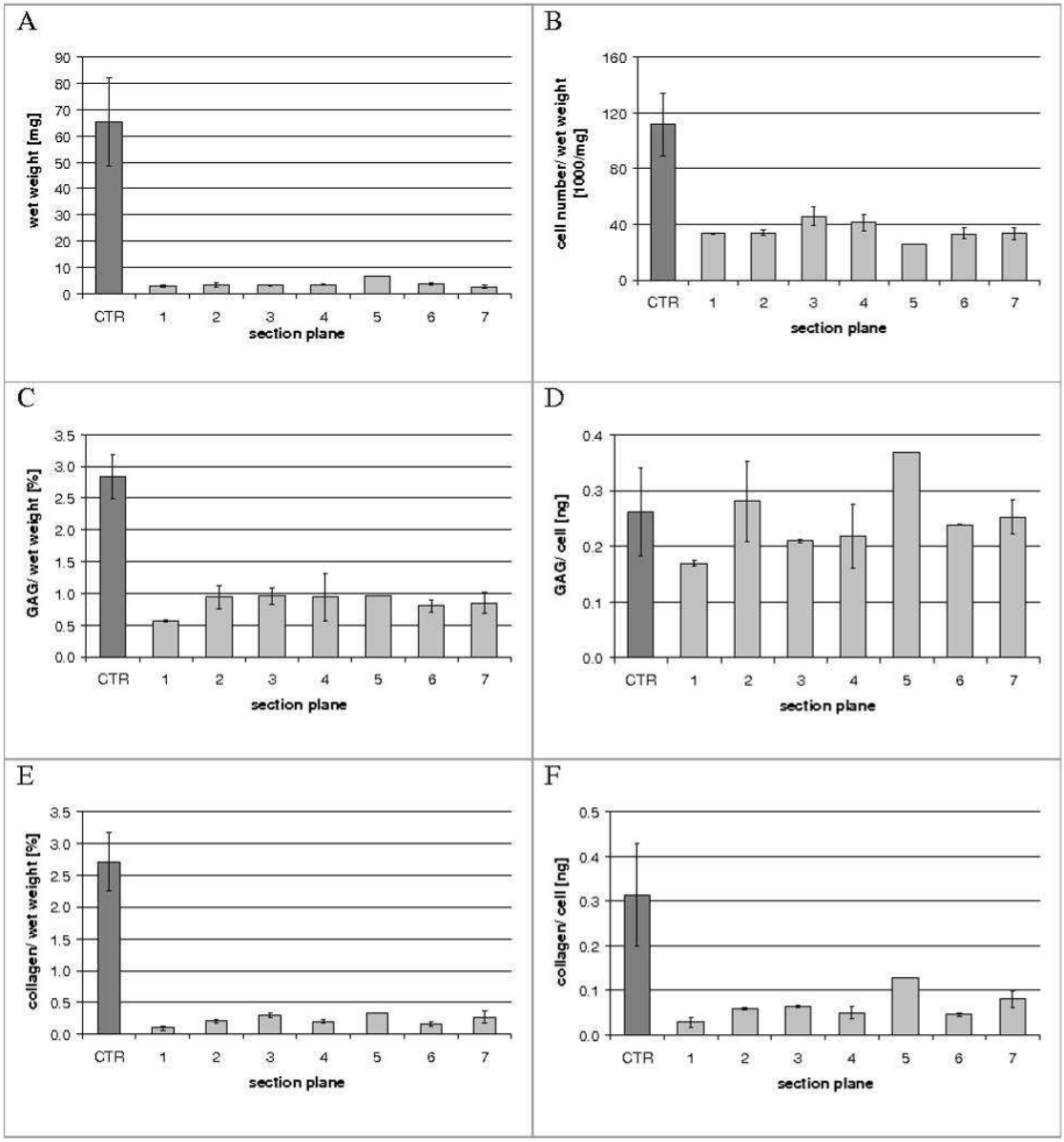


Figure 10: Wet weight (A), cell number (B), GAG content (C-D), and collagen content (E-F) of samples taken from representative section planes (1-7) of the ear-shaped construct after 28 days in culture. Round polyurethane scaffold discs seeded with chondrocytes in fibrin gel served as control (CTR). CTR: Data represents the average ± SD of three independent samples. Samples from the ear: Data represents the average of two or three samples from the respective section plane with the exception of section plane # 3, where only 1 sample could be obtained; error bars represent the minimum and maximum values.

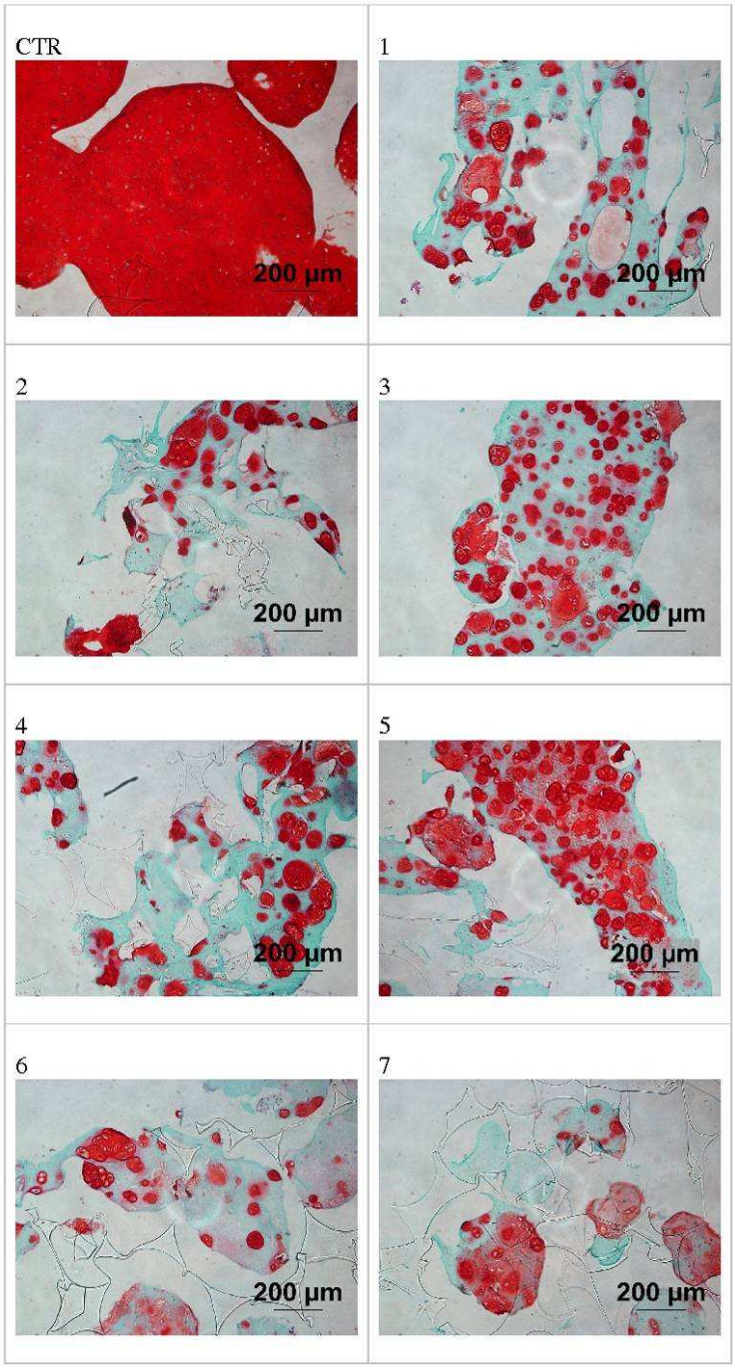


Figure 11: Glycosaminoglycan (GAG) distribution in cross-sections of discs punched out of the representative sections indicated in figure 9 and of control discs (CTR) of round-shaped routine polyurethane scaffolds. GAG was stained red with safranin-O.

Discussion

The first experiment evidenced that it was irrelevant for the development of cartilage tissue, if the chondrocytes were suspended in the fibrinogen or in the thrombin solution before mixture with the respective second component and subsequent injection of the mixture into the PU scaffold. The great benefit of the adjustment of the procedure, i.e., using the thrombin solution for suspending the cells, was an easier processing leading to a more robust procedure. A comparison with the previous procedure demonstrated no difference in outcome (Figs. 4 & 5) and guaranteed comparability with the experiments previously conducted in the group.

In the second experiment, in which PU scaffolds were seeded without fibrin gel, the role of the fibrin gel with respect to cell distribution and structure of the engineered cartilage tissue was enlightened. In comparison with the tissue development and yielded cell numbers in the first experiment, the great advantage of combining fibrin gel and PU scaffold for cartilage tissue engineering became evident in terms of higher retention of chondrocytes and newly synthesized extracellular matrix (Figs. 4 & 5 in comparison with Figs. 6 & 7). Another aspect is the homogeneity of cell and matrix distribution which was given to a high extent when combining fibrin gel and PU scaffold, whereas when seeding the scaffold with a suspension of chondrocytes in culture medium, cells were found to adhere basically to the wall of the pores of the scaffold. Over time the highest concentration of cells remained at the walls of the pores, whereas approaching the centre the density decreased (Fig. 7). Interestingly, this rendered a structure highly resembling the zonal structure displayed in native cartilage [4]. Even though the zonal organization was not intended in this approach towards auricular cartilage engineering, the possibility to mimic zonal organization implies options for further research towards designing cartilage constructs, which may also be used for studies investigating cartilage physiology. Another approach described in literature tried to obtain a

zonal organization by application of bilayered photopolymerized hydrogels, which was used to investigate the effect of the zonal organization on the properties of the yielded tissue [47]. After optimization in terms of handling of the combination of polyurethane scaffold and fibrin gel the system was transferred to the complex 3D structure of the human external ear. In previous studies, other groups also approached reconstruction of the external ear by tissue engineering methods. One group also suspended the chondrocytes in fibrin gel, but instead of the porous scaffold the gel was put between two layers of lyophilized perichondrium [48]. Another group used bioresorbable PGLA-PLLA polymer scaffolds which were seeded with human nasal septal chondrocytes suspended in fibrin gel. This approach rendered solid tissue and substantial neo-cartilage formation with the presence of cartilage specific matrix components after 6 weeks of pre-culture in vitro and subsequent in vivo implantation for 6-12 weeks [49]. Another possibility was represented by using an ear model, produced by using clay modelling, to produce ear-shaped scaffolds of PGA (poly (glycolic acid)), PCL (poly (ε-caprolactone)) and P-4HB (poly (4-hydroxybutyrate)) for culture of chondrocytes isolated from adult sheep ears [41]. Liu et al also employed CAD/CAM to engineer a human ear-shaped cartilage construct. PGA scaffolds coated with PLA to enhance stability of the scaffold were seeded with chondrocytes isolated from newborn articular swine cartilage [50]. None of these studies provided a comprehensive analysis of the whole construct with high spatial resolution in contrast to the investigation presented here. Analyzing samples from different representative parts of the ear-shaped cartilaginous construct allows to judge, if cartilage tissue is really coherent and homogenous. Furthermore with the exemption of the scaffolds applied by Liu et al., the ear-shaped scaffolds applied were very simple and crude in shape. They only resemble the human external ear, but are not shaped with the individual details of the patient. Liu et al. also applied CAD/ CAM to produce the ear-shaped scaffold. Within this study, although in the generated ear-shaped cartilage construct cartilaginous ECM could be detected to some extent throughout the construct (Fig. 11), particularly section plane

1, 3 and 5 a coherent cartilage construct was not achieved in this prototype. Further experiments have to be conducted to elucidate the reasons for the not yet satisfying development of cartilaginous tissue. Pore size and interconnectivity of the scaffold are particularly crucial parameters in cartilage engineering. First of all seeding efficiency may be compromised by an unfavourable pore structure. If the seeding efficiency is too low, a comparably low number of chondrocytes affecting subsequent tissue development. But ECM formation and generation of a coherent cartilaginous tissue can also be directly negatively affected by a too low pore size and interconnectivity. Grad et al., for instance, showed that decreasing pore size did not improve phenotype expression and cartilaginous matrix synthesis of incorporated chondrocytes, but rather restricted nutrient supply [51]. A careful analysis of the scaffold used in this study with special attention paid to its pres structure appears inevitable. This may then facilitate rational changes in the polymer composition of the scaffold and the optimization of the scaffold production process.

Chapter 4

Characterization of Polyurethane Scaffolds for Auricular Reconstruction

Introduction

In Chapter 3, engineering of an ear-shaped cartilage construct applying the combination of fibrin gel and ear-shaped PU scaffolds was described and the results of the first prototype are given and discussed. As this prototype turned out to be very promising, but did not display the development of a really coherent cartilage construct, here possible reasons were investigated in order to allow for optimization. First the seeding efficiency had to be determined and compared to round routine discs. A low seeding efficiency would lead to a low amount of total matrix synthesized in the whole construct due to a low number of chondrocytes synthesizing extracellular matrix (ECM) components. Moreover, the scaffolds had to be further characterized as pore size and interconnectivity have a great impact on seeding efficiency, nutritive supply and retention of the newly synthesized matrix. Highly porous polymeric scaffolds are sometimes associated with the problem of low retention of the newly synthesized extracellular matrix, especially when mechanic stress is applied. On the other hand, a large pore size and an interconnective pore structure are required to ensure adequate access to nutrients. Pore size and interconnectivity are, thus, crucial characteristics of the scaffold. In order to enlighten the impact of pore size on the yielded quality of the cartilaginous construct Grad et al. studied cartilage development in polyurethane scaffolds of three different pore sizes. This study clearly evidenced that decreasing pore size restricted nutrient supply without having any positive effect [51]. Here, scanning electron microscopy (SEM) and seeding efficiency testing were done to characterize the ear-shaped PU scaffolds with spatial resolution.

Results

Several ear-shaped scaffolds with slight variations in polymer composition (all manufactured by polyMaterials AG, Kaufbeuren, Germany) were investigated with regard to seeding efficiency and pore structure. As the results were very similar, here one example is shown in detail. Ear 7 (internal number) was an ear-shaped polyurethane scaffold of the mannitol-based MVI-type. For cell seeding experiments, disc-shaped samples were punched out from various section planes of the ear-shaped construct. The cell seeding efficiency was determined to be very poor, with on average less than 30% of the theoretically possible $2.5*10^6$ cells within the construct.

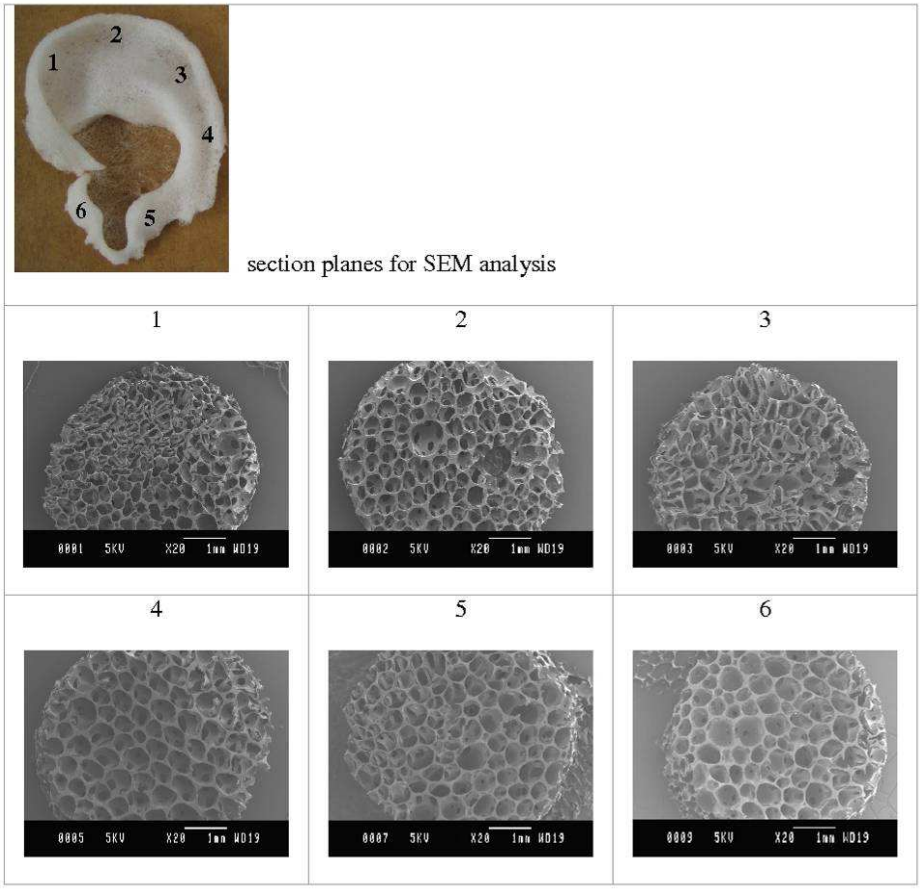


Figure 12: SEM analysis of ear-shaped polyurethane scaffold (MVI type, ear 7).

The unsatisfying results in the seeding efficiency test could be well correlated to SEM images revealing clear deficits in terms of interconnectivity for the tested material (Fig. 12). Nevertheless, the SEM images displayed that the pore structure was homogenous throughout the whole ear-shaped polyurethane scaffold (Fig. 12).

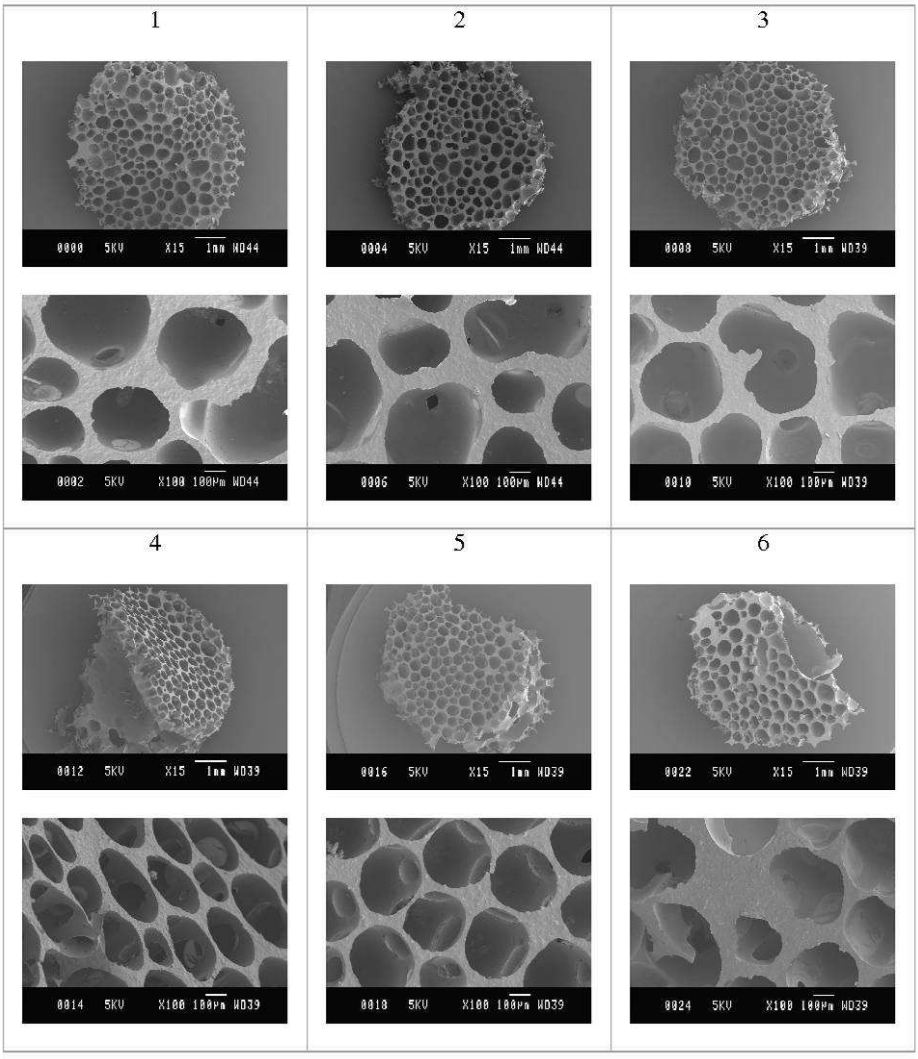


Figure 11: Further SEM images from representative parts of an ear-shaped PU scaffold.

In order to facilitate a deeper insight in the structure of the pores, representative images were obtained in different magnifications (Fig. 13). The larger magnifications revealed that the pores are either connected by very small holes or completely unconnected. Only two out of

six samples (sample # 4 and # 5) showed interconnective pores, whereas the exposure of sample # 6 was characterized by domination of solid polymer. These insights showed the potential but also demanded optimization in order to obtain an interconnective ear-shaped scaffold.

Subsequently produced variants of PU scaffolds were tested using SEM in order to allow conclusions by polyMaterials AG concerning the applied formulations and the resulting pore size and interconnectivity. Simultaneously, physico-chemical treatments to increase interconnectivity were imposed on the scaffolds and evaluated (Table 2). As physico-chemical treatment procedures did neither increase pore size nor interconnectivity, data is not shown here.

#	treatment procedure	evaluation
I	with/without ethanol treatment (70%, 5 min), five times rinsing with PBS, autoclave sterilization, 45 min boiling water	seeding efficiency test
II	45 min sodium hydroxide (Merck, Darmstadt, Germany) solution (3%) at 60°C, thrice 15 min PBS, ethanol (70%, 5 min), five times rinsing with PBS, autoclave sterilization	seeding efficiency
III	ethanol (70%, 5 min), five times rinsing with PBS, autoclave sterilization, 45 min boiling water	seeding efficiency SEM
IV	argon or nitrogen plasma (Reinhausen Plasma GmbH, Regensburg, Germany), ethanol (70%, 5 min), washing with PBS, autoclave sterilization, 45 min boiling water	seeding efficiency, SEM
V	ethanol (70%, 5 min), washing with PBS, freezing in water, 45 min sodium hydroxide solution (3%) at 60° C	SEM
VI	freezing in ethanol, vacuum	SEM
VII	ethanol (70%, 5 min), five times rinsing with PBS, autoclave sterilization, freezing (–80° C), vacuum ($1.2*10^{-2}$ mbar) at -78° C (approx.6h), vacuum overnight after removal of cooling	SEM
VIII	isopropanol (70%, 5-10 min), vacuum (70 mbar, 7min), ventilation, vacuum (70 mbar, 7 min), removal of isopropanol (2 mbar, 10 min), 70° C (10 min) or microwave (3 min)	SEM

Table 2: Applied physico-chemical treatment procedures to increase interconnectivity and pore size of the scaffolds.

Discussion

In general, there are several requirements for scaffolds applied for cartilage tissue engineering. They provide a highly porous, interconnected network, allowing cell growth and fluid exchange for nutrient supply and removal of metabolic products. Furthermore, they have to be biocompatible and bioresorbable with controllable degradation. Suitable surface chemistry for cell attachment, proliferation and differentiation are also essential. Moreover, the scaffold material ought to display mechanical properties suitable for the site of implantation [14, 52].

Pore size and interconnectivity represent especially crucial characteristics of the scaffold as they decisively influence nutrient availability, but also retention of newly synthesized extracellular matrix. SEM analysis of the ear-shaped polyurethane scaffolds revealed that in some areas of the scaffold interconnectivity as well as pore size was satisfying, whereas in other parts of the construct the structure was dominated by solid polymer, small pores and poor interconnectivity. Insufficient interconnectivity precludes a homogenous distribution of the chondrocytes suspended in fibrin gel and consequently the development of a coherent cartilage construct. Low exchange rates of nutrients and metabolic waste further contribute to the development of inferior cartilage tissue. The results obtained here provide a highly probable explanation for the comparatively weak engineered cartilage observed within the ear-shaped construct in Chapter 3. Nevertheless, the scaffold material resisted mechanical as well as thermal treatment, which indicates the stability of the material. This stability is required when implanting the constructs or if mechanical stimuli are already applied during in vitro culture.

In conclusion, the polyurethane scaffolds represent a scaffold material, particularly in the shape of the external human ear, with a great potential for clinical application as being biocompatible and biodegradable. They are also characterized by mechanical stability which

is required for implantation and for form stability. However, so far, the pore structure within the ear-shaped constructs investigated here prevented the development of coherent cartilage tissue.

New material variants of PU scaffolds are currently under investigation.

Chapter 5

Chondrocyte Cell Culture Applying OPF Scaffolds for Auricular Reconstruction

(Manuscript in preparation, c.f. Appendices, Publications to be submitted)

Introduction

Loss of auricular cartilage due to trauma, tumour resection or congenital defects represents a challenge in craniofacial surgery [1, 2]. Due to aesthetic deficits and other disadvantages associated with the currently applied surgical methods for ear reconstruction using autologous costal grafts, tissue engineering is considered to be a promising alternative for the established approaches.

Cartilage tissue engineering using scaffolds and chondrocytes has already been established for a large variety of scaffold materials [3, 9, 14] Custom-made scaffolds for the individual patient seeded with autologous chondrocytes are considered to render aesthetic implants. Rapid prototyping technologies allow the creation of even very complex, individual scaffolds as needed for external ear reconstruction [42-44].

Oligo (poly (ethylene glycol) fumarate- (OPF-) based hydrogels are often used for tissue engineering applications, particularly for release of bioactive molecules like growth factors [53–55], but also as a cell carrier for differentiation and culture of cells [56, 57]. OPF hydrogels are mainly used as injectable biodegradable system [58]. The biocompatibility of pre-crosslinked gels from various OPF-formulations and leachable fractions has been shown in vitro and in vivo [58, 59].

In this study, different compositions of OPF scaffolds were investigated for application in the manufacture of complex 3-dimensional (3D) constructs such as the human external ear. For this purpose, certain mechanical properties are required, which can be influenced by the composition of the used polymer (Polymer and scaffold production were performed within research group by M. Henke.). At first, the influence of the OPF formulation on engineered cartilage tissue was assessed using simple disc-shaped scaffolds. Subsequently, the best suited composition for creating highly complex 3D scaffolds was chosen. The ear-shaped OPF scaffolds were fabricated in a silicone mold, which was created applying rapid prototyping

methods including computer assisted design and manufacture (CAD/ CAM) by KL-Technik (Krailing, Germany). After sterilization by γ-irradiation, the ear-shaped scaffolds were seeded with chondrocytes suspended in fibrin gel in order to evaluate the potential of the material for generation of complex 3D cartilage constructs.

Results

Investigation of Development of Cartilage Tissue in Different OPF Scaffolds

In the first part of the study, the influence of the scaffold formulation on the yielded cartilage tissue was evaluated. Furthermore, the combination of OPF scaffolds with fibrin gel was compared to OPF scaffolds alone with respect to development of cartilaginous tissue. After 28 days of culture, constructs were harvested and analyzed.

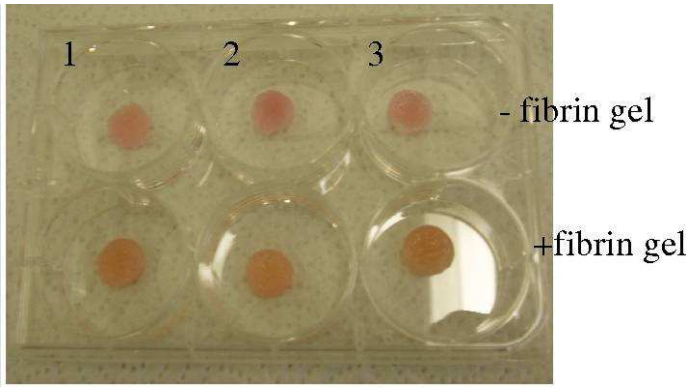


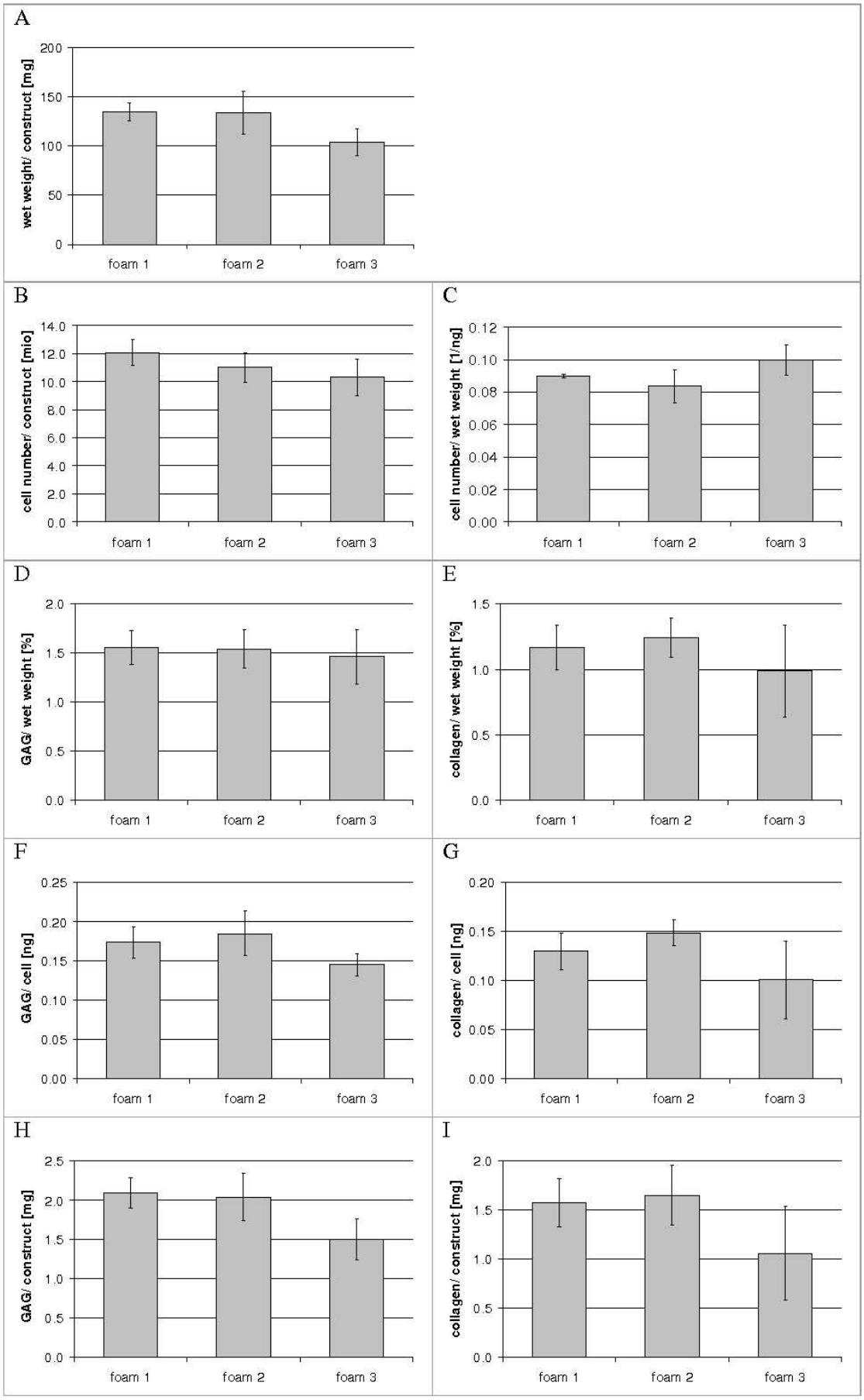
Figure 14: Macroscopic appearance of OPF foams (1 – 3) seeded with chondrocytes (- fibrin gel) and with chondrocytes suspended in fibrin gel (+ fibrin gel) after 28 days in culture.

The macroscopic appearance of the constructs after the culture period was characterized by a higher amount of medium inside the directly seeded OPF scaffolds compared to the scaffolds seeded with chondrocytes suspended in fibrin gel, which appeared more yellowish (Fig. 14).

A higher diffusion of medium into the construct might be due to lower density of extracellular matrix (ECM) in the construct. Comparing the three different scaffold types (foam 1 - 3), no difference could be observed macroscopically

Comparing wet weight (Fig. 15A) and cell number (Fig. 15B,C) no difference was detectable between the three different scaffold types. The relatively low cell number after 28 days, which was equivalent to the calculated cell number initially seeded onto the scaffolds, was due to an inevitable loss of cells during the seeding procedure (Fig. 15B).

Glycosaminoglycan (GAG) content and production (Fig. 15D,F,H) indicated substantial production of cartilage matrix in all three scaffold formulations. The same applied to collagen content and production (Fig. 15E,G,I). Statistical evaluation of the results of the biochemical analysis showed no difference between the three different scaffold formulations could be detected.


A
wet weight/ construct [mg]
200
150
100
50
0
foam 1
foam 2
foam 3
B
cell number/ construct [mio]
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
foam 1
foam 2
foam 3
C
cell number/ wet weight [1/ng]
0.12
0.10
0.08
0.06
0.04
0.02
0.00
foam 1
foam 2
foam 3
D
GAG/ wet weight [%]
2.0
1.5
1.0
0.5
0.0
foam 1
foam 2
foam 3
E
collagen/ wet weight [%]
1.5
1.0
0.5
0.0
foam 1
foam 2
foam 3
F
GAG/ cell [ng]
0.25
0.20
0.15
0.10
0.05
0.00
foam 1
foam 2
foam 3
G
collagen/ cell [ng]
0.20
0.15
0.10
0.05
0.00
foam 1
foam 2
foam 3
H
GAG/ construct [mg]
2.5
2.0
1.5
1.0
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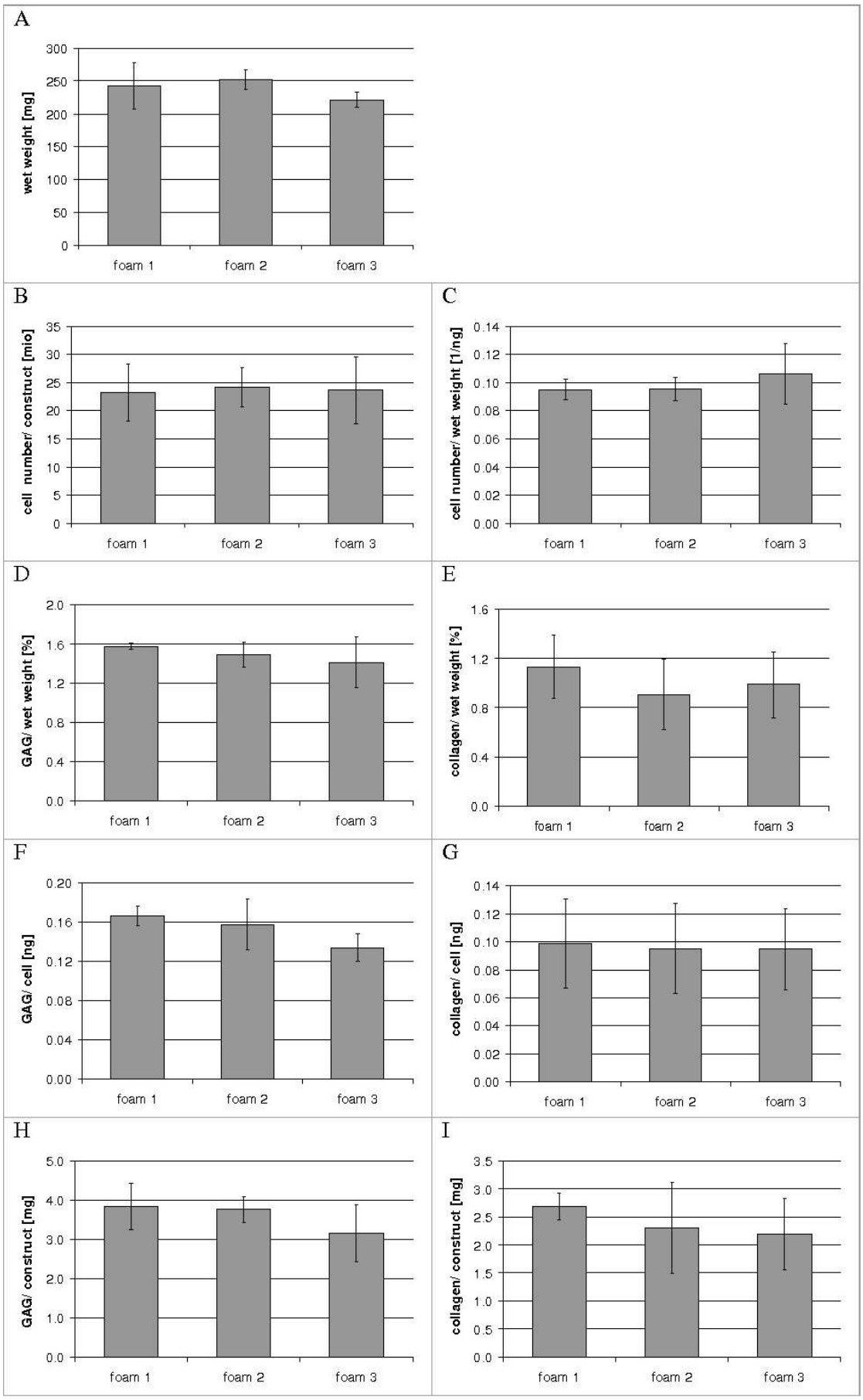
0.0
foam 1
foam 2
foam 3
I
collagen/ construct [mg]
2.0
1.5
1.0
0.5
0.0
foam 1
foam 2
foam 3

Figure 15 (p. 65): Wet weight (A), cell number (B, C), GAG content (D,F,H) and collagen content (E,G,I) of cartilaginous constructs generated with OPF scaffolds (1-3) seeded with primary bovine chondrocytes after 28 days in culture. Data represents the average ± SD of three independent constructs.

In a second study the development of cartilaginous tissue was evaluated when combining fibrin gel and OPF scaffolds.

Wet weight of the constructs seeded with chondrocytes in fibrin gel was much higher than of the constructs directly seeded with chondrocytes suspended in CCM (approximately 100 mg higher in each group) (Fig. 16A). Cell number (Fig. 16B,C) was also approximately 100% higher than cell number in the corresponding samples of OPF scaffolds directly seeded with chondrocytes. The cell number reached two times the initial cell number seeded onto the scaffold by insertion of fibrin gel with suspended chondrocytes.

Analysis of GAG production (Fig. 16) and GAG content (Fig. 16D,H) proved the development of substantial amounts of cartilage matrix. Collagen content (Fig. 16E,I) and collagen production (Fig. 16G) also indicated the development of cartilage tissue over the period of 4 weeks. Comparing the results of the different foam formulations no difference could be detected. Comparing the yielded results for GAG and collagen production (GAG/ cell and collagen/ cell) of the combination constructs with the constructs seeded without fibrin gel, there was also no difference detectable between the constructs seeded with and without fibrin gel. However, as approximately a two-fold increase in cell number was reached in the OPF-fibrin-combination, there was also approximately a two fold-increase observed in GAG and collagen (Figs.15 & 16).


A
wet weight [mg]
300
250
200
150
100
50
0
foam 1
foam 2
foam 3
B
cell number/ construct [mio]
35
30
25
20
15
10
5
0
foam 1
foam 2
foam 3
C
cell number/ wet weight [1/ng]
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
foam 1
foam 2
foam 3
D
GAG/ wet weight [%]
2.0
1.6
1.2
0.8
0.4
0.0
foam 1
foam 2
foam 3
E
collagen/ wet weight [%]
1.6
1.2
0.8
0.4
0.0
foam 1
foam 2
foam 3
F
GAG/ cell [ng]
0.20
0.16
0.12
0.08
0.04
0.00
foam 1
foam 2
foam 3
G
collagen/ cell [ng]
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
foam 1
foam 2
foam 3
H
GAG/ construct [mg]
5.0
4.0
3.0
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1.0
0.0
foam 1
foam 2
foam 3
I
collagen/ construct [mg]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
foam 1
foam 2
foam 3

Figure 16 (p. 67): Wet weight (A), cell number (B,C), GAG content (D,F,H) and collagen content (E,G,I) of cartilaginous constructs generated with OPF scaffolds (1-3) seeded with primary bovine chondrocytes in fibrin gel after 28 days in culture. Data represents the average ± SD of three independent constructs.

Staining for GAG on cross-sections of the constructs proved a predominantly homogenous and substantial production of cartilaginous matrix throughout the constructs seeded with chondrocytes suspended in CCM (Fig. 17). OPF hydrogels seeded with chondrocytes suspended in fibrin gel displayed large areas intensely stained red, although areas with faint safranin-O staining were also detected (Fig. 17). Similar to the results of the biochemical analysis no difference between foam 1 to 3 was detectable, neither when directly seeded nor in case of seeding with chondrocytes suspended in fibrin gel. Fibrous tissue, however, could only be detected at the edges of single constructs as a margin with a faint green staining (Fig. 17 foam 1 + fibrin gel, foam 3 + fibrin gel).

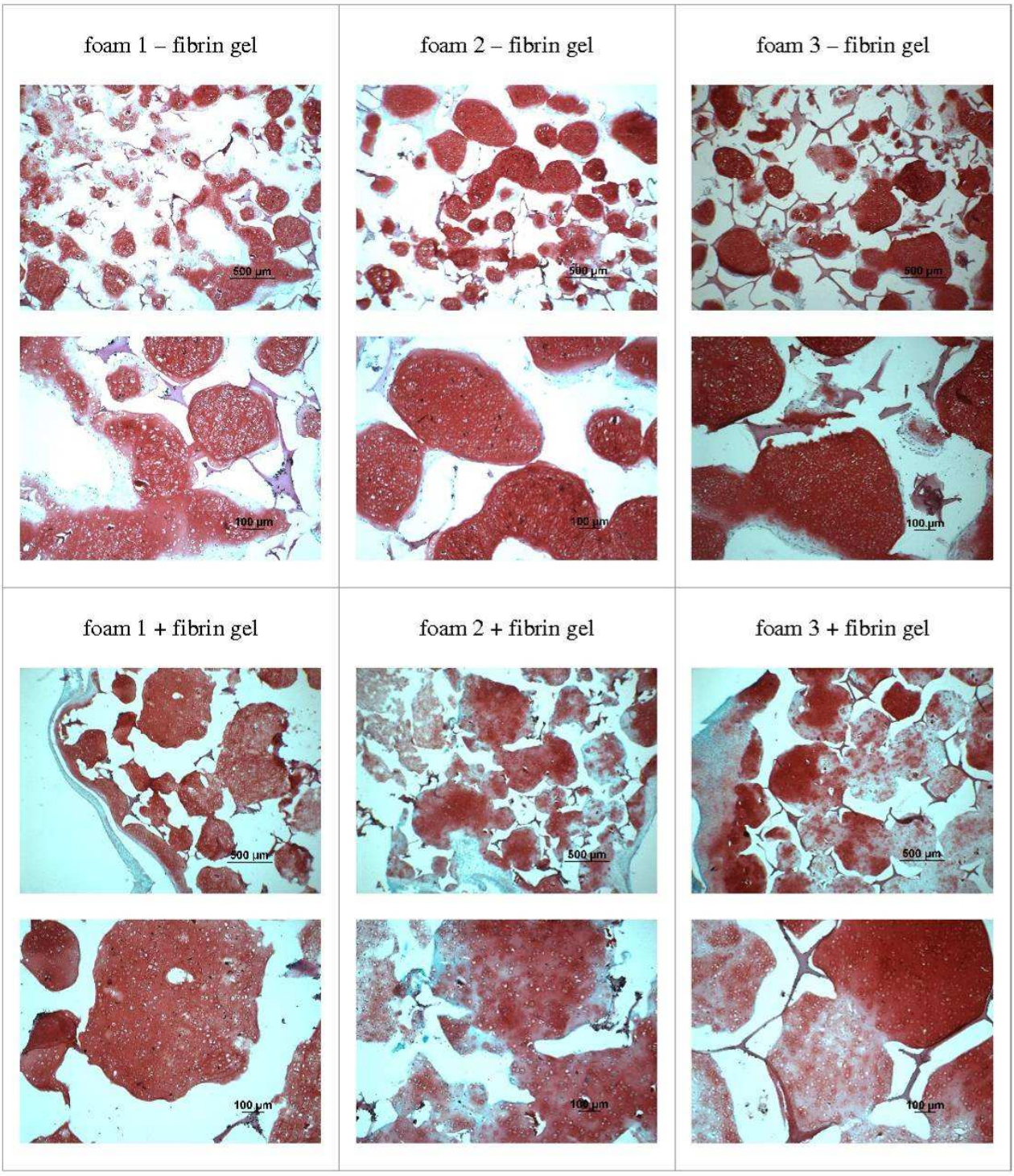

Figure 17: Glycosaminoglycan (GAG) distribution in cross-sections of OPF scaffolds (foam 1 – 3) seeded with chondrocytes (-fibrin gel) or with chondrocytes in fibrin gel (+ fibrin gel) respectively. GAG was stained red with safranin-O.

Ear-shaped Cartilage Construct

According to the results of the first experiments with OPF discs a variety of scaffold formulations was available for cartilage engineering without affecting the development of cartilaginous tissue. Foam 3 turned out to be best suited for the generation of the complex 3D structures of the human external ear and was chosen for the subsequent approach to engineer a cartilage construct in the shape of the human external ear. The composition of foam 3 was foamed in the silicone mold and then seeded with chondrocytes suspended in fibrin gel. The combination with the fibrin gel was chosen due to a higher mechanical stability of the whole construct and a higher cell retention resulting in a higher seeding efficiency.

After 28 days in culture an ear-shaped construct could be harvested, which under macroscopic examination had the appearance of cartilage (Fig. 18) and proved to be very elastic and mechanically stable during the harvesting procedure. During gross examination using forceps, it could be bent and folded up, which was completely reversible after removing the mechanical force with regaining its original shape After 28 days in culture the construct reached a wet weight of nearly 10 g.

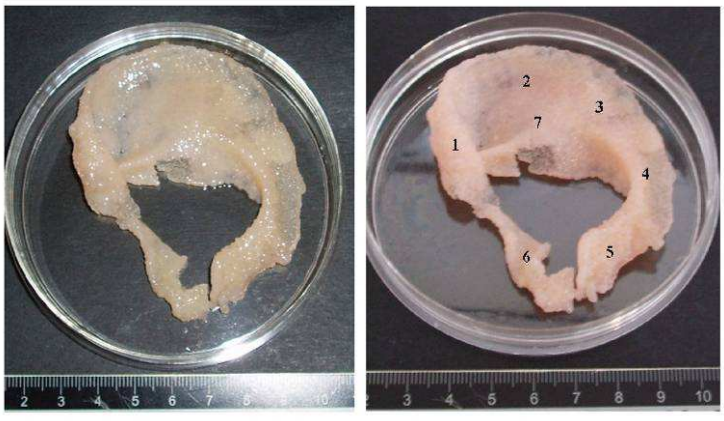

Figure 18: Ear-shaped OPF scaffold seeded with primary bovine chondrocytes in fibrin gel after 28 days in culture. Localization of the samples taken from representative section planes of the construct for biochemical and histological evaluation (1-7).

In order to gain an overview over the homogeneity of the development of cartilage tissue within the whole construct, samples of representative areas were taken (Fig 18 – right, section plane 1-7) and subjected to biochemical and histological examination. Wet weight slightly differed between the discs from the respective section planes, as the construct was not even, thus the thickness of the samples also varied (Fig 19A). Thus cell number was normalized to wet weight (Fig. 19B). Over the culture period substantial amounts of extracellular matrix components were produced as indicated by an average of 1.85 % of GAG content per wet weight and a collagen content per wet weight of 1.27% (Table 4) without large variations between the section planes analyzed (Fig. 19C,E). The activity in synthesizing matrix components expressed as GAG per cell (Fig. 19D) and collagen per cell (Fig. 19F), respectively, did not show large variations depending on the location of the sample and reached satisfying levels. The total amount of GAG and collagen in the whole construct was calculated considering the wet weight of the whole construct and the wet weight of the discs cut out of the construct for biochemical analysis (Table 3).

	mean	standard deviation
wet weight ear-shaped construct [mg]	9343.4	-
cell number [mio]	686.3	117.0
cell number/ wet weight [1/ng]	0.073	0.013
GAG/ wet weight [%]	1.850	0.218
GAG/ cell [ng]	0.256	0.037
GAG per construct [mg]	172.93	20.37
collagen/ wet weight [%]	1.266	0.271
collagen/ cell [1/ng]	0.189	0.038
collagen per construct [mg]	122.30	28.70

Table 3: Summary of the biochemical analysis of the ear-shaped cartilage construct after 28 days in culture

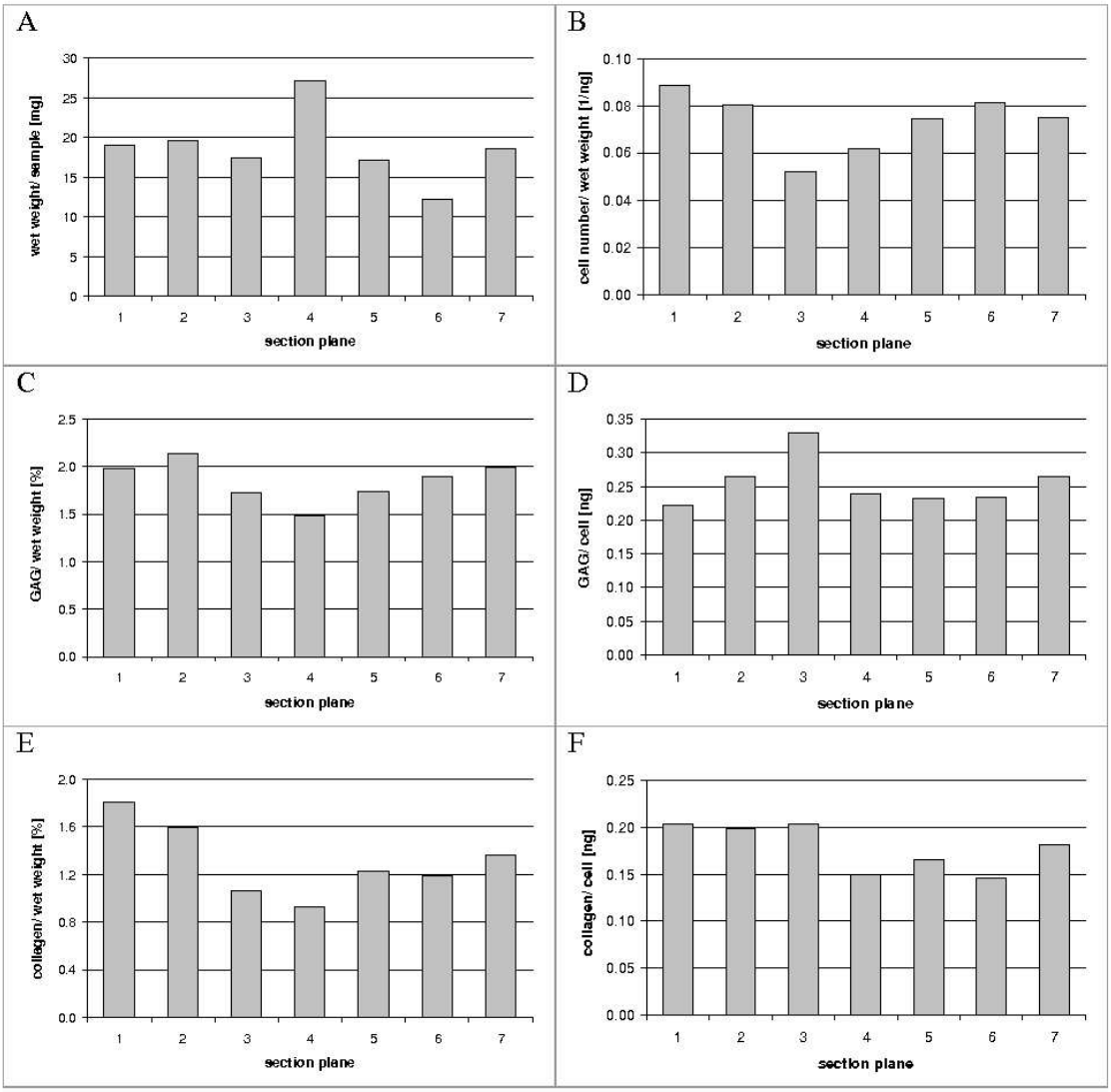

Figure 19: Wet weight (A), cell number (B), GAG content (C,D) and collagen content (E,F) of samples taken from representative section planes of the construct as indicated in figure 18.

In order to evaluate the distribution of GAG within the construct cross-sections from the different section planes were stained with safranin-O and fast green. All samples displayed an intense and almost homogenous red staining indicating matrix rich in cartilage specific GAG. The space between the pores filled with cartilage (red) was partially due to the cutting procedure during which the different parts were torn apart (Fig. 20). Only in section plane 2 and 5, there were pores partially stained green, where fibrous tissue could be found. In section plane 5 there were some pores visible displaying green staining, a phenomenon which might

be due to air bubbles in the gel during the seeding procedure, i.e. the pores were incompletely filled with fibrin gel and suspended chondrocytes.

Discussion

In the first part of the study the general suitability of the OPF scaffolds for cartilage tissue engineering, also in combination with fibrin gel, was investigated as well as the impact of the formulation on tissue development. In contrast to the approach combining fibrin gel and polyurethane scaffolds (Chapter 3) in the investigation two hydrogels were combined. The comparison of cartilage tissue development in the three different OPF foams rendered no statistically significant difference between the OPF formulations. With each formulation a coherent cartilage tissue could be achieved (Fig. 17). Few single pores were not completely stained in the same intense red colour (Fig. 17 foam 1/ 2/ 3 + fibrin gel). This phenomenon was attributed to air bubbles in the gel during the seeding procedure, as this was only observed in the constructs seeded with chondrocytes in fibrin gel. The air in the fibrin gel apparently inhibited a complete filling of the respective pores with the gel containing the cells. Hence in this area less ECM could be detected.

For further investigations foam 3 was chosen, as the three different OPF formulations showed no difference with regard to cartilage development (Figs. 16 & 17) and foam 3 turned out to be the best suited for the foaming process during the generation of scaffolds with a more complex structure. Thus, in the subsequent approach the formulation of foam 3 was foamed in a silicone mold, which had the shape of a human external ear.

The combination of the scaffold with fibrin gel was chosen to guarantee mechanical stability and a high retention of cells during the seeding procedure and of the newly synthesized matrix during the culture period. Especially the latter advantage has already been shown for other systems like combining polyurethane scaffolds and fibrin gel [24].

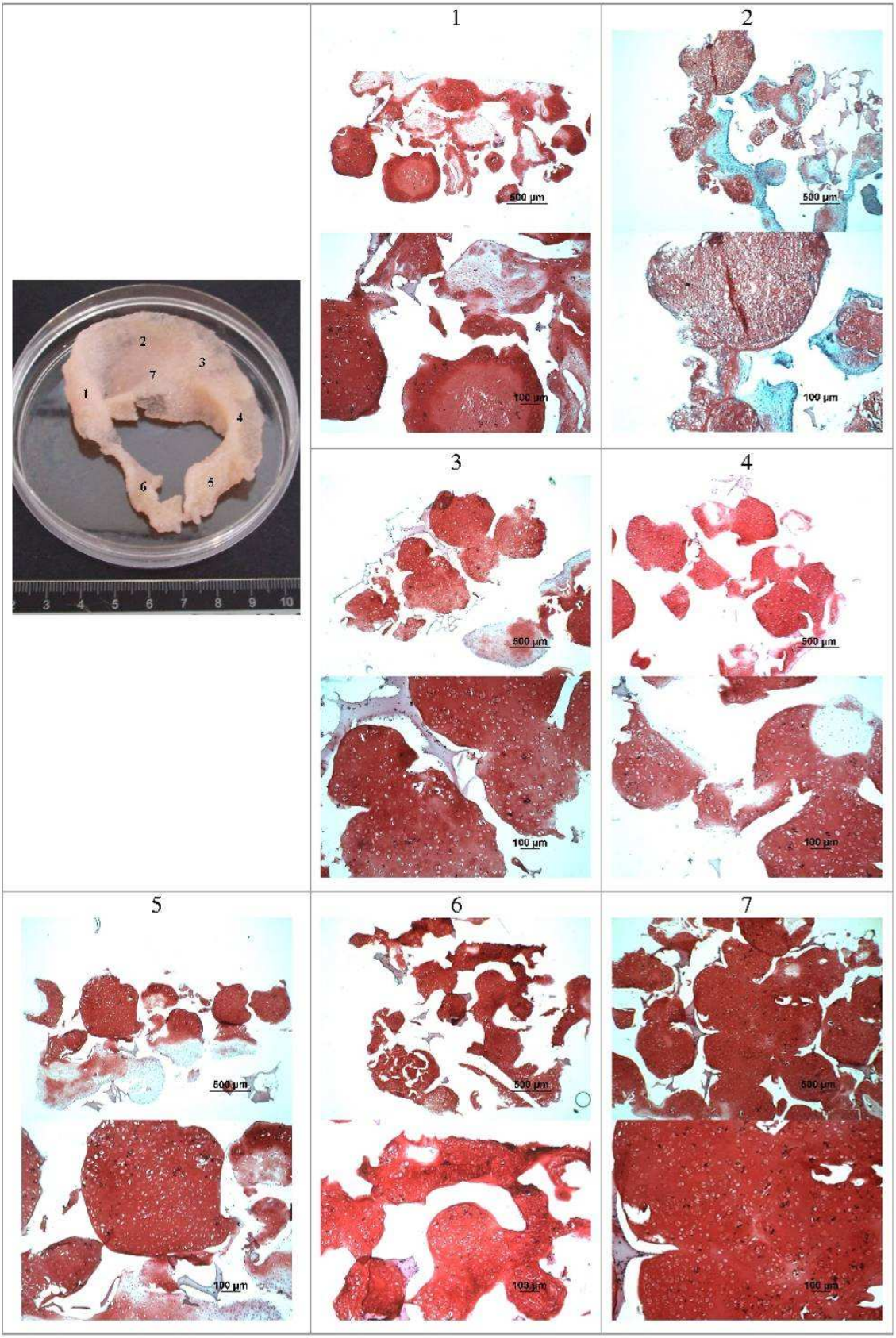

Figure 20: GAG distribution in cross-sections of samples taken from indicated sections planes of the ear-shaped cartilage construct after 28 days in culture. GAG was stained red with safranin-O.

After 28 days in culture, the ear-shaped construct had the typical appearance of cartilage and was elastic and resisted mechanical forces (bending, folding) during the harvesting procedure and gross examination using forceps. Biochemical analysis showed substantial amounts of ECM components throughout the construct (Fig. 19, Table 3). Histological evaluation demonstrated a coherent cartilage tissue throughout the construct with a mainly homogenous and intense staining for GAG in cross-sections of the majority of discs that were punched out from representative parts of the scaffold (Fig. 20).

Other groups also approached reconstruction of the external ear by tissue engineering [41, 48-50] applying a variety of biomaterials (c.f. Chapter 3). Liu et al. also applied CAD/ CAM and reported the only ear-shaped scaffold that had a detailed structure similar to one presented in this study. However, none of the cited studies provided a comprehensive analysis of developed cartilage in the whole ear-shaped construct with spatial resolution. Consequently, investigation of homogeneity and coherence of the engineered cartilaginous tissue was not considered in these studies in contrast to the study presented here.

In summary, here we successfully demonstrate the engineering of a proto-type of a very complex and custom-made ear-shaped cartilage construct. Samples taken from representative areas of the scaffold showed substantial accumulation of ECM (Fig. 20). Variation of the results of the biochemical evaluation of the construct depending on the location of the samples revealed the need to optimize the seeding procedure. After optimization of the seeding procedure and possibly also of the culture conditions, with respect to growth factor supplementation or dynamic cultivation further experiments have to be undertaken with human chondrocytes in order to proceed towards clinical application. Human, autologous cartilage implants of clinically relevant dimension also demand further research to solve the problem of cell source, i.e., the limited availability of autologous chondrocytes. Methods to expand cells under conservation of their cartilaginous phenotype or options for redifferentiation after expansion in a 2-dimensional environment, which easily leads to rapid

dedifferentiation [60], are to be investigated. As an alternative, chondrogenically differentiated mesenchymal progenitor cells may be utilized (see also Chapter 8).

Chapter 6

Towards Osteochondral Constructs

Introduction

Osteochondral defects affect the articular cartilage but also the underlying subchondral bone of the joint. They are usually caused by trauma or osteochondritis dissecans. Osteochondral defects often cause mechanical instability of the joint and are therefore associated with the risk to induce osteoarthritic degenerative disease. To date a widely accepted treatment procedure for osteochondral defects is still missing [61]. Although in clinical use autologous osteochondral constructs turned out to be promising, they are accompanied by several limitations. Tissue engineering of osteochondral grafts allows the generation of individually tailored constructs of defined dimension and shape [62]. Furthermore co-culture is a powerful tool to study cellular interactions [63].

There are several strategies to engineer osteochondral constructs. Concerning the applied scaffold one option is to use a scaffold for the bone part in combination with a scaffold-free cartilage-part. Moreover different scaffolds for bone and for cartilage are chosen and combined at the time of implantation. In case of applying a single scaffold two approaches are possible, either the application of a single heterogeneous, bilayered or of a single homogenous scaffold for both, the bone and the cartilage component. The choice of the cell source also implies a variety of options. Scaffolds can be loaded with a single cell source having chondrogenic capacity or with two cell sources having either chondrogenic or osteogenic potential. Another possibility is the application of cells which have both, chondrogenic as well as osteogenic differentiation capacity. Cell-free scaffolds have also been employed [62].

A wide range of materials is available for scaffolds used for osteochondral grafts, biological and synthetic materials. Perichondrium, alginate, chitosan, periost, DBM (demineralised bone matrix) and allogenic/ xenogenic bone serve as examples for biological materials. These materials facilitate tissue development as they comprise good osteoinductive as well as biomechanical properties. DBM for instance contains growth factors and supports osteogenic

differentiation. Synthetic materials applied include PLA (polylactic acid), PLGA (polylactic – co- glycolic acid), PU (polyurethane), CDHA (calcium-deficient hydroxyapatite), β-TCP (tricalciumphosphate). Ceramics like hydroxyapatite or β-TCP consist of minerals of the natural bone matrix [64].

Within a joint project (‘Regenerative Implants’, Bavarian Research Foundation ‘Bayerische Forschungstiftung’) involving several research groups and companies, our group was responsible for the chondrogenic culture within an osteochondral construct. In order to yield an optimal environment for the respective cell type, well established scaffold-materials were chosen for the bone as well as for the cartilage component and combined in a single, bilayered scaffold. Biphasic scaffolds for osteochondral grafts have been proposed by various groups [65–68]. To engineer the cartilage part the established concept of seeding chondrocytes suspended in long-term stable fibrin gels in PU scaffolds [24] was chosen. For the bone part a hydroxyapatite–based scaffold was prepared by dispense-plotting a special rapid prototyping method. Hydroxyapatite-based scaffolds were already successfully used for bone tissue engineering approaches [69]. In order to obtain a single, bilayered scaffold the hydroxyapatite based composite was directly plotted on the polyurethane scaffold. The PU-part was seeded with chondrocytes in fibrin gel. After pre-culture for one week the composite-part was seeded with GFP-labelled human BMSC. In the first part of the study a method for selective seeding of the cartilaginous part had to be established. The following experiments were conducted applying both cell types to investigate their compatibility and cell distribution to evaluate the potential of the system for generation of osteochondral grafts for a longer culture period in a bioreactor.

Results

Establishment of a Selective Seeding Procedure

To reach a selective seeding of the polyurethane (PU)-part of the bilayered scaffold consisting of a PU-part for the development of cartilage tissue and a composite-part plotted directly on the surface of the PU scaffold for engineering bone, a method to radially insert the fibrin gel with the chondrocytes into the scaffold was established (c.f. Chapter 2 “Materials and Methods”, Fig. 3). Two different types of bilayered scaffold were employed. The first type was characterized by a membrane separating PU- and composite-part (“with membrane”). The membrane was introduced during the production process of the PU scaffold and the composite was later plotted directly on the membrane. The second type was produced without the separating membrane (“open”). In this case the composite was directly plotted on the open pores of the PU scaffold and the development of a membrane was inhibited to a certain extent during the fabrication of the PU scaffold.

The application of a cell-free fibrin gel stained with bromophenolblue permitted a first estimation of the success in selective insertion of the fibrin gel into the PU-part (Fig. 21). The images of the constructs with stained fibrin gel taken with the stereo-magnifier demonstrated an evenly blue stained PU-part and a composite-part, which remained mainly unaffected by the blue fibrin gel.

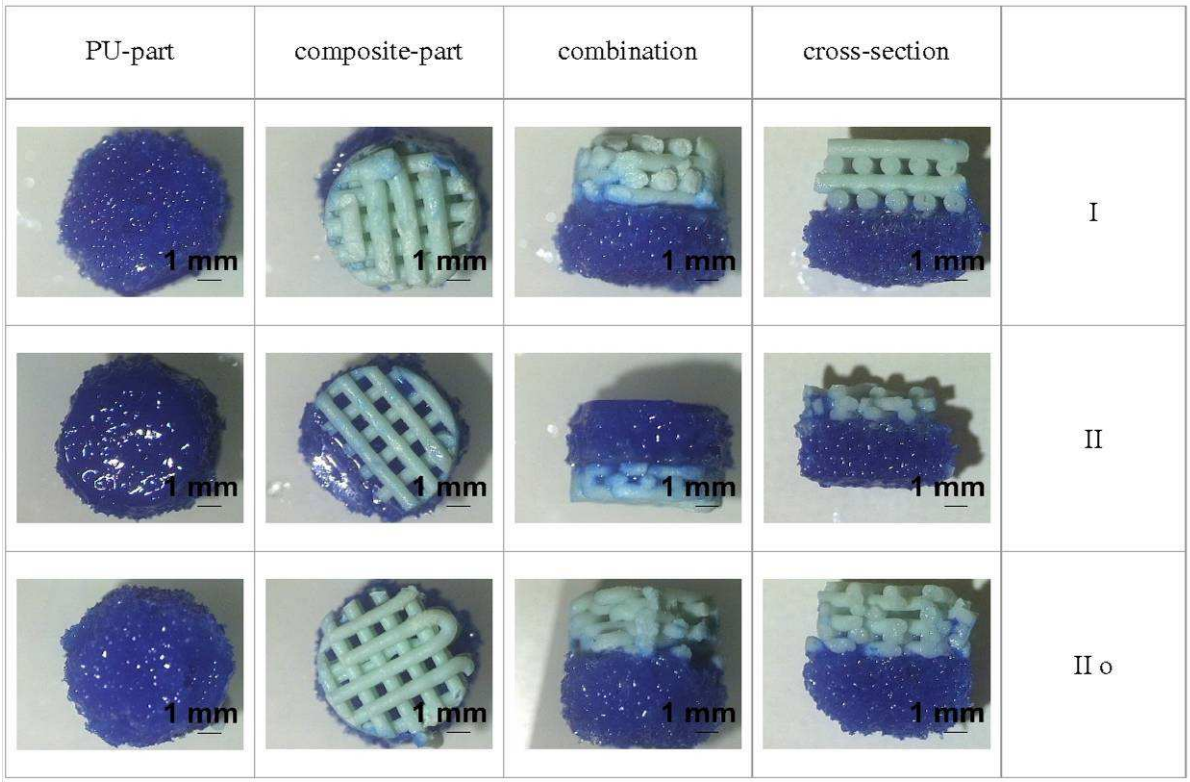

Figure 21: Selectivity of gel insertion into bilayered scaffolds visualized by addition of bromophenolblue to the fibrin gel. Two batches of scaffolds were investigated. Two different types can be distinguished. I, II represent scaffolds, which were produced by plotting the composite part on the membrane covering the PU scaffold ("with membrane"), whereas in case of II o the composite-part was plotted on the scaffold on open pores ("open").

For a first cell culture experiment, bovine chondrocytes suspended in fibrin gel were seeded in the PU-part. After 21 days cultured in vitro, the ratio of chondrocytes in the PU-part and in the composite-part was determined (Fig. 22A). The majority of the cells could be detected in the PU-part, thus a certain degree of selectivity could be realized, however, for a co-culture the number of chondrocytes in the composite-part, supposed for the GFP-labelled hBMSC, had to be reduced. The cell number per wet weight (Fig. 22B) was also determined, but was of minor value due to the differences concerning the weight of the scaffold material. Unexpectedly, the membrane between the two scaffold parts did also not favour selectivity during the seeding procedure (Fig. 22A). Considering the activity of the chondrocytes concerning production of extracellular matrix (ECM) components, i.e., glycosaminoglycans

(GAG) (Fig. 22C) and collagen (Fig. 22D), no difference between both types of bilayered scaffolds ("with membrane" and "open") could be detected.

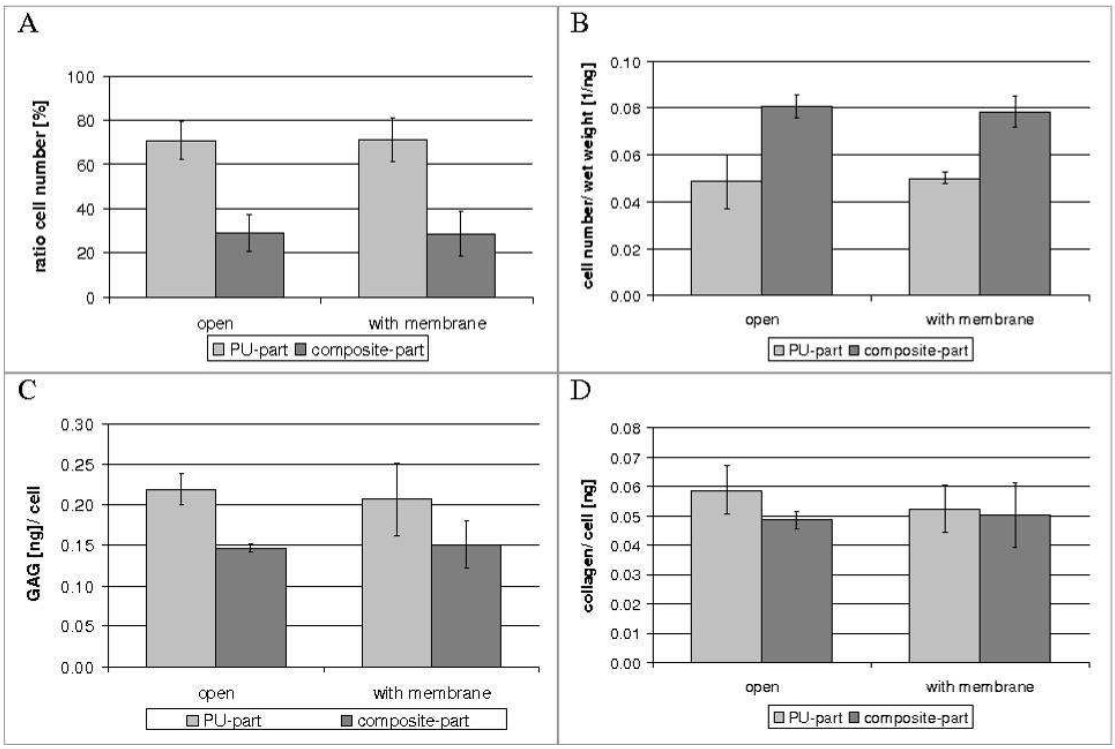

Figure 22: Biochemical analysis of bilayered scaffold, seeded with bovine chondrocytes in fibrin gel selectively in the PU-part of the scaffold, after 21 days in culture. Selectivity was analyzed by calculating the ratio of chondrocytes in the PU-part and in the composite-part (A). Matrix production was analyzed by determination of GAG- (C) and collagen – (D) production. Data represents the average ± SD of three independent constructs.

Homogeneity of GAG distribution in the PU-part and possible undesirable and unintended matrix in the composite part was analyzed by safranin-O staining of cross-sections after 21 days in culture (Fig. 23). Regarding the stained cross-sections, in the area of the composite part an intense and homogeneous safranin-O staining indicated a coherent cartilage matrix in the part of the construct intended for the bone-part. This was the case in both experimental groups. In the PU-part intense red staining of GAG indicated production of substantial cartilage tissue in both groups. The empty space between the pores may be due to the cutting

process during sample preparation. The pictures of the transition zone in both groups also displayed ECM accumulation particularly towards the composite part, which is the lower layer in the images.

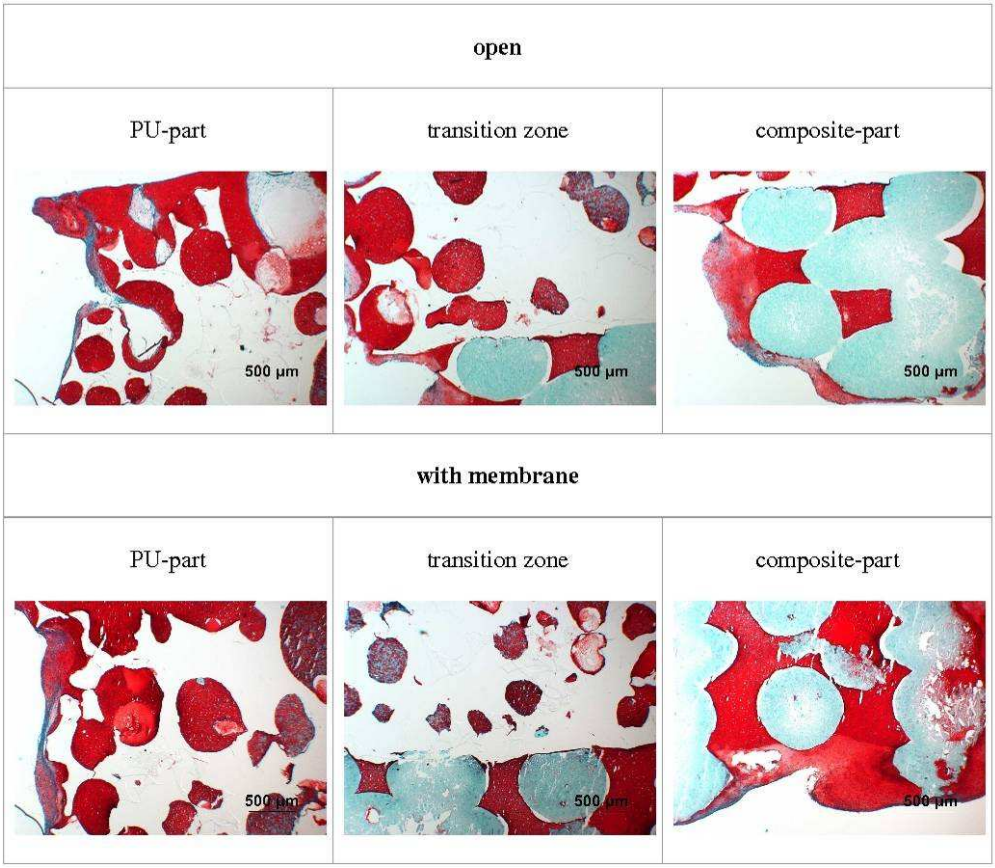

Figure 23: Glycosaminoglycan (GAG) distribution in cross-sections of bilayered scaffolds after 21 days in culture. Safranin-O stained GAG red.

Thus, histological evaluation also showed that there was no difference between the two types of bilayered scaffolds and substantial amounts of chondrocytes and consequently also of produced extracellular matrix could be detected in the composite part. Histological examination also documented that the membrane did not enhance selectivity of the seeding procedure.

A first real co-culture resulted in similar findings. After one week of pre-cultivation of the constructs with chondrocytes in the PU-part, the composite-part was seeded with GFP-

labelled hBMSC and co-cultured for 3 days to evaluate compatibility. DAPI (2-(4-amidinophenyl)-1H indole-6-carboxamidine) staining was conducted to analyze distribution of chondrocytes (blue fluorescence) and GFP-labelled hBMSC (green fluorescence) in the cross-section. A considerable number of chondrocytes could be detected by blue fluorescence in the composite-part (Fig. 24A). The live-dead-assay displayed many fluorescing cells particularly in the PU-part of the bilayered scaffold indicating a high amount of dead cells (Fig. 24B).

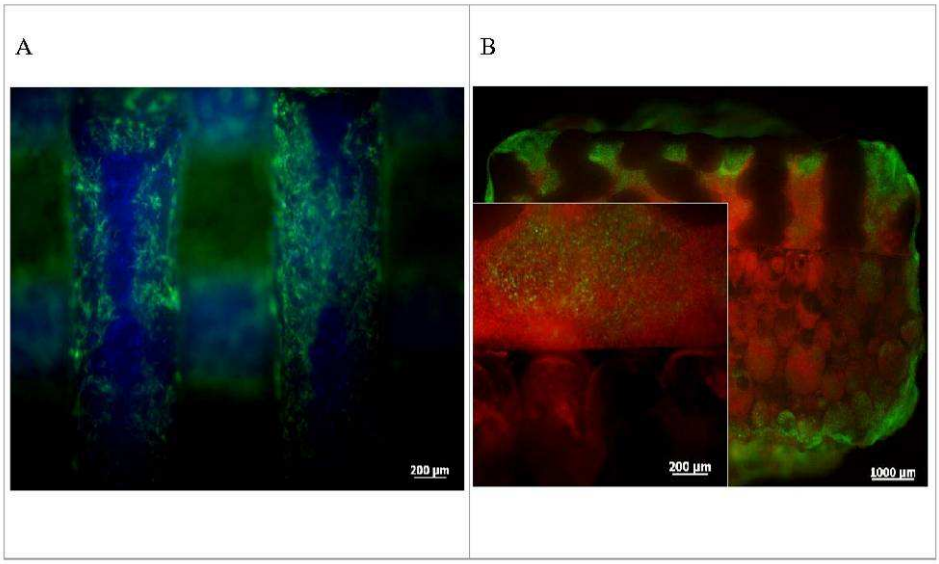

Figure 24: After pre-cultivation for 1 week and subsequent seeding with GFP-labelled hMSC (green fluorescence) cross-sections were stained with DAPI to visualize distribution of chondrocytes (blue fluorescence) and GFP-labelled hBMSC (green fluorescence) (left). Viability of chondrocytes was investigated by live-dead assay with propidium iodide (red fluorescence of dead cells) and fluorescein-diacetate (green fluorescence of viable cells) (right). (Seeding with hBMSC, co-culture and staining was conducted by Dr. I. Drosse, research group Prof. M.Schieker, LMU, Munich, Germany.)

In order to overcome the problem of reduced viability the height of the PU-part was reduced from 4 mm to 2 mm. The initial cell density seeded into the PU-part was reduced to half of the amount applied in the first experiment and the volume of gel inserted was additionally

reduced to 80% of the volume of the PU-scaffold part, i.e., the calculated initial number of chondrocytes seeded per construct amounted to $3.7*10^6$ cells. Cell distribution was analyzed 24 hours after seeding, on day 3 and day 8 to explore, if the high number of chondrocytes located in the composite-part was due to little selectivity during the seeding procedure or due to cell migration.

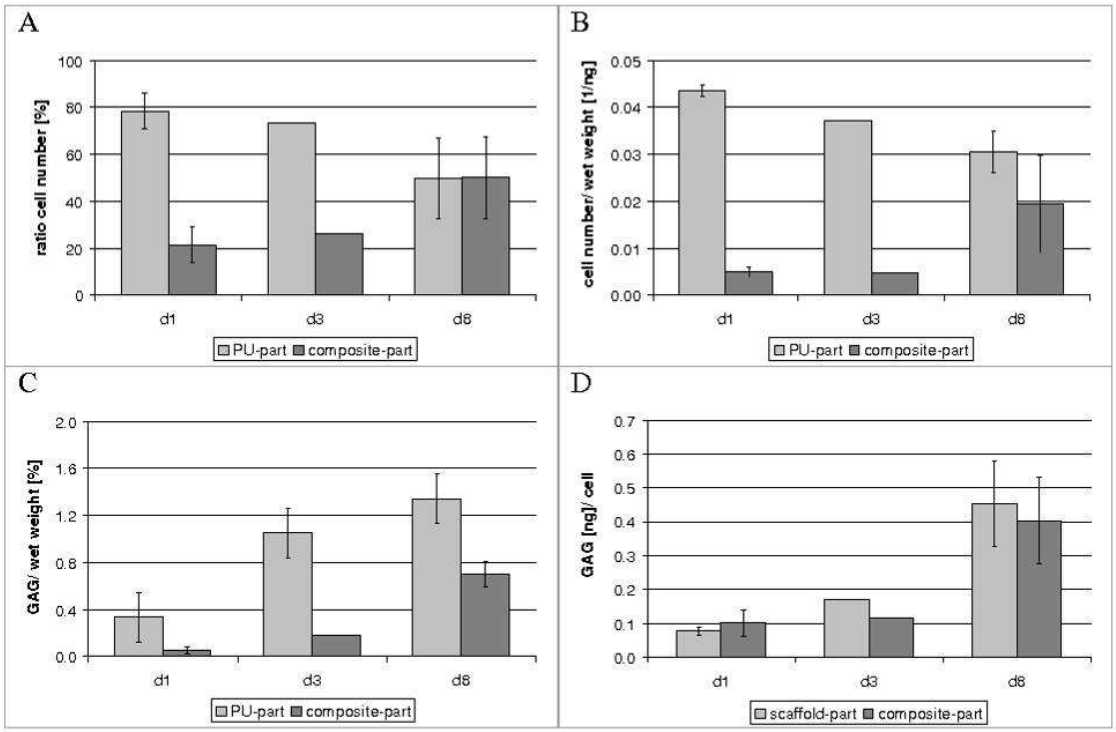

Figure 25: Selectivity of the seeding procedure and cell distribution during a culture period of 8 days (intended pre-cultivation time till seeding with GFP-labelled hBMSC) determined by DNA assay (A, B). Matrix production of the cells in the different parts of the bilayered scaffolds was analyzed by GAG assay from day 1 up to day 8 (C, D). Data represents the average of three independent constructs ± SD with the exception of day 3, which was only represented by 2 constructs.

24 hours after seeding the bilayered scaffolds, approximately 20 % of the total cell number could be detected in the composite part (Fig. 25A), consequently the seeding procedure had to be declared as preferential, but not as selective as intended. Up to day 8 the ratio of chondrocytes in the composite-part increased to almost 50%. In both parts of the bilayered

scaffold GAG was deposited (Fig. 25C) and GAG production per cell was similar in both parts of the bilayered scaffold (Fig. 25D).

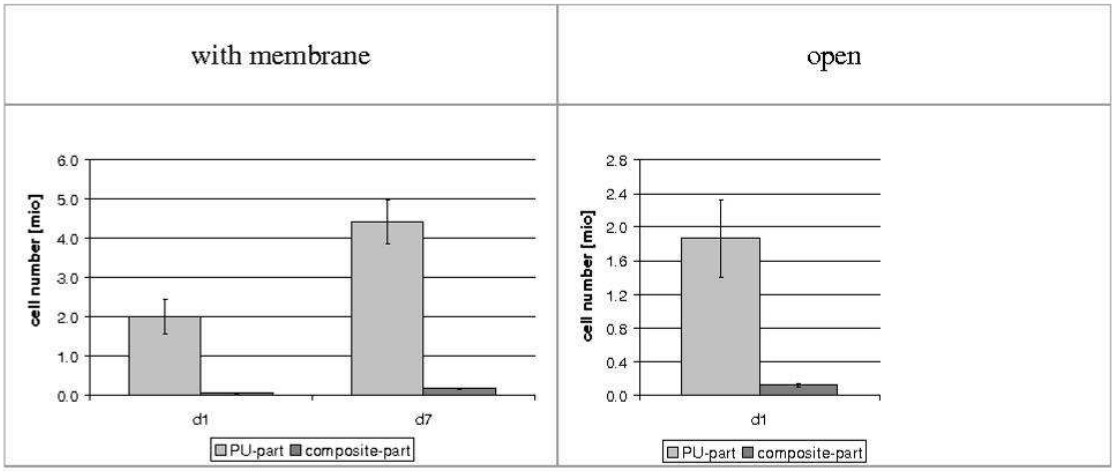

Figure 26: Selectivity of seeding the PU-part of the bilayered scaffold, while the composite part was blocked with a thermo-reversible gelatine gel. In case of the constructs "with membrane" the composite was plotted on the membrane covering the surface of the PU scaffold. The other variant indicated by "open" implied a PU-part without a membrane on the surface, thus, the composite was plotted on the open pores of the PU disc. Data represents the average of three independent constructs ± SD.

As the results described above were not satisfying for the intended purpose due to deficits in selectivity, the effect of blocking the composite-part with a thermo-reversible gelatine gel during the seeding procedure was investigated (c.f. Chapter 2). Blocking the composite-part with the thermo-reversible gelatine gel yielded a highly selective seeding of exclusively the PU-part of the bilayered scaffold using the scaffolds with ("with membrane") and without ("open") a membrane between PU- and composite-part (Fig. 26). After 24 hours the ratio of chondrocytes in the composite-part accounted for 2.4% in the scaffolds with membrane and 5.9% in the scaffolds with the composite plotted on the PU-part without a membrane. Thus, remarkable increase in selectivity could be reached applying the thermo-reversible gelatine gel with little difference between the two types of bilayered scaffolds. Also after 7 days in

culture, only a negligible amount of chondrocytes was detected in the composite part (3.7%) (Fig. 26).

In order to gain an impression of the interconnectivity of the PU-part of the different scaffolds, which is crucial for providing nutrients, scanning electron microscopy was conducted. The PU scaffold with membrane was characterized by very small pores without interconnections between the pores (Fig. 27).

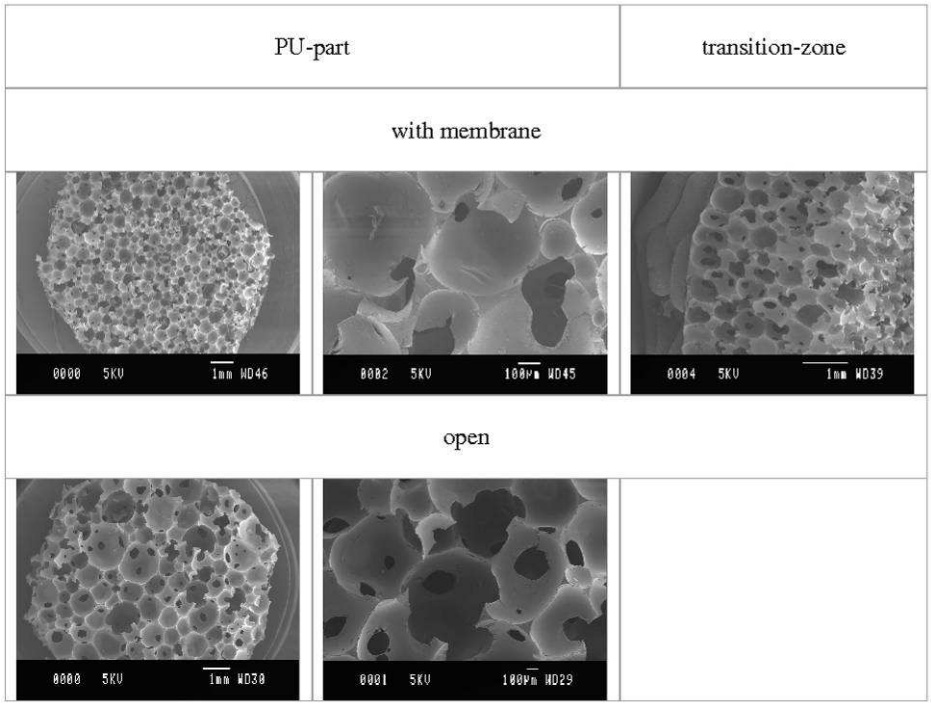

Figure 27: SEM images taken from the PU-part of the bilayered scaffolds to show the difference of pore size and interconnectivity between PU scaffold with a membrane on the surface ("with membrane") or without a membrane ("open").

Due to the fact that the production of a membrane on the surface of the PU scaffold during the foaming process resulted in a very low interconnectivity and small pores, this variant was regarded as inappropriate for further application in chondrocyte cell culture. Moreover, the membrane did not promote selectivity in the seeding procedure of the bilayered scaffolds.

Consequently, for the following co-culture experiment bilayered scaffolds without a membrane were selected.

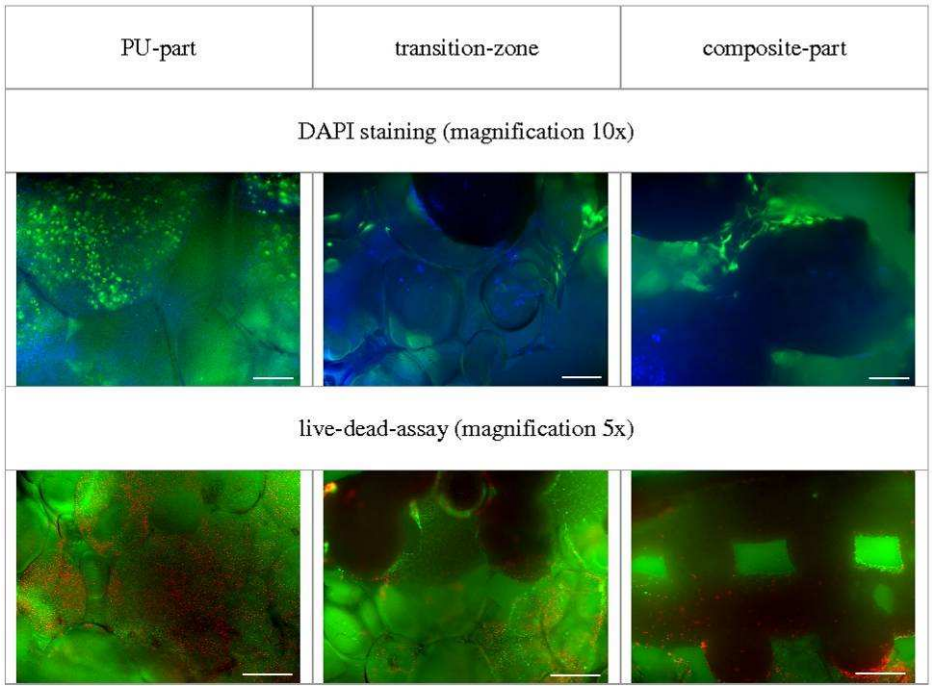

Figure 28: DAPI staining (blue fluorescence of nuclei) of the cross-sections of the bilayered scaffold seeded with chondrocytes and after 7 days pre-cultivation with GFP-labelled hBMSC (green fluorescence) was conducted to visualize the distribution of the two cell types in the two parts of the scaffold as well as in the transition zone after 3 days of co-culture. A live-dead-assay was also performed to test viability of the cells in the different parts of the construct after 3 days of co-culture. Living cells appear green (staining with fluorescein-diacetate) and dead cells were stained with propidium iodide (red). (Seeding with hBMSC, co-culture and staining were conducted by Dr. I. Drosse, research group Prof. M.Schieker, LMU, Munich, Germany.)

In a second co-culture experiment, chondrocytes were suspended in fibrin gel and seeded into the PU-part using the optimized seeding method, i.e., after blocking the composite part with the thermo-reversible gelatine gel. Constructs were pre-cultured for 7 days and then were seeded with GFP-labelled hBMSC. After a short culture of 3 days cell distribution and viability were investigated. The cross-sections stained with DAPI showed that some green-fluorescent hBMSC could be found in the PU-part and a small amount of blue-fluorescent

chondrocytes could be detected in the composite-part (Fig. 28). The DAPI staining clearly demonstrated an increase in selectivity compared to the first co-culture (Fig. 24 & Fig. 28). Cross-sections of the bilayered constructs stained with propidium-iodide and fluorescein-diacetate revealed some dead cells in the bilayered construct particularly in the PU-part (Fig. 28).

Discussion

The engineering of osteochondral constructs was approached employing bilayered scaffolds with two different parts intended for cartilage and bone engineering, respectively. A method to selectively seed the PU-part of the bilayered scaffolds with chondrocytes could be established. The ratio of chondrocytes detectable after 24 hours in the composite-part could be reduced by temporary application of a thermo-reversible gelatine gel from 20% (Fig. 25A) to 2.4% with membrane and 5.9% (Fig. 26) without a membrane between the PU- and the composite-part.

The large increase in selectivity yielded by temporary blockage of the composite was also apparent in the cross-sections of the bilayered scaffold stained with DAPI after 3 days of real co-culture of chondrocytes and hBMSC (Fig. 28). Only a small number of blue fluorescent cells, i.e. of chondrocytes, was detected in the composite part. The scaffold without a membrane between the two different parts yielded results very similar to the membrane containing scaffolds, i.e., the membrane on the surface of the PU-part, which originally was supposed to increase selectivity and to inhibit cell migration, did not additionally favour selectivity, when blocking the composite-part with the thermo-reversible gelatine gel during the seeding procedure (Fig. 26).

This was advantageous as the generation of the membrane during the production process of the PU scaffold was associated with decreasing pore-size and interconnectivity (Fig. 27).

Interconnectivity and a certain dimension of the pore, in turn are vital for nutritive supply of the cells inside the scaffold and, thus, for tissue development [51].

Comparing the expansion of the number of chondrocytes in the composite part without temporary blockage of the composite part (Fig. 26A) with the very low increase from day 1 to day 7, when the composite part was blocked with the thermo-reversible gelatine gel (Fig. 26), the high increase of cells in the composite part is probably ascribable to proliferation not to migration of the cells.

A similar concept with regard to scaffold design for osteochondral constructs was chosen by Schek et al. Two bonded cylinders were used to form a bilayered scaffold comprising a cylinder of hydroxyapatite (HA) and another one of poly-L-lactic acid (PLA). In order to inhibit infiltration during seeding and cell migration during culture a thin polyglycolic acid (PGA) film coated the poly (lactide acid) (PLA) cylinder. The seeding was conducted in two steps. First bone morphogenetic protein-7 (BMP-7) transfected human gingival fibroblasts suspended in a fibrinogen solution were seeded onto the HA-part and immediately placed on a drop of thrombin solution to start gelation. Then the PLA-part was seeded with chondrocytes. In this study, the centre of the polymer-part also displayed only a low safranin-O staining. Cartilage tissue could also be found in the HA-cylinder indicating that the PGA barrier failed to prevent infiltration of the ceramic part by chondrocytes. The PGA film was also supposed to be the reason for deficits in the development of the interface as it also inhibited interaction of osteoblasts and chondrocytes. Mineralized cartilage however could be detected at the interface [70].

Towards the successful engineering of osteochondral constructs, further experiments with a longer co-culture-period have to be conducted to evaluate development of coherent cartilage, bone and a bone-cartilage interface. In order to improve the nutritive situation in the polymeric part of the bilayered scaffolds a higher degree of interconnectivity would be advantageous. Culture conditions may be improved by the use of bioreactors that can provide

controlled medium flow and can impose mechanical load [71]. For example, it has been shown that cyclic hydrostatic pressure can enhance chondrogenic matrix production of human mesenchymal progenitors differentiated in vitro. These results also suggested that mechanical loading might play a vital role in cartilage development [72]. In order to provide optimal conditions for co-culture two-chamber bioreactors may be advantageous [73].

Chapter 7

Synergistic Effects of Growth and Differentiation Factor – 5 (GDF-5) & Insulin

Published in parts in:

Synergistic Effects of Growth and Differentiation Factor-5 (GDF-5) and Insulin on Expanded Chondrocytes in a 3-D Environment
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Introduction

Cartilage tissue engineering is a promising approach as a treatment for cartilage defects in the joint [5, 13], but also in craniofacial surgery, e.g., for auricular reconstruction [37, 41]. One of the great challenges in this field is to overcome the limitations concerning the amount of autologous harvestable chondrocytes. One possibility is to expand the chondrocytes in 2-dimensional (2D) environment before culture on a 3-dimensional (3D) cell carrier. Expansion in 2D, however, is accompanied by rapid dedifferentiation, associated with. for example, predominant expression of type I collagen [60] rendering the chondrocytes less suitable for cartilage tissue engineering purposes [5, 74]. Thus, growth factors are applied either already during expansion of the chondrocytes [75–78] or during subsequent 3D culture [75, 78–80] to improve the quality of the generated tissue.

Studies conducted in the group demonstrated synergistic effects of the protein growth and differentiation factor-5 (GDF-5) in combination with insulin, on bovine chondrocytes on poly (glycolic acid) (PGA) scaffolds. GDF-5 belongs to the bone morphogenetic protein (BMP) subfamily. BMPs are members of the transforming growth factor-β superfamily and were identified as inducers of bone and cartilage formation *in vivo* [81]. GDF-5 or cartilage derived morphogenetic protein-1 (CDMP-1), which is used synonymously, is a growth factor involved in limb formation [82–84] and appears also in the stage of mesenchymal condensation and throughout the cartilaginous cores of the developing long bones of bovine embryos [85]. GDF-5 has also been postulated to be involved in the maintenance of healthy cartilage and regenerative response in diseased tissue as it was found in the superficial layer of normal cartilage and throughout osteoarthritic cartilage [86]. Insulin has previously been shown to have distinct anabolic effects on engineered cartilaginous constructs from primary cells similar to those of insulin-like growth factor-I (IGF-I) [27, 87].

In the preceding study mentioned above the effects of GDF-5 either alone or in combination with insulin were investigated. In case of primary chondrocytes, the application of GDF-5 combined with insulin resulted in an increase of wet weight and cell number without affecting the production of glycosaminoglycans (GAG) and collagen per cell. When the clinically more relevant expanded chondrocytes were used, supplementation with GDF-5 or insulin alone led to only very small constructs. However, supplementation with the combination of GDF-5 (0.01 and 0.1µg/ml) and insulin (2.5 µg/ml) during cartilage development affected the engineered made from expanded chondrocytes in a synergistic manner, leading to substantially increased production of cartilaginous extracellular matrix [88, 89].

In the studies presented here the impact of the application of GDF-5 alone or in combination with insulin already during chondrocytes expansion was investigated with bovine chondrocytes in fibrin gels as 3D cultures system [23]. Furthermore, the transferability of the results obtained with expanded juvenile bovine chondrocytes in PGA scaffolds to expand adult human chondrocytes in PGA scaffolds to expand adult human chondrocytes in a 3D pellet culture system, which has also been previously successfully employer for cartilage engineering [90, 91]. By using different culture systems, also independency of the synergism from the chosen 3D system could be examined. In a last preliminary experiment, the effect of GDF-5 or insulin, either alone or in combination, on adipose-derived stem cells was investigated in pellet culture, as adipose-derived stem cells represent an attractive alternative cell source for cartilage tissue engineering applications (see also Chapter 8).

Results

Effect of GDF-5 or GDF-5 in Combination with Insulin Applied during Expansion

In the first part of the study of GDF-5 (0.1 µg/ml) (G 0.1) alone or in combination with insulin (2.5 µg/ml) (G 0.1 +I) was applied during the expansion of bovine chondrocytes and

the effect on tissue quality yielded during expansion of bovine chondrocytes and the effect on tissue quality yielded during subsequent 3D culture in fibrin on tissue quality yielded during subsequent 3D culture in fibrin gel was investigates. The concentration of the protein was based on previous studies [88]. Fibrin gel culture was also conducted under three different conditions, i.e., control conditions without supplementation, supplementation with GDF-5 (G 0.1) alone or in combination with insulin (G 0.1+I). A flow chart showing the experimental design is given in Chapter 2 (Fig. 3). After 21 days in fibrin gel culture the groups which received the combination of GDF-5 and insulin displayed the typical appearance of cartilage and retained the initial construct size of 5 mm in diameter.

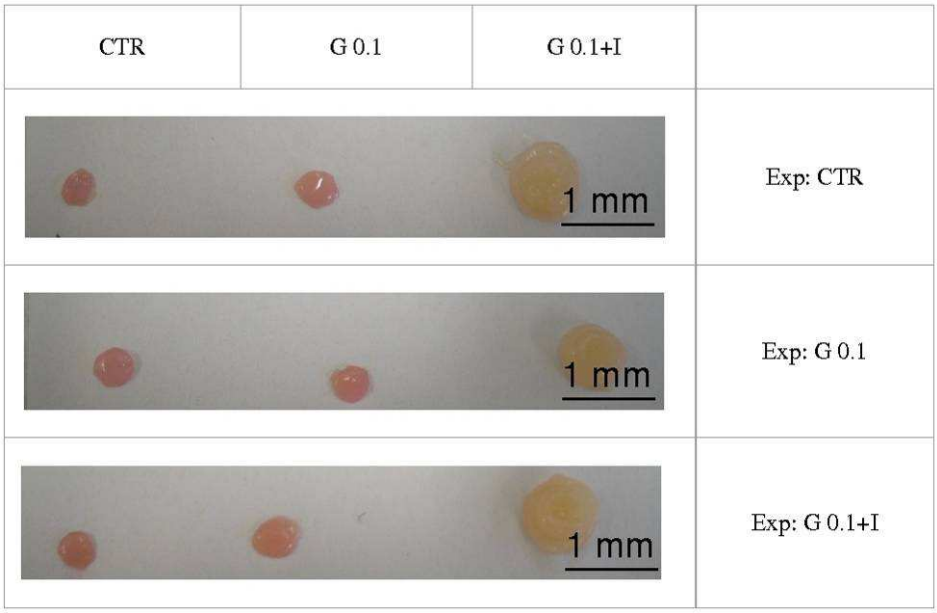

Figure 29: Macroscopic appearance of fibrin gels seeded with expanded (P2) bovine chondrocytes after 21 days in culture without factor supplementation (CTR), supplementation with GDF-5 at a concentration of 0.1µg/ml (G 0.1) or the combination of GDF-5 (0.1µg/ml) and insulin (2.5µg/ml) (G 0.1+I). Before seeding, cells were expanded under different conditions. Exp: CTR: expansion without supplementation; Exp: G 0.1: expansion with GDF-5 supplementation of 0.1 µg/ml; Exp: G-0.1 +I: expansion with supplementation with the combination of GDF-5 (0.1 µg/ml) and insulin (2.5 µg/ml).

The constructs were mechanically stable suggesting the production of substantial amount of extracellular matrix (ECM). In this group there was no obvious difference between the different expansion conditions.

Fibrin gels cultured under control conditions or under supplementation with GDF-5 alone (0.1µg/ml) decreased dramatically in size and were red in colour indicating high diffusion of medium and low amount of cartilaginous ECM (Fig. 29). This was consistent with the mechanical instability of the constructs, which were soft and gel-like. In these two groups there was also no difference apparent between the different expansion conditions.

Biochemical analysis displayed a much higher wet weight (approximately 10 – to 20-fold higher wet weight) of the constructs cultured under supplementation with the combination of GDF-5 and insulin, compared to culture without supplementation with the combination of GDF-5 and insulin, compared to culture without supplementation or with addition of GDF-5 alone (Fig. 30A). The same applied to the absolute cell number per construct, which was approximately 10-fold higher in the combination group as in the control (CTR) and in the GDF-5 (G 0.1) group (Fig. 30B). GAG was hardly detectable in the control groups as well as in the GDF-5 (G 0.1) groups irrespective of the expansion conditions. In contrast, the groups cultured under GDF-5 and insulin supplementation (0.1+I) displayed substantial amounts of GAG (Fig. 30C).

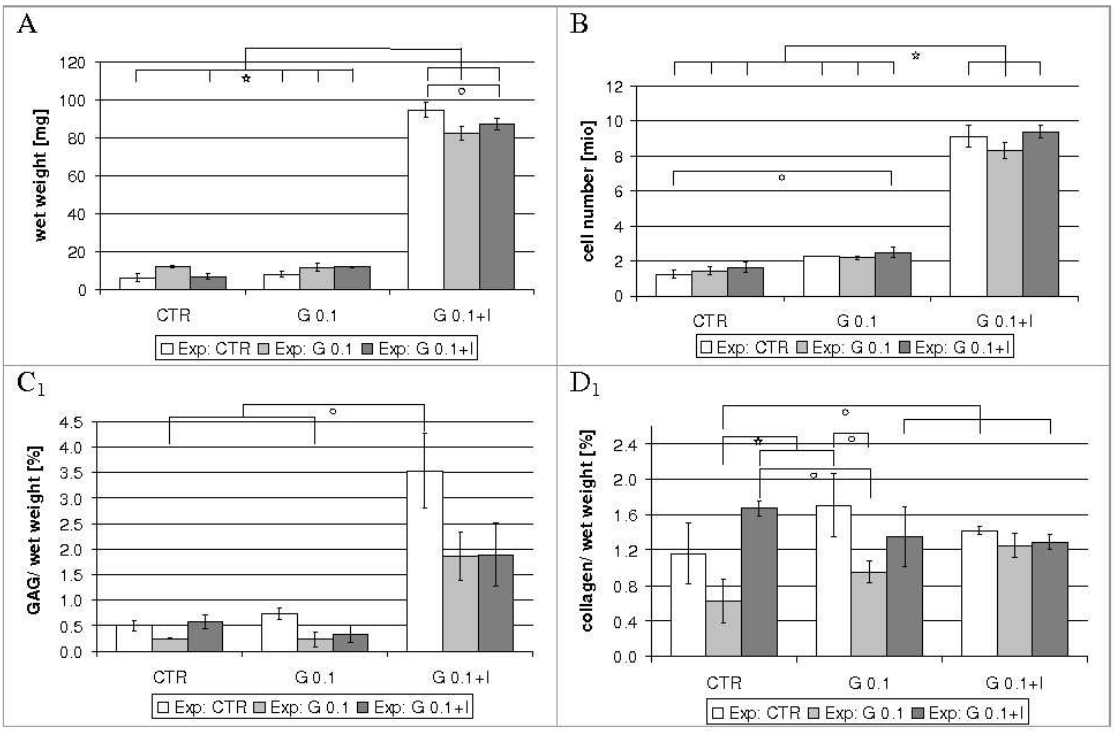

Figure 30: Wet weight (A) cell number (B), glycosaminoglycan (GAG) (C) content, and collagen content (D) of fibrin gel constructs after 21 days of culture. Fibrin gels were seeded with passage 2 chondrocytes expanded under different conditions: control (Exp.: CTR), GDF-5 (0.1 µg/ml) (Exp: G 0.1) supplementation or addition of the combination of GDF-5 (0.1 µg/ml) and insulin (2.5 µg/ml). Fibrin gels were cultured under the following conditions: control (CTR), GDF-5 supplementation (0.1 µg/ml) (G 0.1) or addition of GDF-5 (0.1 µg/ml) and insulin (2.5 µg/ml) (G 0.1 +I). Data represents the average ± SD of three independent constructs. Statistically significant differences between the groups are denoted by symbol (○) ($p < 0.05$) or asterisk (*) ($p<0.01$). Before analysis normality test was conducted. In case of failure (indicated by $_1$) Kruskal Wallis analysis of variance on ranks with subsequent Tukey test was performed.

Comparing, for example, constructs seeded with cells expanded under control conditions (Exp: CTR) and cultured in 3D in CCM without further supplements (Exp. CTR – CTR) with constructs applying cells expanded also under control conditions (Exp: CTR), but cultured with GDF-5 and insulin supplementation in fibrin gel culture (Exp. CTR – G 0.1+I), the latter one showed the 8-fold GAG content per wet weight compared to control constructs (Exp: CTR-CTR). Comparing matrix production within the group receiving the factor combination

during 3D culture, the preceding expansion under control conditions turned out to be more favourable than expansion under GDF-5 (Exp.: G 0,1) supplementation or addition of the combination of both proteins, GDF-5 and insulin (Exp. G 0.1+I) (Fig. 30). Considering the collagen content, the groups did not defer much with regard to collagen per wet weight (Fig. 30 D).

GAG distribution was analyzed by staining cross-sections of the fibrin gel constructs with safranin-O (Fig. 31). Only the constructs which received CCM supplemented with the combination of GDF-5 and insulin (G 0.1+I) during fibrin culture displayed homogenous and intense red staining indicating substantial amounts of GAG throughout the whole construct. Constructs, cultured either in CCM without supplements or GDF-5 supplementation alone were stained green, indicating the dominance of fibrous tissue and the absence of GAG. Comparing the sub-groups of the group, which was cultured with CCM supplemented with the combination of the proteins during 3D culture (G 0.1 +I), the most intense and homogeneous red staining could be detected in the cross-sections of the fibrin gels seeded with passage 2 chondrocytes expanded under control conditions (Exp: CTR).

Biochemical as well as histological evaluation of the constructs showed no beneficial effect of applying GDF-5 alone or in combination with insulin during the expansion of the chondrocytes on tissue quality attainable during subsequent 3D culture.

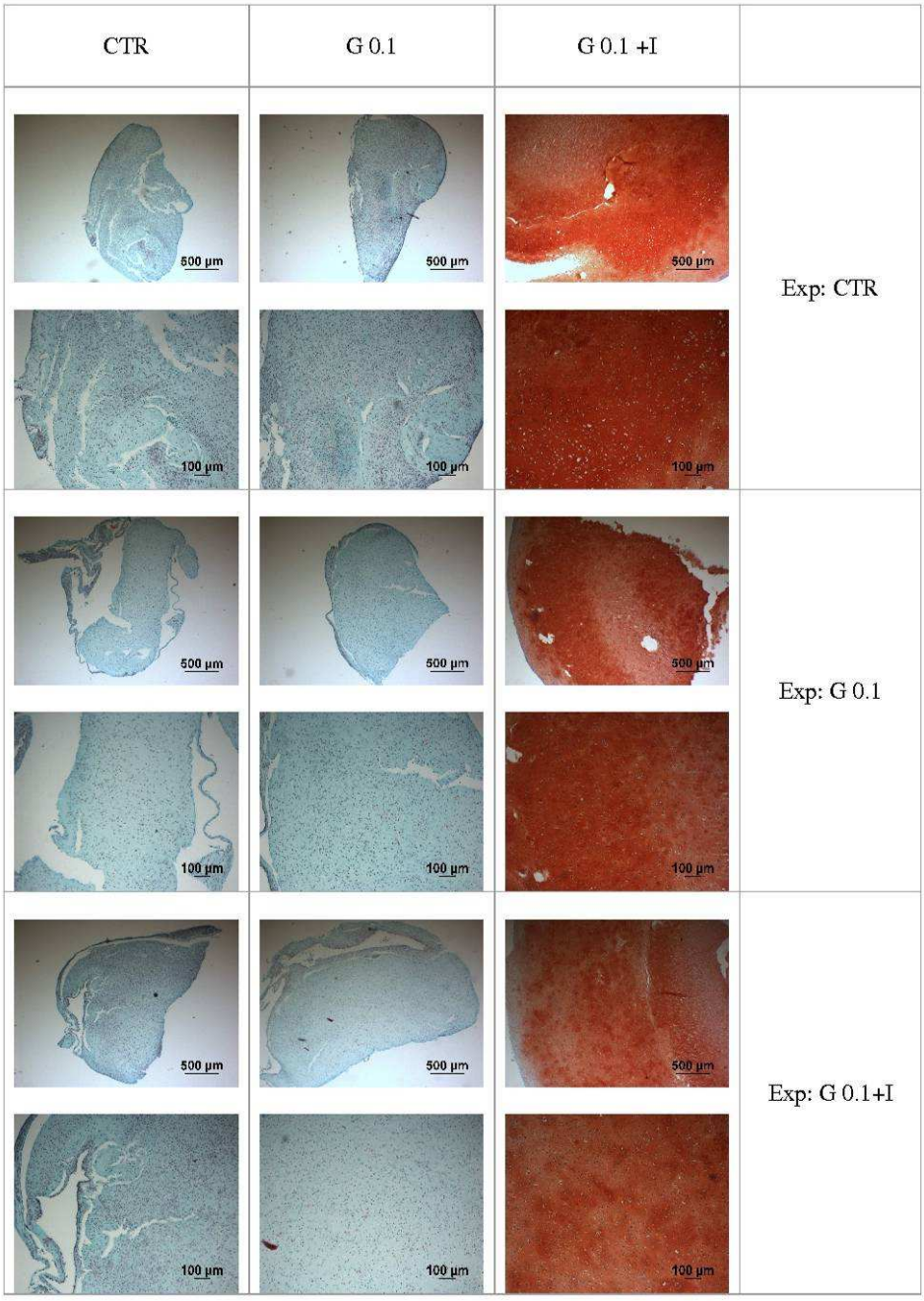

Figure 31: Glycosaminoglycan (GAG) distribution in cross-sections of fibrin gels seeded with passage 2 chondrocytes after 21 days in culture. GAG stained red with safranin-O. The columns divide the different conditions during 3D culture in fibrin gel: control (CTR), with GDF-5 (0.1µg/ml) (G 0.1) supplementation or addition of GDF-5 (0.1 µg/ml) combined with insulin (2.5 µg/ml) (G 0.1+I). The first row shows constructs seeded with passage 2 (P2) chondrocytes expanded under control conditions (Exp: CTR), the second one with P2 chondrocytes expanded under GDF-5 supplementation (0.1 µg/ml; Exp: G 0.1), the last row with P2 chondrocytes expanded under supplementation with GDF-5 (0.1 µg/ml) and insulin (2.5 µg/ml) (Exp: G 0.1+I).

Synergistic Effect of GDF-5 and Insulin on Expanded (P2) Human Chondrocytes

In order to investigate the possible clinical relevance of the combination of GDF-5 and insulin the effect of insulin (2.5 µg/ml) (I 2.5) and GDF-5 (0.1 µg/ml) alone or in combination (G 0.1+I) on tissue quality yielded with expanded (passage 2) adult human chondrocytes was studied in a pellet culture system.

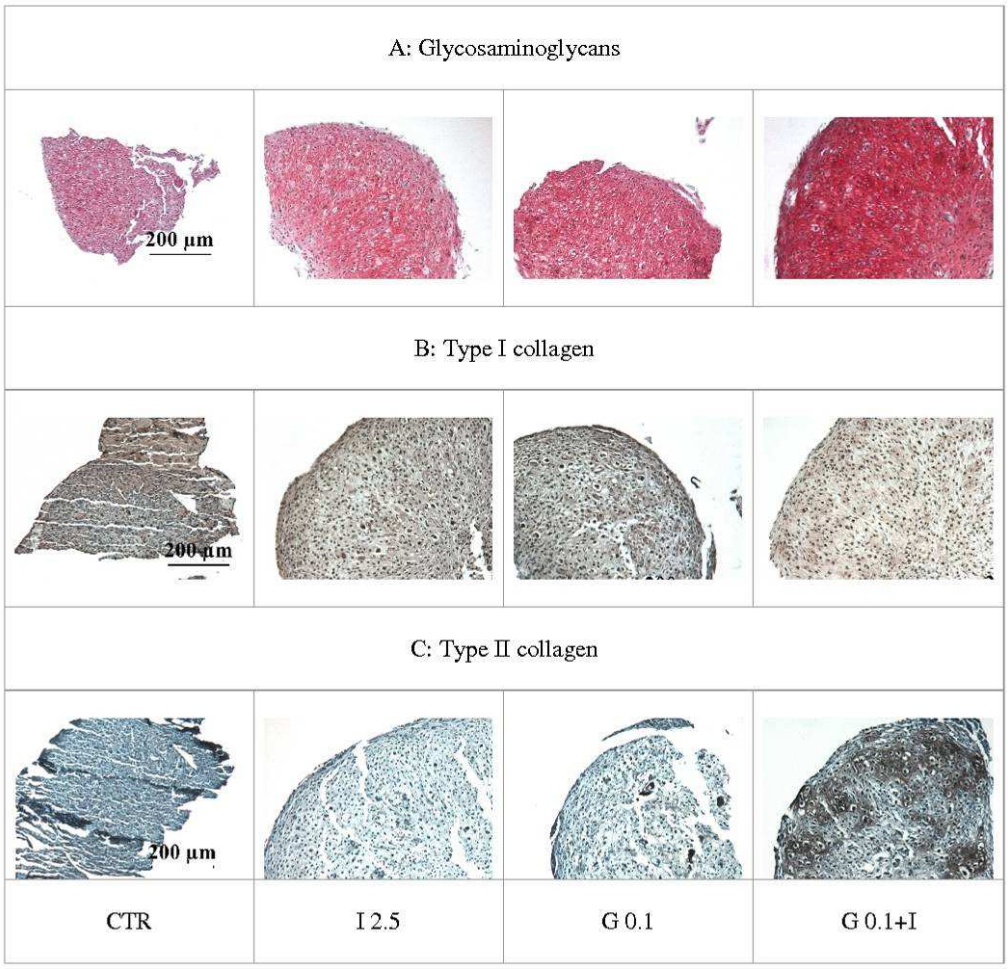

Figure 32: Cross-sections of pellets generated with human passage 2 chondrocytes after 3 weeks of culture. Distribution of glycosaminoglycans (GAG) (A), GAG was stained red with safranin-O. Type I collagen (B) and type II collagen (C) distribution, collagens were immunohistochemically stained employing type I and type II collagens antibody respectively (brown stain).

The study with human adult passage 2 chondrocytes rendered similar results as obtained with expanded bovine chondrocytes. Application of proteins resulted in increased pellet size as compared to controls. Control pellets and pellets receiving single proteins exhibited a homogeneous, but weak safranin-O staining for GAG (Fig. 32A). The combination of GDF-5 and insulin resulted in a distinctly stronger staining for GAG in large areas of the pellet.

Control constructs were stained positive for type I collagen (Fig. 32B). Constructs cultured under supplementation with insulin (2.5 µg/ml) (I 2.5) or GDF-5 (0.1 µg/ml) (G 0.1) also displayed a positive staining for type I collagen staining, however, was less intense compared to the control group. The combined application of GDF-5 and insulin resulted in a distinctly lighter staining for type I collagen compared to the other experimental groups. Complementary results were observed by type II collagen immunohistochemical staining. Neither control constructs nor constructs receiving either insulin or GDF-5 alone showed the presence of type II collagen. In clear contrast, under the supplementation with the combination of GDF-5 and insulin (G 0.1+I), a strong type II collagen staining was detected.

The results obtained by quantitative real-time RT-PCR were consistent with the histological and immunohistochemical staining. Type I collagen mRNA was upregulated by insulin, as compared to controls (3.6-fold), whereas it was not affected by GDF-5 applied alone or in combination with insulin (Fig. 33A). In contrast, application of insulin alone led to a distinct down-regulation of type II collagen mRNA expression compared to control (33-fold), and the combination of GDF-5 and insulin resulted in a tremendous up-regulation of type II collagen (115-fold, Fig. 33B).

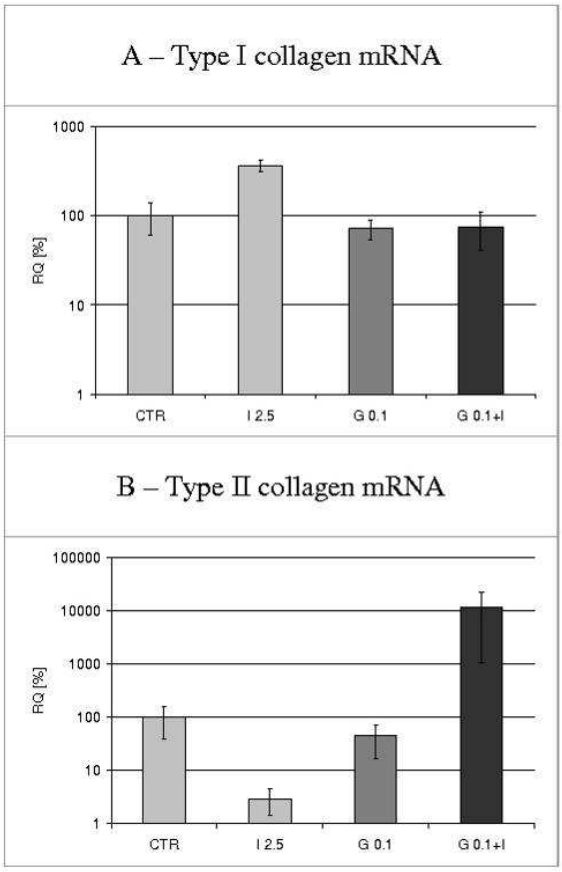

Figure 33: Relative quantification using RT-PCR of type I (A) and type II (B) collagen mRNA expression on day 21 of pellet culture with human passage 2 chondrocytes. Data represents the average of two or three independent constructs, error bars indicate the minimum and the maximum values. Expression levels of target genes were normalized to the expression of GAPDH serving as housekeeping gene. Expression levels were further normalized to expression levels of the control samples (set as 100%).

Effect of GDF-5 and Insulin on Chondrogenic Differentiation of Human Adipose Tissue Derived Stem Cells (ASC)

In a last, preliminary experiment the effect of GDF-5 and insulin alone or in combination on chondrogenic differentiation was examined. The application of GDF-5 (0.1 µg/ml) (G 0.1) or insulin (2.5 µg/ml) alone or in combination did not exert an effect on cell number after 21 days in pellet culture (Fig. 34A). Supplementation of CCM with either factor alone or in combination yielded a higher GAG production per cell compared to the control without factor

supplementation (Fig. 34B). Highest increase in GAG production per cell could be achieved applying GDF-5 alone at 0.1 µg/ml (approximately 3-fold higher production in the GDF-5 group compared to control). Substantial amounts of GAG, however, could not be detected. In accordance to the results of the biochemical analysis, the histology did not show a considerable red staining of the cross-sections of the pellets, indicating no sufficient GAG production. Cross-sections displayed green colour which demonstrated the development of fibrous tissue. Only around few cells a faint red corona could be detected, particularly in the group which received supplementation with GDF-5 and insulin (G 0.1+I) (Fig. 35).

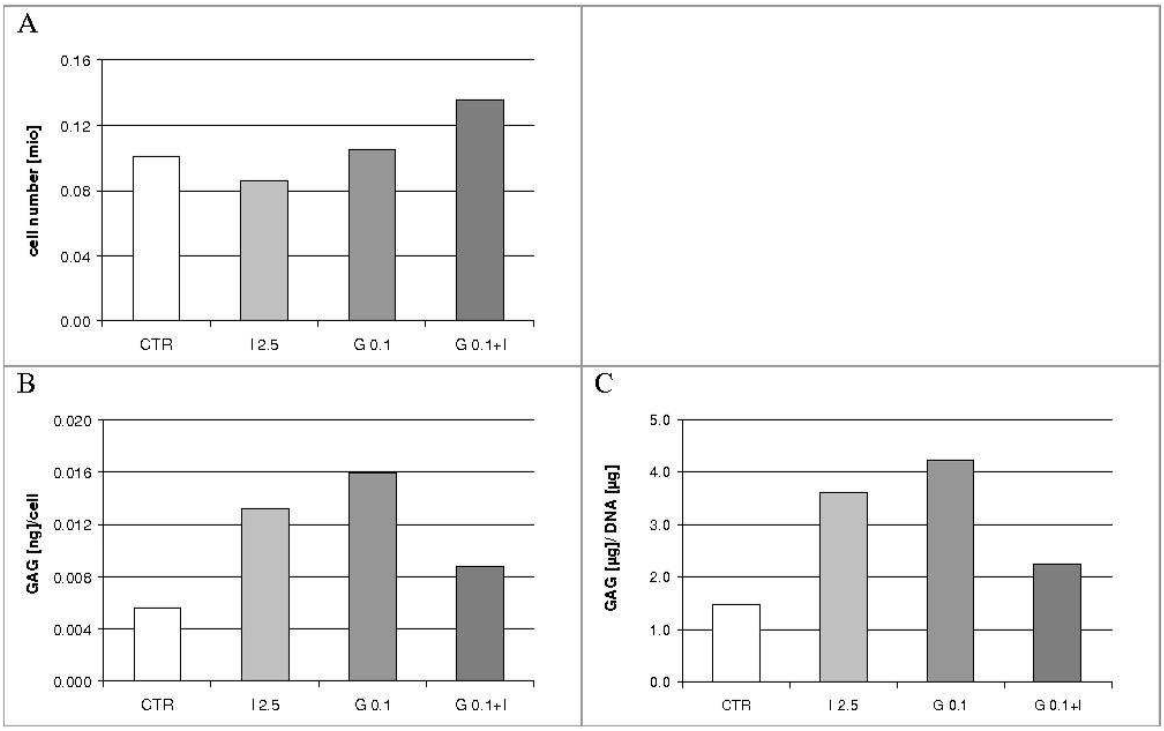

Figure 34: Cell number (A) and GAG content (B) of ASC pellets after 21 days in culture. Pellets were either cultured under control conditions (CTR), in CCM supplemented with GDF-5 (0.1µg/ml) (G 0.1) or with insulin (2.5 µg/ml) (I 2.5) alone or with supplementation with the combination of both proteins (G 0.1 +I). For each group 3 individual pellets were pooled. GAG [ng]/ cell as well as GAG [µg]/ DNA [µg] are shown here for better comparison with literature (c.f. discussion).

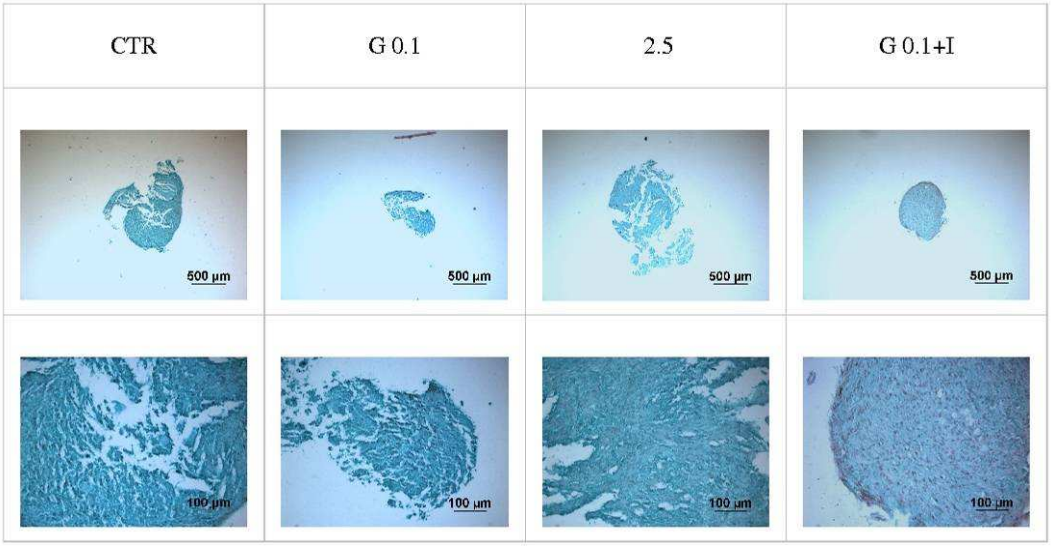

Figure 35: Cross-sections of pellets with ASC after 21 days in culture. Pellets were cultured under control conditions (CTR), with GDF-5 (0.1 µg/ml) (G 0.1) or insulin (2.5 µg/ml) (I 2.5) supplementation or the combination of both GDF-5 and insulin (G 0.1 +I) was added to CCM. Cross-sections were stained with safranin-O, which stains glycosaminoglycans red.

Discussion

GDF-5 is known to play an essential role in embryonic chondrogenesis and limb formation [81, 92, 93]. There is evidence that GDF-5 takes part in the complex cascade of bone fracture healing, for example by playing a central role in chondrogenesis and maturation of chondrocytes [94–96]. In adult bovine and human cartilage, GDF-5 was found to be expressed in both normal and osteoarthritic tissue [79]. However, the specific functions of GDF-5 in adult cartilage are still to be elucidated. Nevertheless, GDF-5 represents an interesting candidate molecule for cartilage engineering applications, as suggested in several studies using different in vitro and in vivo models [97–100].

Engineering cartilaginous constructs, the use of in vitro expanded chondrocytes is still one of the major obstacles. In order to improve constructs made from passaged chondrocytes, different approaches have employed various growth factors. The synergistic effect of GDF-5

and insulin exerted on expanded (passage 2) bovine chondrocytes had previously been proven in the group [88, 89]. The experiments presented here were conducted applying GDF-5 and insulin in the concentrations which had turned out to be most effective [27, 88, 89].

In the first study, GDF-5 alone or in combination with insulin was applied during expansion of bovine chondrocytes and the effects on tissue quality attainable in subsequent 3D fibrin gel culture employing the differently expanded (passage 2) chondrocytes were investigated. The fact that the application of GDF-5 alone or in combination in the expansion phase with insulin did not exert a positive effect on subsequent development of cartilaginous tissue in fibrin gel (Figs. 30 & 31) could be considered as a clue towards the mechanism of action of these proteins. They seemed not to be able to inhibit dedifferentiation, but to have a favourable impact on redifferentiation processes occurring during 3D culture. For other growth factors, differential results were found. For example, the application of FGF-2 during expansion of bovine articular chondrocytes resulted in a reduced expression of fibroblast markers and a higher responsiveness to BMP-2 applied during PGA culture. The application of BMP-2 during expansion in 2D, however, did not exert this positive effect [78], similar to the results observed in our study employing GDF-5 also being a member of the BMP-family. Jakob et al. also investigated the effect of several proteins applied during expansion (i.e., EGF, FGF-2, TGF-β1, PDGFbb, TGF-β1/ FGF-2). All used factors increased dedifferentiation as well as cell proliferation, but only FGF-2, EGF and PDGFbb application during expansion in 2D resulted in increased redifferentiation in pellet culture [75].

The results previously obtained with juvenile bovine cells [88, 89] were supported by experiments employing adult human chondrocytes: Again, only the combination of GDF-5 and insulin led to type II collagen expression, as detected by immunohistochemistry, at the same time reducing expression of type I collagen, compared to either factor alone (Fig.32). Type II collagen expression was also dramatically upregulated for the combination at the mRNA level (Fig. 33). Whereas no other studies on passaged chondrocytes are currently

available, previously, it has been reported that GDF-5 stimulated the ECM response of fetal rat calvarial cells towards the chondrogenic phenotype [101]. Interestingly, supplementation of GDF-5 alone led to only a slight upregulation of type II collagen expression and had no effect on type I collagen in mesenchymal stem cells, whereas a combination of GDF-5 and TGF-β1 distinctly increased type II collagen and reduced type I collagen expression [102]. In accordance to the previous studies with bovine chondrocytes[88, 89], in the presented study neither factor alone was able to overcome the dedifferentiation resulting from chondrocyte expansion; however, the combination of GDF-5 and insulin led to cartilaginous constructs with cells more actively producing ECM, and with a distinctly improved collagen subtype content. The demonstration of the synergistic redifferentiating effects of GDF-5 and insulin also for adult human chondrocytes further adds to the clinical relevance of the findings concerning the synergistic effect of GDF-5 and insulin.

Various 3D culture systems were chosen for the investigation of the synergistic effects of GDF-5 and insulin. In previous studies, where the synergistic effects were described for expanded bovine chondrocytes, PGA scaffolds were applied [88, 89]. In fibrin gel culture as well as in the pellet culture system, the 3D culture systems used in the study presented here, synergistic effects of GDF-5 and insulin were also exerted on expanded bovine and human chondrocytes. The synergistic effects of GDF-5 and insulin are thus independent from the chosen 3D system.

Due to the convincing results with expanded human adult chondrocytes the question arose, if besides a synergistic effect during redifferentiation of dedifferentiated chondrocytes a synergistic effect during chondrogenic differentiation of human mesenchymal is also exerted by GDF-5 and insulin. Human adipose derived stem cells were chosen, as they are characterized by an easy availability with little burden for the patient, the possibility to obtain large amounts thereof [25] and their described chondrogenic potential [103–107]. In a first preliminary study, GDF-5 (G 0.1) and insulin (I2.5) alone as well as the combination thereof

were added to complete chondrocyte medium in order to observe possible chondrogenic differentiation. In contrast to expanded chondrocytes, no synergism was found in the study with ASC. The highest degree of chondrogenic differentiation, when taking into account GAG production as an indicator for the production of cartilaginous extracellular matrix (ECM), could be reached by GDF-5 (Fig. 35). However, as to be expected, compared to our results with expanded chondrocytes, only very low GAG production was observed with ASC within the three weeks of culture. In order to assess the potential of GDF-5 for stem cell differentiation, comparison of the results with other studies, attempting to differentiate ASC towards a chondrogenic phenotype have to be taken into account. Feng et al. for example compared the extent of chondrogenic differentiation under TGF-β and GDF-5 supplementation versus control as well as the chondrogenic differentiation induced by transfection with an adenoviral vector expressing GDF-5 (Ad-GDF-5). The highest degree of chondrogenic differentiation measured as GAG production (GAG [µg]/ DNA [µg]) was yielded by GDF-5 supplementation at the same concentration applied in the study presented here. Comparing the results in both studies, the same level of GAG production was reached (approximately 3.5 and 4.2 respectively) [108]. TGF-β1 was also employed for chondrogenic differentiation of ASC. The results in GAG production were also comparable to the degree of differentiation achieved here [1]. Nevertheless histological examination (Fig. 43) clearly revealed the great discrepancy between the obtained results and the requirements, when aiming at clinical application. In order to overcome the reduced capacity of ASC to differentiate towards a chondrogenic phenotype, media compositions are to be optimized. Comparing ASC and BMSC concerning their capability to differentiate towards a chondrogenic phenotype most of the direct comparisons found a lower chondrogenic differentiation capacity for ASC than for BMSC (c.f. Chapter 8). In order to distinctly improve the comparatively inferior chondrogenic potential of ASC, the reasons for the lower chondrogenic capability as well as possible optimizations of standard culture conditions were

investigated. For example, the application of higher concentrations of TGF-β2 in combination with IGF-I resulted in a cartilaginous differentiation of ASC comparable to BMSC differentiated by standard protocol [109].

Another group was able to show that in contrast to BMSC, ASC exhibited a reduced expression of BMP-2/-4/-6 mRNA and did not express TGF-β-receptor-I protein. Consequently, an increase in the concentration of TGF-β did not result in improved chondrogenesis. A treatment with BMP-6, however, induced TGF-β- receptor-I expression and in combination with TGF-β resulted in a gene expression profile in ASC similar to BMSC during chondrogenic differentiation [110].

With regard to the main part of the presented study, i.e., the redifferentiation of expanded chondrocytes, it also appears desirable to further elucidate the mechanism of action involved in the detected synergism of GDF-5 and insulin. Viewed together with results previously obtained, it is remarkable that there were clear differences between the primary and the passaged chondrocyte cultures [88, 89]. Whereas for the primary cells, beneficial effects on construct size were clearly ascribable to increased proliferation, for the passaged chondrocytes the distinct redifferentiating effects were elicited on the cellular level independent of proliferation (increased ECM production per cell, shift in collagen subtype expression on mRNA and protein level) [88, 89]. One possible explanation for the redifferentiating effects on the passaged chondrocytes is an upregulation of receptor densities. GDF-5 transmits its signals through binding to two different serine-/threonine-kinase receptors forming a heterodimeric complex, that is, type II (either ActR-II, ActR-IÍB, or BMPR-II) and type I (BMPR-IB) [111–113]. As described for other growth factors in skeletal development such as TGF-β1, FGF-2, and PDGF-AB [102, 114], insulin may induce expression of BMPR-IB in turn enhancing the signal transduction of GDF-5. Furthermore, there is evidence that dedifferentiated cells pass through similar stages when redifferentiating,

as cells do in chondrogenesis [91]. Consequently, other possible mechanisms for the synergistic effects include transcriptional cross-talk as elicited by morphogens and growth factors during chondrogenesis. In our study, cross-talk between Smad and MAP kinase pathways activated by GDF-5 and insulin may have been involved in the observed effects [113, 115, 116]. The fact that GDF-5 decisively modulates the response to another cartilage-effective protein contributes to the emerging picture of the role GDF-5 apparently plays in chondrogenesis and cartilage physiology.

Chapter 8

Differentiation Capacity of Adipose - Derived Stem Cells (ASC) and Bone Marrow Derived Stem Cells (BMSC) towards Fat, Bone and Cartilage in Direct Comparison Review

(Manuscript in preparation, c.f. Appendices, Publications to be submitted)

Introduction

Stromal cells from bone marrow and adipose tissue represent an attractive source of adult progenitor cells particularly for regenerative medicine in tissue engineering approaches. Moreover, they constitute a fascinating model system for the investigation of differentiation processes. The most obvious use is in the field of orthopaedics due to their proven ability to differentiate towards cartilage and bone. The chondrogenic potential of bone marrow-derived mesenchymal stem cells (BMSC) has been shown in several studies [117–125] as well as the osteogenic potential [121, 123, 126–129]. Due to their multilineage potential BMSC can also differentiate, among other lineages, towards adipocytes [123, 126, 128]. The procurement of bone marrow, however, is limited and associated with a procedure which may be painful and often demands general or spinal anaesthesia. BMSC are usually obtained from bone marrow aspirates by density grade centrifugation. Moreover, the cell yield is not very high thus demanding in vitro expansion.

Adipose tissue constitutes another source for mesenchymal stem cells, which can be obtained in large quantities and under local anaesthesia [25]. Since the publication of the first work demonstrating the multilineage potential of stem cells derived from adipose tissue [25, 26], several groups have investigated the differentiation capacity of these cells. For example, their capability to differentiate towards a chondrogenic [103–107], osteogenic [130–132] and neuronal [133, 134] phenotype has been demonstrated. Several terms have been used synonymously for adipose-derived stem cells. For reasons of harmonization and simplification the International Fat Applied Technology Society agreed to employ the term adipose tissue-derived stem cells (ASC) for isolated, plastic-adherent multipotent cell population from adipose tissue [135]. According to this agreement the same nomenclature will be used in this article. ASC applied in the regarded studies were in brief isolated by

digestion of the adipose tissue with collagenase, subsequent filtration and centrifugation and removal of blood cells and then seeded in culture flask or dishes for further expansion.

As mentioned above, numerous studies have shown the multilineage potential of ASC and BMSC. However, there is still a lively ongoing discussion among researchers in the field of regenerative medicine on the preferred cell type for use in specific applications. By now, there is a considerable number of investigations available that have directly compared ASC and BMSC concerning their chondrogenic, osteogenic or adipogenic differentiation capability under different culture conditions and with sometimes varying outcome. The aim of this review is to summarize and evaluate these studies in order to facilitate more rational conclusions on the choice of stem cells for a specific regenerative approach. Future aspects of research into ASC and BMSC application are discussed.

The differentiation capacity towards a chondrogenic phenotype is of particular interest due to the limited availability of autologous harvestable chondrocytes for cartilage tissue engineering applications.

Comparison of the Chondrogenic Potential of ASC and BMSC

Methods to effectively differentiate mesenchymal stem cells towards a chondrogenic phenotype may solve one of the major problems in cartilage tissue engineering: the limited number of autologous, harvestable cells. As a 3-dimensional (3D) environment generally favours the chondrogenic phenotype, in many studies chondrogenic differentiation is conducted in a 3D environment. The studies directly comparing ASC and BMSC with regard to chondrogenic differentiation are summarized in Table 4. The extent of chondrogenic differentiation is evaluated by investigation of the amount of typical components of cartilaginous extracellular matrix (ECM), like collagen or glycosaminoglycans, which can be easily quantified by spectrophotometric assays. The distribution of glycosaminoglycans can

be visualized by safranin O or toluidine blue staining. Synthesis of the characteristic type II collagen is assessed by immunohistochemical staining. But also mRNA of typical chondrogenic markers, like type II collagen (COL2A1), aggrecan and cartilage oligomeric protein (COMP), can reveal if chondrogenic differentiation was successful. Moreover, the expression of type X collagen, a marker for hypertrophic chondrocytes, is often analyzed. A high expression of type X collagen indicates a differentiation beyond the mature chondrocytes towards a mineralized tissue, which is generally not intended for cartilage tissue engineering purposes.

In Vitro Studies

Although 3D culture systems are favoured for chondrogenic differentiation, the chondrogenic capacity or gene expression profiles of both ASC and BMSC were assessed in monolayer (c.f. study by Winter [18] et al and study by Liu et al. [22]) in some investigations. Standard chondrogenic induction in monolayer resulted in an up-regulation of cartilage markers and expansion related genes. The differentiation, however, remained incomplete. A shift to the 3D pellet culture system in combination resulted in an improved differentiation of BMSC to a molecular phenotype highly resembling that of cartilage, whereas with ASC displayed a distinctly delayed and decreased COL2A1 expression [18].

In other studies, directly comparing morphology chondrogenically induced BMSC in 3D environment displayed round chondrocyte morphology, whereas ASC stayed small and fusiform [19, 136]. Pellets of ASC remained smaller in size compared to those generated from BMSC. Macroscopically BMSC had a higher chondrogenic potential and morphologically resembled chondrocytes, whereas ASC acquired a morphology resembling that of fibroblast [21].

Staining of sulfated glycosaminoglycans with safranin O or toluidine blue proved a distinct proteoglycan production by chondrogenically induced BMSC in 3D culture, whereas only

faint staining was reached in ASC cultures indicating low matrix production [19, 21, 137–139].

Ref.	Outcome	Medium composition	Culture System & Period	Passage #	
				ASC	BMSC
[136]	ASC << BMSC	DMEM/ F12, 1% insulin-transferrin-selenium (ITS), 10^{-7} M dexamethasone, 50 mM ascorbate-2-phosphate, 50 µM L-proline, 1 mM sodium pyruvate, 5 ng/ml TGF-β2, 100 ng/ml IGF-1	pellet; fibrin gel - 28 d	P3	P3
[22]		DMEM, 10% FBS, 2 mM L-glutamine, 10 ng/ml TGF-β1, 6.25 µg/ml insulin, 50 nM ascorbate-2-phosphate, 1% antibiotic/ antimycotic	culture flask - 14 d (gene expr. profiling)	P2	P2
	ASC << BMSC	DMEM, 10% FBS, 2 mM L-glutamine, 10 ng/ml TGF-β3, 10^{-7} M dexamethasone, 50 µg/ml ascorbate-2-phosphate, 40 µg/ml proline, 100 µg/ml pyruvate, 50 mg/ml ITS^+ premix (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid)	pellet culture - 14 d (comparison of chondrogenic potential)		
[18]	ASC ≈ BMSC	DMEM-HG, 10% FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 µg/ml selenious acid, 0.1 µM dexamethasone, 0.17 mM ascorbic acid-2-phosphate, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/ml bovine serum albumin, 10 µg/ml TGF-β3	culture flask - 14 d	-	-
	ASC << BMSC		spheroid culture - 28 d		
[140]	ASC < BMSC	DMEM, 10% FCS, 10 ng/ml TGF-β1, 6.25 µg/ml insulin, 1% antibiotic/ antimycotic	pellet culture - 14 d	P3-P6	P3-P6
[141]	ASC ≈ BMSC	DMEM-HG, 1% FCS, 10 ng/ml TGF-β1, 0.5 µg/ml insulin, 50 µM ascorbic acid	pellet culture - 21 d	P3-P5	P3-P5
[142]	ASC < BMSC	DMEM-HG, 0.1 µM dexamethasone, 0.17mM ascorbate-2-phophate, 1% insulin-transferrin-sodium selenite supplement, 100 ng/ml rhBMP-2	pellet culture - 21 d	P2-P4	P2-P4
[21]	ASC << BMSC	DMEM-HG, 500 ng/ml BMP-2, 10 ng/ml TGF-β3, 10^{-7} M dexamethasone, 50 µg/ml ascorbate-2-phosphate, 40 µg/ml proline, 100 µg/ml pyruvate, 50 mg/ml, ITS^+ premix	pellet culture - 21 d	P1	P1
[143]	ASC < BMSC	DMEM-HG, 1% ITS^+ premix, 1 mM pyruvate, 73.5 mg/ml ascorbate-2-phosphate	pellet culture - 28 d	P1	P1
[137]	ASC < BMSC	DMEM-HG, 500 ng/ml BMP-2, 10 ng/ml TGF-β3, 10^{-7} M dexamethasone, 50 µg/ml ascorbate-2-phosphate, 40 ng/ml proline, 100 ng/ml pyruvate, 1:100 diluted ITS^+ premix (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid	pellet culture - 21 d	P2	P2
[139]	ASC < BMSC	DMEM-HG, 2% FCS, 100 ng/ml BMP-2 or AdBMP-2 (500 pfu/cell), 100 nM dexamethasone, 1xBSA/ linoleic acid, 1x ITS premix, 50 µg/ml ascorbate-2-phosphate, 100 U/ml penicillin/streptomycin	pellet culture - 14 d	P2	P2
[138]	ASC << BMSC	DMEM-HG, 10% FCS?, 1% antibiotic/ antifungal solution, 100 nM dexamethasone, 50 mg/ml ascorbate-2-phophate, ITS^+ premix, with/ without 10 ng/ml TGF-β3, 10 ng/ml rhBMP-6	pellet culture - 21 d	P2	P2

[144]	ASC ≈ BMSC	Complete chondrogenic differentiation medium (Cambrex) incl. 10 ng/ml TGF-β	pellet culture - 20 d	P1	P2
[145]	ASC << BMSC	DMEM-HG, 1% ITS^{+}, 0.1 µM dexamethasone, 37.5 µg/ml ascorbate-2-phosphate, with/ without 10 ng/ml rhTGF-β1	agarose hydrogels/ self-assembling peptide - 21 d	P2	P2
[19]	ASC << BMSC	DMEM-HG, 4 mM proline, 50 µg/ml ascorbic acid, 1% ITS-premix, 1mM sodium pyruvate, 0.1 µM dexamethasone, 10 ng/ml TGF-β3		P2	P2
[146]	ASC << BMSC	DMEM-HG, 0.1mg/ml sodium pyruvate, 0.1mg/ml L – glutamine, 0.1 mg/ml pyridoxine hydrochloride, 1 % penicillin/ streptomycin, ITS^{+} premix (6.25 mg/ml each), 10^{-7}M dexamethasone, 10 ngTGF-β1		P3	P3
[109]	ASC << BMSC	DMEM/F12, 1% ITS premix, 10^{-7} M dexamethasone, 50 µM ascorbate-2-phosphate, 50 µM L-proline, 1 mM sodium pyruvate ASC: 5 ng/ml TGF-β2/ 5 ng/ml TGF-β2, 100 ng/ml IGF-I/ 15 ng/ml TGF-β2/ 15 mg/ml TGF-β2, 300 ng/ml IGF-I/ 25 ng/ml TGF-β2/ 25 ng/ml TGF-β2, 500 ng/ ml IGF-I BMSC: 5 ng/ml TGF-β2		P3	P3
[110]	ASC < BMSC	DMEM-HG, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid, 100 nM dexamethasone, 0.17 mM ascorbic acid-2-phosphate, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 BSA, supplements: 10 ng/ml TGF-β3, BMP-2/-4/-6/-7, IGF-I, FGFa, bFGF, PTHrP		P2-P6	P2-P6

Table 4: Overview of the studies directly comparing the chondrogenic differentiation capacity of ASC and BMSC in vitro.

In some studies the results of histological examination of matrix production and distributions were consistent with the results of biochemical analysis of GAG synthesis, which was significantly higher for treated BMSC compared to treated ASC [19], whereas another group found no significant difference in GAG production normalized to total DNA concentration [138].

Immunohistochemical staining of the harvested constructs revealed a type II collagen-rich matrix in BMSC cultures in contrast to ASC cultures, where only a faint staining [139, 146] or even no staining [19, 136, 138, 145] was detected.

The results of the analysis of gene expression were consistent with the results of histological and immunohistochemical analysis, although there were minor differences in the results depending on the respective study.

Mehlhorn et al. compared the expression levels of chondrogenic markers expressed by induced ASC and BMSC on day 14. Detected chondrogenic marker expression displayed the superiority of BMSC to differentiate towards a chondrogenic phenotype [146]. Chondrogenic induction resulted in an up-regulation of type II collagen mRNA in both cell types compared to unstimulated cells, whereas up-regulation was significantly higher in chondrogenic BMSC cultures [22, 139-141]. Afizah et al found a much higher and earlier increase in type II collagen expression [19]. Liu et al also detected a higher increase in type II and X collagen expression in BMSC cultures compared to ASC cultures after chondrogenic induction, aggrecan expression was only detectable in BMSC culture [22]. In other studies type II collagen expression was only detectable in BMSC, but not in ASC [145]. Expression of type X collagen in BMSC cultures indicated that BMSC tend to hypertrophic chondrogenic differentiation, which is not intended for cartilage tissue engineering.

Integrin-mediated signalling has emerged to play an important role in chondrogenic differentiation and maintenance of a chondrogenic phenotype. There were no differences detected in integrin expression between the two cell types [144].

In Vivo Studies

Chondrogenic capability was also analyzed in vivo in animal models. Table 5 summarizes the design and the outcome of the respective studies. TGF-β1-transduced ASC and BMSC were injected intraarticularly and the two groups stained similarly positive for type II collagen after 21 days [142]. Untreated ASC seeded in a collagen gel and transplanted into a full-thickness osteochondral defect resulted in poor cartilage matrix visualized by a faint toluidine blue staining, whereas the corresponding group with untreated BMSC, which displayed an intense blue staining [137]. ASC or BMSC, infected with adenoviral vector expressing bone morphogenetic protein-2 (AdBMP-2) and seeded in fibrin glue before subsequent

transplantation into partial thickness defects in the patellar groove, showed significant differences concerning adherence to the bordering walls of the defect.

Ref.	Outcome	Animal Model	Implant Generation	in vivo culture period	Passage #	
					ASC	BMSC
[142]	ASC ≈ BMSC	immunodeficient mouse; injection of cells intra-articularly	cells transfected with TGF-β1	- 21d	P2 – P4	P2 – P4
[137]	ASC << BMSC	rabbit, full thickness osteochondral defect (femur) filled with cell/ collagen gel composites	cells in collagen gel, α-MEM, 20% FBS for 1d before transplantation	- 28/ 84 d	P2	P2
[139]	ASC << BMSC	rat, partial thickness defect in patellar groove, pellet transplanted into defect	cells transfected with AdBMP-2, generation of cell pellet of infected & non-infected cells at a ratio of 2:1	- 14 d	P2	P2

Table 5: Overview of the studies directly comparing the chondrogenic differentiation of ASC and BMSC in vivo.

In the groups with BMSC most of the lesions were completely filled with cartilaginous repair tissue, whereas repair tissue deriving from ASC rarely filled the defects completely. Moreover AdBMP-2 infected BMSC displayed a positive toluidine blue and type II collagen staining, whereas AdBMP-2 infected ASC produced predominantly fibrous repair tissue lacking proteoglycans and being predominantly composed of type I collagen [139].

Although the studies in principle demonstrate the chondrogenic capacity of ASC, none of the studies directly comparing ASC and BMSC showed a higher chondrogenic differentiation capacity of ASC and only few studies showed similar outcome. Noel et al concluded that ASC and BMSC represent fundamentally different cell types and with different commitment [147]. In order to investigate into and possibly overcome the inferiority of ASC in the ability to differentiate towards a chondrogenic phenotype further studies were conducted. A successful approach by Kim and Im was to apply higher concentrations of TGF-β2 in combination with IGF-I. Culturing ASC under the mentioned conditions resulted in a

chondrogenic differentiation comparable to BMSC under standard conditions [109]. Hennig et al investigated the mRNA expression of several BMPs and expression of TGF-β-receptor-I-protein to elucidate the lower chondrogenic capacity of ASC, partially contradictory to the findings of Kim and Im. In comparison to BMSC, ASC showed a reduced expression of BMP-2/-4/-6 mRNA and, in contrast to BMSC, did not express TGF-β-receptor-I protein. Consequently an increase in the concentration of TGF-β did not result in improved chondrogenesis. A treatment with BMP-6, however, induced TGF-β- receptor-I expression and in combination with TGF-β resulted in a gene expression profile in ASC similar to BMSC during chondrogenic differentiation [110]. Comparing the two studies, one has to consider that Kim and Im investigated the effect of higher concentrations of TGF-β, also in combination with IGF-I. They did not further analyze possible changes on the receptor level. Hennig et al, on the other hand, approached the problem by testing different growth factors and the combination thereof. Thereby, the expression of TGF-β-receptor-I-protein was analyzed. Possible positive effects of increasing the TGF-β concentrations were also investigated based on type II collagen synthesis. In contrast to Kim and Im, no type II collagen was detectable even when applying high concentrations of TGF-β in contrast to Kim and Im. Kim and Im, however, did not elucidate possible impact of IGF-I on TGF-β-receptor-I-protein and did not consider possible synergistic effects of TGF-β and IGF-I, as observed for growth and differentiation factor-5 (GDF-5), which also is a member of the TGF-superfamily, and insulin, which displays great similarities to IGF-I [88].

Comparison of the Osteogenic Potential of ASC and BMSC

A distinct osteogenic potential has also been demonstrated for both BMSC and ASC. In contrast to the studies analyzing chondrogenic differentiation, the majority of the investigations directly comparing osteogenic differentiation of BSMC and ASC were only

conducted in 2-dimensional environment (studies summarized in Table 6). Osteogenic differentiation is commonly evaluated by analysis of alkaline phosphatase (ALP) activity, matrix mineralization, which can be visualized by von Kossa staining or staining with alizarin S, and additionally calcium deposition assay. Another possibility to assess osteogenic differentiation is to analyze the expression of osteogenic markers like Runx2, osteopontin, osteonectin, osteocalcin and osteomodulin or alkaline phosphatase.

In Vitro Studies

After osteogenic induction in vitro (media composition given in Table 6), in some studies similar matrix mineralization was detected by von Kossa or alizarin S staining indicating that both BMSC and ASC possess osteogenic potential [18, 141, 148, 149]. In other studies the intensity of the staining as a measure for the amount of calcium deposition revealed, that BMSC had accumulated more calcium in consequence of osteogenic induction [21, 22, 136, 150, 151]. A higher degree of matrix mineralization in BMSC cultures was also evidenced by stronger calcein fluorescence in matrix. Calcium quantification on day 14 as wells as on day 28 indicated higher calcium deposition in BMSC groups than in ASC groups [152].

Ref.	Outcome	Medium composition	Culture Period	Passage #	
				ASC	BMSC
[136]	ASC << BMSC	DMEM/ F12, 1% FCS, 0.1 µM dexamethasone, 50 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 1% antibiotic/antimycotic	- 21 d	P3	P3
[22]	ASC << BMSC	DMEM, 10% FBS, 2mM glutamine, 0.1µM dexamethasone, 50 µM acorbate-2-phosphate, 10 mM β-glycerophosphate, 1% antibiotic/ antimycotic	- 14 d	P2	P2
[18]	ASC = BMSC	MSC growth medium, 0.1 µM dexamethasone, 0.05 mM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate	- 14 d	-	-
[140]	ASC < BMSC	DMEM, 10% FBS, 10 mM β-glycerophosphate, 0.01 µM 1,25 dihydroxyvitamin D3, 50 µM ascorbate-2-phosphate, 100 U/ml penicillin/ streptomycin	- 7 d	P3 & P6	P3 & P6

[141]	ASC ≈ BMSC	IMDM, 15% FCS, 0.1 µM dexamethasone, 10mM β-glycerophosphate, 100 µM ascorbate	- 21 d	P3-P5	P3-P5
[142]	ASC < BMSC	DMEM-HG, 10% FBS, 0.1 µM β-glycerophosphate, 3mM NaH_2PO_4, 50 µg/ml ascorbic acid	- 21 d	P2-P4	P2-P4
[21]	ASC < BMSC	α-MEM, 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, 10^{-9} M dexamethasone, 20 mM β-glycerol phosphate, 50 µg/ml ascorbate-2-phosphate	14 d pre-culture, 21 d osteogenic medium	P1	P1
[153]	ASC ≈ BMSC	DMEM, 10% FCS, 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate BMP-4 transduced cells	- 14 d	P3 & P7	P3 & P7
[152]	ASC < BMSC	MEM, Earle's salts & L-Gln, 15% FBS, 1% antibiotics, 10 mM β-glycerophosphate, 0.28 mM ascorbic acid-2-phosphate, 10 nM dexamethasone	- 14 d	-	-
[154]	ASC ≈ BMSC	DMEM lg, 10% FCS, 3mM L-glutamine, 100 U/ml penicillin/ 1000 U/ml streptomycin, 100 nM dexamethasone, 0.2 mM L-ascorbic acid-2-phosphate, 10 mM β-glycerophosphate	- 21 d	P3-P6	P3-P6
[149]	ASC < BMSC	DMEM, 15% FBS, 0.1µM dexamethasone, 50 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate, antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin)	- 28 d	P5	P3
[155]	ASC < BMSC	α-MEM, 20% FCS, P/S, 2 mM glutamine, 1 mM sodium glycerophosphate, 50µM L-ascorbate, 10^{-8}M dexamethasone (in most cases)	- 28 d	P3	P3
[150]	ASC < BMSC	α-MEM, 10% FBS, 10 mM β-glycerol phosphate, 50 µg/ml L-ascorbic acid, 10 nM dexamethasone	- 28 d	P2-P3	P2-P3
[151]	ASC < BMSC	DMEM-HG, 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbic acid-2-phosphate, 10% FBS	- 28 d	P3	P3
[148]	ASC ≈ BMSC	DMEM, 10% FBS, 1% antibiotic/ antimycotic, 0.1 µM dexamethasone, 50 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate	7 d pre-cultivation, 21 osteogenic medium	P4	P4
[156]	-	ASC: DMEM, 10% FBS, 1% antibiotic/ antimycotic, 0.1 µM dexamethasone, 10 mM β-glycerophosphate, 50 µM ascorbate-2-phosphate BMSC: commercial osteogenic induction medium (Clonetics, Cambrex, BioSciences, Baltimore, MD): MSC growth medium, dexamethasone, L-glutamine, ascorbate-2-phosphate, β-glycerophosphate, penicillin/ streptomycin	- 21 d	-	-

Table 6: Overview of the studies directly comparing the osteogenic differentiation capacity of ASC and BMSC in vitro. Studies were conducted in "D with one exception - Hattori et al cultured and differentiated the cells on atelocollagen honeycomb-shaped scaffolds with a membrane-seal (ACHMS scaffold) [149].

ALP activity assays resulted in higher ALP activity in osteogenically induced BMSC cultures in comparison with corresponding ASC cultures [136, 140, 152, 155]. This is also reflected by

higher levels of alkaline phosphatase expression [141]. Besides ALP expressions other osteogenic markers were analyzed. Significantly more osteocalcin was detectable in differentiated BMSC cultures in comparison to the corresponding ASC cultures [152]. Niemeyer et al. reported increases in ALP and osteocalcin expression in both osteogenic ASC and BMSC cultures, without statistically significant differences between the cell types [154]. In another study, much higher levels of extracellular matrix genes like osteomodulin (OMD) were expressed by osteogenically induced BMSC compared to ASC. The same also applied for numerous other osteogenic markers analyzed [22]. Noel et al also found a stronger up-regulation of osteocalcin mRNA in differentiated ASC than in differentiated BMSC cultures. In contrast osteogenic differentiation resulted in a higher up-regulation of osterix and alkaline phosphatase mRNA in BMSC cultures than in the corresponding ASC cultures [142].

Furthermore, osteogenic induction of BMSC and ASC was reported to result in a considerable down-regulation of various extracellular matrix- (ECM-) associated genes over the culture period. Interestingly, certain genes were only down-regulated in ASC (e.g., COL1A2, COL3A1), whereas expression of others were only decreased in BMSC (e.g., COL2A1, COL6A1) [156].

In a special approach, transduction of BMSC and ASC with an adenoviral vector expressing bone morphogenetic protein-4 (Ad-BMP-4) resulted in a stronger deposition of calcified extracellular matrix by ASC in comparison with BMSC at day 14. ASC cultures, however also simultaneously developed fat tissue. Concerning ALP expression a tremendous increase was reported for both transduced BMSC and ASC, without a difference between ASC and BMSC cultures. On day 3, Runx2 mRNA, and on day 7 and 14, bone sialoprotein were more strongly up-regulated in differentiated Ad-BMP-4 transduced BMSC compared to ASC, whereas osteocalcin mRNA was more strongly up-regulated in differentiated Ad-BMP-4 transduced ASC in comparison to BMSC. Cells transduced with adenovirus expressing enhanced green fluorescent protein (AdEGFP) and non-induced cells served as control.

Neither AdEGFP transduced cells nor non-induced cells displayed any sings of osteogenic differentiation during the whole culture period [153].

In Vivo Studies

The osteogenic potential of stem cells derived from bone marrow or fat tissue was also investigated in vivo. The experimental design of the analyzed studies is given in table 7.

Ref.	Outcome	Animal Model	Implant Generation	in vivo culture period	Passage #	
					ASC	BMSC
[153]	ASC ≈ BMSC	rabbit, full thickness defect (cranial) treated with AdBMP-4 transduced cells in fibrin gel	AdBMP-4 transduced cells	- 84 d	P3 & P7	P3 & P7
[157]	ASC < BMSC	sheep, implantation in critical size defect (tibia)	hydroxyapatite coated collagen scaffolds seeded with the cells, 1 d precultivation in expansion medium	- 182 d	P2 - P4	P2 – P4
[149]	ASC > BMSC	nude mouse, s.c. implantation of osteogenic induced ACHMS- cell constructs	ACHMS scaffolds seeded with cells, 14 days pre-culture in osteogenic medium	- 28 d	P5	P3
[152]	ASC << BMSC	rat, subcutaneous implantation	hydroxyapatite discs placed in cell suspension overnight for cell attachment	- 42 d	P0	P0

Table 7: Overview of the studies directly comparing the osteogenic differentiation capacity of ASC and BMSC in vivo.

Osteogenically induced BSMC and ASC cultured for 2 weeks within atelocollagen honeycomb-shaped scaffolds with a membrane-seal (ACHMS) in osteogenic medium were transplanted s.c. in nude mice for 4 weeks. Subsequent analysis of the yielded calcification displayed substantial calcium and phosphate deposits at the surface. Scaffold calcification could be detected in osteogenically induced ASC- as well as BMSC-seeded scaffolds. In contrast to the results of the in vitro study, which displayed no difference between ASC and BMSC seeded ACHMS scaffolds, the calcification level, however, seemed to be higher in

ASC cultures than in BSMC seeded scaffolds [149]. In another study hydroxyapatite (HA) scaffolds seeded with either BMSC or ASC were implanted after incubation for cell attachment. After 6 weeks histological analysis of the implants showed that more calcified areas in the BMSC/ HA composites than in the ASC/ HA composites, in which no bone formation could be detected [152]. Other approaches investigated the osteogenic capability of scaffolds seeded with osteogenically induced BMSC and ASC, respectively, for bone repair in vivo. BMSC or ASC were seeded on mineralized collagen sponges, which were implanted into critical size defects of sheep tibia. Significant superiority of the BSMC-group versus the ASC-group could be detected between week 12 and 26: in the ASC-group there was no significant bone formation compared to d1 at any point of time, whereas all BMSC-implants displayed a bridging of the defect. In this study, addition of plasma to the scaffold, when it was seeded with ASC elicited a positive effect and reduced the inferiority of ASC in osteogenic differentiation compared to BMSC [157]. Ad-BMP4-transduced MSC from bone and from adipose tissue were seeded in fibrin gel matrix and then implanted into full thickness cranial defects in rabbits. In both group, similarly substantial levels of bone regeneration in radiographic examination and the histological analysis were observed [153].

Taken together, in general the surveyed studies, BMSC display a superiority concerning osteogenic differentiation. Some differences between the studies concerning the measure of difference in the osteogenic differentiation potential may be attributed to differences in the applied culture conditions and also to the kind of results on which the respective conclusion was based on. Particularly histological findings allow only semi - quantitative estimations.

Comparison of the Adipogenic Potential of ASC and BMSC

As described above, also the adipogenic potential of ASC as well as of BMSC has been confirmed in several studies. As for osteogenic differentiation, the majority of the investigations the directly comparing adipogenic differentiation of BMSC and ASC were only conducted in 2-dimensional (2D) environment (studies summarized in table 8). Only in one study different scaffold materials were used to investigate the adipogenic differentiation of ASC and BMSC [158]. Adipogenic differentiation can be visualized by staining the lipids with a hydrophobic dye, e.g., red oil O. Moreover key transcription factors in adipogenesis like peroxisome proliferator-acitvated receptor γ (PPARγ) and C/EFBα and late markers of adipogenesis such as lipoprotein lipase (LPL), involved in the lipid exchange programme indicate adipogenic differentiation.

In Vitro Studies

In adipogenic medium ASC and BMSC both display lipid rich vacuoles stained positive with red oil O [18, 21, 22, 140-142, 148] indicating that stem cells from both origins are capable of differentiating towards the adipogenic lineage.

Ref.	Outcome	Medium Composition	Culture Period	Passage #	
				ASC	BMSC
[22]	ASC > BMSC	DMEM, 10% FCS, 0.5 mM isobutyl methylxanthine, 1 µM dexamethasone, 10 µM insulin, 200 µM indomethacin, 1% antibiotic/ antimycotic	- 14 d	P2	P2
[18]	ASC > BMSC	DMEM-HG, 10% FCS, 0.01 mg/ml insulin, 1µM dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methyl xanthine, 100 U/ml penicillin, 100 µg/ml streptomycin	- 14 d	-	-
[140]	ASC > BMSC	DMEM, 10% FBS, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine, 10 µg/ml rh insulin, 100 U/ml penicillin/ streptomycin	- 14 d	P3 & P6	P3 & P6

[141]	ASC ≈ BMSC	IMDM (Iscove's modified Dulbecco's medium), 15% FCS, 1 µM dexamethasone, 5 µg/ml insulin, 60 µM indomethacin	- 21 d	P3-P5	P3-P5
[142]	ASC ≈ BMSC	DMEM/ F12, 10% FBS, 1 µM dexamethasone, 0.5 mM isobutyl-methylxanthine, 60 µM indomethacin for 3 days, then DMEM/ F12, 10% FCS	- 21 d	P2-P4	P2-P4
[21]	ASC > BMSC	α-MEM, 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin = control medium for 14 days, then control medium + 10^{-7} M dexamethasone, 0.5 mM isobutyl-1-methyl xanthine, 50 µM indomethacin	14 d pre-cultivation, 21 d adipogenic medium	P1	P1
[150]	ASC > BMSC	ITT-medium: DME/Ham's F12, 0.05 µM insulin, 0.2 nM 3,5,3'-triiodthyronine, 100 nM transferrin, 17 µM calcium panthotenate, 33 µM biotin, 100 nM dexamethasone	- 28 d	P2-P3	P2-P3
[151]	ASC > BMSC	DMEM-HG, 1.0 µM dexamethasone, 0.5 mM isobutylmethylxanthine, 10µM insulin, 200 µM indomethacin, 10% FBS, 1% penicillin G/ streptomycin, 5.0 mg/l amphotericin B	- 28 d	P3	P3
[148]	ASC ≈ BMSC	DMEM, 10% FBS, 1% antibiotic/ antimycotic, 0.5 mM isobutyl-methyl xanthine, 1 µM dexamethasone, 10 µM insulin, 200 µM indomethacin	7 d pre-cultivation, 21 d adipogenic medium	P4	P4
[158]	ASC ≈ BMSC	DMEM, 10 % FCS; 0.1 mM non-essential amino acids, 100 U/ml penicillin, 1000 U/ml streptomycin, 0.2% fungizone, 0.5 mM 3-isobutyl-1-methyl-xanthine, 1µM dexamethasone, 5 µg/ml insulin, 50 µM indomethacin	21 d cultivation on silk fibroin, collagen or PGA scaffolds in adipogenic medium	P2	P2

Table 8: Overview of the studies directly comparing the adipogenic differentiation capacity of ASC and BMSC in vitro.

The adipogenic conditions applied in various studies are given in table 9, which indicates great similarities concerning adipogenic media composition. Table 9 also provides a summary of the results comparing the differentiation potential of ASC and BMSC towards an adipogenic phenotype.

Although the capability of both BMSC and ASC to differentiate towards an adipogenic phenotype was shown, in some studies the ratio of red stained area to the total area of the cells

was significantly higher in ASC than in BMSC [140, 150], or the number of donors displaying red oil O positive colonies was higher for ASC than for BMSC [21]. Monaco et al showed that the extent of lipid accumulation per differentiated cell was lower in adipogenic BMSC cultures than in the respective ASC culture till day 21, but became equal on day 28. On day 28 however the percentage of BMSC, which differentiated to adipocytes, was lower in BMSC cultures compared to ASC cultures [151]. Other groups stated a similar rate of red oil O positive colonies [141, 148]. In one study, expression of PPARγ and LPL was significantly higher in induced ASC than in BMSC [140] suggesting a higher potential of ASC compared to BMSC towards an adipogenic phenotype, whereas in another study an increase in mRNA encoding for PPARγ and LPL was reported for both ASC and BMSC to a similar extent [142].

Activity of the lipogenic enzyme GPDH was found to be similarly increased in induced ASC and BMSC [148]. Liu et al. found genes involved in lipid metabolism like LPL, FABP4 and PLIN, were all also up-regulated. Among these genes, those related to energy reserve and cholesterol metabolism were expressed at higher levels in ASC compared to expression in BMSC after adipogenic differentiation [22] - Strong induction of LPL and adiponectin in adipogenic cultures of BMSC and ASC was also observed by Winter et al., whereby expression levels of LPL in ASC were significantly higher than in BMSC [18].

Mauney et al investigated the adipogenic differentiation of ASC and BMSC in a 3-dimensional environment. Four different porous 3D scaffold types were applied; aqueous or 1,1,1,3,3,3-hexafluoro-2-propanol based silk fibroin scaffolds, poly(glycolic acid) (PGA) and collagen scaffolds. After 21 days of adipogenic culture both BMSC- and ASC- cultures displayed substantial amounts of lipid accumulating cells, detected by red oil O staining. In comparison, uninduced cultures displayed a distinctly less intense staining. Both cell types also showed - to a similar extent - a significant up-regulation of mRNA levels for adipogenic markers, such as PPARγ [158].

According to these results, in general ASC were shown to be superior to BMSC with regard to differentiation towards an adipogenic phenotype in 2D culture.

In Vivo Studies

The in vivo adipogenic differentiation of BMSC and ASC in direct comparison so far has not been intensively investigated. In one study, after adipogenic differentiation in vitro for 28 days, different scaffolds seeded with BMSC or ASC were transplanted into bilateral muscle pouches in athymic rats (Table 9). Non-seeded scaffolds served as control constructs. After 4 weeks in vivo, in silk fibroin constructs seeded with BMSC and ASC lipid accumulating cells were detected, whereby BMSC and ASC showed similar lipid accumulation [158].

Ref.	Outcome	Animal Model	Implant Generation	in vivo culture period	Passage #	
					BMSC	ASC
[158]	ASC ≈ BMSC	muscle pouch model, rat (athymic, nude); implantation into bilateral muscle pouches within the rectus abdominus muscles	silk fibroin, collagen or PLA scaffolds seeded with BMSC or ASC, adipogenic culture for 21 days (c.f. Table 9)	28 d	P2	P2

Table 9: Overview of the study directly comparing the adipogenic differentiation capacity of ASC and BMSC in vivo.

Summary and Conclusions

Despite the proven potential to differentiate towards the chondrogenic, osteogenic and adipogenic lineage, distinct differences in the extent of differentiation could be observed, when directly comparing ASC and BMSC. ASC turned out to be superior compared to BMSC concerning differentiation towards an adipogenic phenotype, whereas BSMC displayed a greater osteogenic and chondrogenic potential.

In Table 4 to 9, the outcome of each comparative study is summarized. While for all three lineages a clear overall trend regarding the superior cell type was obvious, there were also studies in which both types were similar. The differences between the cited studies may be attributed to different expansion levels of the cells applied as well as the application of different culture conditions (including growth factor supplementation). Furthermore, both cell populations are heterogeneous thus comprising a mixture of cells which might differ in differentiation capability between studies [159].

While ASC apparently have a lower inherent potential to differentiate towards the chondrogenic and osteogenic lineage, applying ASC for cartilage and bone tissue engineering would still be of great interest due to their easier accessibility and the larger amount of harvestable cells. First approaches were undertaken to overcome the reduced chondrogenic capacity of ASC compared to BMSC. Modifications of standard differentiation protocols might be very promising. For this purpose, further research has to be undertaken to enlighten the differences of BMSC and ASC concerning signalling and receptor expression. This may enable to rationally enhance the differentiation potential of ASC, which would render them a promising alternative to BMSC.

Chapter 9

Summary & Conclusions

Ear-shaped Cartilage Constructs (Chapter 3-5)

Loss of auricular cartilage due to trauma, tumour resection or congenital defects remains a challenge in craniofacial surgery [1, 2]. For example, the incidence of microtia is 100 to 150 cases per year in Germany [39]. Surgical procedures, such as costal cartilage grafts for reconstruction of the external part of the ear applying autologous tissue represent the state of the art [40]. As the cosmetic results, however, are often unsatisfactory and donor site morbidity as well as multiple surgical procedures are associated with this approach, tissue engineering is considered to be a promising alternative.

Cartilage tissue engineering using scaffolds and chondrocytes has already been established for several scaffold materials [3, 14]. For auricular tissue engineering, the goal is to apply custom-made scaffolds for the individual patient and to seed them with autologous chondrocytes in order to render an aesthetic autologous implant. Rapid prototyping allows creation of even very complex, individual scaffolds for tissue engineering purposes [42-44].

In the presented work, two different materials were applied for the generation of ear-shaped cartilage constructs (Chapter 3-5). As part of the work within the research consortium ‘Regenerative Implants’ (Bavarian Research Foundation), the successful application of polycaprolactone-based polyurethane (PU) scaffolds [28] in combination with long-term stable fibrin gel [24] was transferred to the complex shape of the human external ear. Cartilage development within the construct was analyzed with spatial resolution (Chapter 3). Partial deficits in the generation of coherent cartilaginous tissue could be correlated to lacking interconnectivity of the porous scaffold structure. To optimize the ear-shaped PU scaffolds, in the complex shape of the external several material formulations as well as various treatment procedures were investigated with regard to their impact on pore size and interconnectivity (Chapter 4).

Oligo (poly (ethylene glycol)) fumarate (OPF), as a second scaffolding material, was also employed for the generation of ear-shaped cartilage constructs in combination with fibrin gel. As with the PU scaffolds, the generation of a very detailed and aesthetic ear-shaped constructs succeeded. Here, spatially resolved analysis demonstrated homogenous and coherent cartilaginous tissue in large parts of construct, indicated by intense and homogenous safranin-O staining as well as biochemical results displaying the production of substantial amounts of extracellular matrix (Chapter 5).

Towards Osteochondral Constructs (Chapter 6)

Osteochondral defects affect the articular cartilage, but also the underlying subchondral bone of the joint. To date, a widely accepted treatment procedure for osteochondral defects is still missing [61]. Tissue engineering of osteochondral grafts has been proposed for the generation of individually tailored constructs of defined dimension and shape [62].

In order to yield an optimized environment for the respective cell type, well established scaffold materials were chosen for the bone as well as for the cartilage component and combined in a single, bilayered scaffold. To engineer the cartilage-part the concept of seeding chondrocytes suspended in long-term stable fibrin gels in PU scaffolds [24] was chosen. For the bone part to be seeded with BMSC, a hydroxyapatite-based composite scaffold was prepared by dispense plotting, a specific rapid prototyping method. The overall goal was to establish a system applicable for the co-culture of cartilage and bone applying the materials which were already successfully applied in the project ‘Regenerative Implants’. Here a seeding procedure was established which facilitated a selective seeding of the part intended for cartilage with chondrocytes in fibrin gel. The selectivity of the seeding procedure is a prerequisite for the success of the concept, as otherwise the composite part would not be left free for subsequent seeding with BMSC . First short-term co-culture of BMSC and bovine

chondrocytes evidenced the effectiveness of the method to selectively seed the respective part of the bilayered scaffold. Having established this system, a powerful tool for further research concerning optimization of co-culture conditions and the time range for pre-cultivation before transplantation is available.

Synergistic Effects of GDF-5 and Insulin – Aspects of (Re-) Differentiation of Chondrocytes and ASC (Chapter 7-8)

One of the major challenges in cartilage engineering is constituted by the limited number of harvestable autologous chondrocytes. Frequently used expansion in 2-dimensional (2D) environment, however, is often accompanied by rapid dedifferentiation, associated with, for example, predominant expression of type I collagen [60] rendering the cell less suitable for tissue engineering purposes [5, 74]. Growth factors have been applied either already during expansion of the chondrocytes [75–78] or during subsequent 3-dimensional (3D) culture [75, 78–80] to improve the quality of the generated tissue.

Studies conducted in the group have previously demonstrated synergistic redifferentiating effects of the protein growth and differentiation factor-5 (GDF-5) and insulin particularly on expanded bovine chondrocytes on PGA scaffolds [88, 89]. Here, further studies were conducted in different 3D culture systems (fibrin gel, pellet). Besides juvenile bovine chondrocytes, adult human chondrocytes were also applied. The synergistic effect of GDF-5 and insulin could also be proven in fibrin gel and pellet culture, thus, the impact can be considered to be independent of the chosen 3D culture system. The application of GDF-5 alone or in combination with insulin during the expansion in 2D did not yield an enhancement of cartilage development in subsequent 3D culture.

The study conducted with expanded adult human chondrocytes revealed synergistic effects of GDF-5 and insulin very similar to those seen for bovine juvenile cells. Redifferentiation was

indicated by intense safranin-O staining and tremendous up-regulation of the cartilage marker type II collagen as seen in the immunohistochemistry. Furthermore, this up-regulation was also detected on mRNA level. These results imply the possible clinical relevance of applying the combination of GDF-5 and insulin for redifferentiation of expanded chondrocytes and thus dedifferentiated autologous cells and represent an option to overcome the problem of the limited availability of harvestable autologous cells (Chapter 7).

As an alternative cell source, ASC may be used for cartilage tissue engineering due to their multilineage potential [25, 26]. Here, a first preliminary experiment was conducted investigating the effects of GDF-5 and insulin on ASC were tested. Interestingly the combination of GDF-5 and insulin did not exert synergistic differentiating effects on human ASC in contrast to expanded chondrocytes (Chapter 7).

ASC can be obtained in large quantities and under local anaesthesia. In contrast, procurement of BMSC is limited and may be associated with a painful procedure. The question, which represents the preferred cell type for use in specific applications, is still controversially discussed by researchers. Reviewing the studies directly comparing the differentiation capacity of ASC and BMSC towards a chondrogenic, osteogenic and adipogenic phenotype was supposed to enable more rational conclusions (Chapter 8). Despite the proven multilineage potential of both cell types, distinct differences were observed. Whereas ASC appeared to have a have a higher potential for adipogenic differentiation, they generally exhibited a lower potential to differentiate towards cartilage and bone. Nevertheless, due to the advantages concerning availability and procurement ASC still are an interesting cell source for cartilage tissue engineering. First approaches were undertaken to enlighten the reasons for the inferior chondrogenic capacity and to increase chondrogenic differentiation of ASC [110, 109]. Further research has to be conducted to elucidate the differences between BMCS and ASC, also concerning signalling and gene expression, in order to modify standard differentiation protocols to fully exploit the potential of ACS also for cartilage engineering.

References

[1] Rotter, N., Haisch, A. and Bucheler, M. (2005) Cartilage and bone tissue engineering for reconstructive head and neck surgery. European Archives of Otorhinolaryngology 262, 539–545.

[2] Renner, G. and Lane, R. (2004) Auricular reconstruction: An update. Current Opinion in Otolaryngology & Head and Neck Surgery 12, 277–280.

[3] Temenoff, J.S. and Mikos, A.G. (2000) Review: Tissue engineering for regeneration of articular cartilage. Biomaterials 21, 431–440.

[4] Poole, A.R., Kojima, T., Yasuda, T., Mwale, F., Kobayashi, M. and Laverty, S. (2001) Composition and structure of articular cartilage: a template for tissue repair. Clinical Orthopaedics and Related Research 391, S26-S33.

[5] Goldring, M.B. (2006) Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. Best Practice & Research, Clinical Rheumatology 20, 1003–1025.

[6] Hall A C, Horwitz E R and Wilkins R J (1996) The cellular physiology of articular cartilage. Experimental Physiology 81, 535-45.

[7] Aigner, T., Sachse, A., Gebhard, P.M. and Roach, H.I. (2006) Osteoarthritis: Pathobiology - targets and ways for therapeutic intervention. Advanced Drug Delivery Reviews 58, 128–149.

[8] Archer, C.W. and Francis-West, P. (2003) The chondrocyte. The International Journal of Biochemistry & Cell Biology 35, 401–404.

[9] Chung, C. and Burdick, J.A. (2008) Engineering cartilage tissue. Advanced Drug Delivery Reviews 60, 243–262.

[10] Vinatier, C., Magne, D., Moreau, A., Gauthier, O., Malard, O., Vignes-Colombeix, C., Daculsi, G., Weiss, P. and Guicheux, J. (2006) Engineering cartilage with human nasal chondrocytes and a silanized hydroxypropyl methylcellulose hydrogel. Journal of Biomedical Materials Research, Part A 80A, 66–74.

[11] Darling, E.M. and Athanasiou, K.A. (2005) Rapid phenotypic changes in passaged articular chondrocyte subpopulations. Journal of Orthopaedic Research 23, 425–432.

[12] Vinatier, C., Mrugala, D., Jorgensen, C., Guicheux, J. and Noël, D. (2009) Cartilage engineering: a crucial combination of cells, biomaterials and biofactors. Trends in Biotechnology 27, 307–314.

[13] Nesic, D., Whiteside, R., Brittberg, M., Wendt, D., Martin, I. and Mainil-Varlet, P. (2006) Cartilage tissue engineering for degenerative joint disease. Advanced Drug Delivery Reviews 58, 300–322.

[14] Hutmacher, D.W. (2000) Scaffolds in tissue engineering bone and cartilage. Biomaterials 21, 2529–2543.

[15] Lavik, E. and Langer, R. (2004) Tissue engineering: current state and perspectives. Applied Microbiology and Biotechnology 65, 1–8.

[16] Csaki, C., Schneider, P.R.A. and Shakibaei, M. (2008) Mesenchymal stem cells as a potential pool for cartilage tissue engineering. Annals of Anatomy - Anatomischer Anzeiger 190, 395–412.

[17] Pelttari, K., Steck, E. and Richter, W. (2008) The use of mesenchymal stem cells for chondrogenesis. Injury 39, 58–65.

[18] Winter, A., Breit, S., Parsch, D., Benz, K., Steck, E., Hauner, H., Weber, R.M., Ewerbeck, V. and Richter, W. (2003) Cartilage-like gene expression in differentiated human stem cell spheroids: A comparison of bone marrow-derived and adipose tissue-derived stromal cells. Arthritis And Rheumatism 48, 418–429.

[19] Afizah, H., Yang, Z., Hui, J.H.P., Ouyang, H.-W. and Lee, E.-H. (2007) A Comparison between the chondrogenic potential of human bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs) taken from the same donors. Tissue Engineering 13, 659–666.

[20] Indrawattana, N., Chen, G., Tadokoro, M., Shann, L.H., Ohgushi, H., Tateishi, T., Tanaka, J. and Bunyaratvej, A. (2004) Growth factor combination for chondrogenic induction from human mesenchymal stem cell. Biochemical & Biophysical Research Communications 320, 914–919.

[21] Sakaguchi, Y., Sekiya, I., Yagishita, K. and Muneta, T. (2005) Comparison of human stem cells derived from various mesenchymal tissues: Superiority of synovium as a cell Liu, T.M., Martina, M., Hutmacher, D.W., Hui, J.H.P., Lee, E.H. and Lim, B. (2007) Identification of Common Pathways Mediating Differentiation of Bone Marrow- and Adipose Tissue-Derived Human Mesenchymal Stem Cells into Three Mesenchymal Lineages. Stem Cells 25, 750–760.

[23] Eyrich, D., Brandl, F., Appel, B., Wiese, H., Maier, G., Wenzel, M., Staudenmaier, R., Goepferich, A. and Blunk, T. (2007) Long-term stable fibrin gels for cartilage engineering. Biomaterials 28, 55–65.

[24] Eyrich, D., Wiese,H., Maier, G., Skodacek.D., Appel, B., Sarhan, H., Tessmar, J., Staudenmaier, R., Wenzel, M.M., Goepferich, A., Blunk, T. (2007) In vitro and in vivo cartilage engineering using a combination of chondrocyte-seeded long-term stable fibrin gels and polycaprolactone-based polyurethane scaffolds. Tissue Engineering 13, 2207–2218.

[25] Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P. and Hedrick, M.H. (2001) Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies. Tissue Engineering 7, 211–228.

[26] Zuk, P.A., Zhu, M., Ashjian, P., Ugarte, D.A. de, Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P. and Hedrick, M.H. (2002) Human adipose tissue is a source of multipotent stem cells. Molecular Biology of the Cell 13, 4279–4295.

[27] Kellner, K., Schulz, M.B., Gopferich, A. and Blunk, T. (2001) Insulin in tissue engineering of cartilage: a potential model system for growth factor application. Journal of Drug Targeting 9, 439–448.

[28] Wiese, H. and Maier, G. (2006) Open-pored polyurethane foam without skin formation, formulation for the production thereof and use thereof as a carrier material for cell and tissue cultures or medicaments, patent (WO 2006032501 A1). PCT Int. Appl. 37 p.

[29] Henke, M., Brandl, F., Goepferich, A.M. and Tessmar, J.K. (2010) Size-dependent release of fluorescent macromolecules and nanoparticles from radically cross-linked hydrogels. European Journal of Pharmaceutics and Biopharmaceutics 74, 184–192.

[30] Joly-Duhamel, C., Hellio, D., Ajdari, A. and Djabourov, M. (2002) All gelatin networks: 2. The master curve for elasticity. Langmuir 18, 7158–7166.

[31] Kim, Y.J., Sah, R.L., Doong, J.Y. and Grodzinsky, A.J. (1988) Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 174, 168–176.

[32] Farndale, R.W., Buttle, D.J. and Barrett, A.J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochimica et Biophysica Acta (BBA) - General Subjects 883, 173–177.

[33] Taylor, K. and Jeffree, G. (1969) A new basic metachromatic dye, 1,9-Dimethyl Methylene Blue. The Histochemical Journal 1, 199–204.

[34] Woessner, J.F. (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of Biochemistry and Biophysics 93, 440–447.

[35] Hollander, A.P., Heathfield, T.F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C. and Poole, A.R. (1994) Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. The Journal of Clinical Investigation 93, 1722–1732.

[36] Martin, I., Obradovic, B., Freed, L.E. and Vunjak-Novakovic, G. (1999) Method for Quantitative analysis of glycosaminoglycan distribution in cultured natural and engineered cartilage. Annals of Biomedical Engineering 27, 656–662.

[37] Ciorba, A. and Martini, A. (2006) Tissue engineering and cartilage regeneration for auricular reconstruction. International Journal of Pediatric Otorhinolaryngology 70, 1507–1515.

[38] Beahm, E.K. and Walton, R.L. (2002) Auricular reconstruction for microtia: part I. Anatomy, embryology, and clinical evaluation. Plastic and Reconstructive Surgery 109, 2473–2482.

[39] Katzbach, R., Klaiber, S., Nitsch, S., Steffen, A. and Frenzel, H. (2006) Auricular reconstruction for severe microtia. Schedule of treatment, operative strategy, and modifications. HNO 54, 493–511.

[40] Walton, R.L. and Beahm, E.K. (2002) Auricular reconstruction for microtia: Part II. Surgical techniques. Plastic and Reconstructive Surgery 110, 234–249.

[41] Shieh, S.J., Terada, S. and Vacanti, J.P. (2004) Tissue engineering auricular reconstruction: in vitro and in vivo studies. Biomaterials 25, 1545–1557.

[42] Landers, R., Pfister, A., Hübner, U., John, H., Schmelzeisen, R. and Mülhaupt, R. (2002) Fabrication of soft tissue engineering scaffolds by means of rapid prototyping techniques. Journal of Materials Science 37, 3107–3116.

[43] Dean, D., Min, K.-J. and Bond, A. (2003) Computer aided design of large-format prefabricated cranial plates. The Journal of Craniofacial Surgery 14, 819-832.

[44] Hutmacher, D.W., Sittinger, M. and Risbud, M.V. (2004) Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems. Trends in Biotechnology 22, 354–362.

[45] Grad, S., Kupcsik, L., Gorna, K., Gogolewski, S. and Alini, M. (2003) The use of biodegradable polyurethane scaffolds for cartilage tissue engineering: potential and limitations. Biomaterials 24, 5163–5171.

[46] Eyrich Daniela, Gopferich Achim and Blunk Torsten (2006) Fibrin in tissue engineering. Advances in Experimental Medicine and Biology 585, 379-392.

[47] Sharma, B., Williams, C.G., Kim, T.K., Sun, D., Malik, A., Khan, M., Leong, K. and Elisseeff, J.H. (2007) Designing Zonal Organization into Tissue-Engineered Cartilage. Tissue Engineering 13, 405–414.

[48] Xu, J.W., Johnson, T.S., Motarjem, P.M., Peretti, G.M., Randolph, M.A. and Yaremchuk, M.J. (2005) Tissue-engineered flexible ear-shaped cartilage. Plastic and Reconstructive Surgery 115, 1633–1641.

[49] Haisch, A., Klaring, S., Groger, A., Gebert, C. and Sittinger, M.A. (2002) A tissue-engineering model for the manufacture of auricular-shaped cartilage implants. European Archives of Otorhinolaryngology 259, 316-321.

[50] Liu, Y., Zhang, L., Zhou, G., Li, Q., Liu, W., Yu, Z., Luo, X., Jiang, T., Zhang, W. and Cao, Y. (2010) In vitro engineering of human ear-shaped cartilage assisted with CAD/CAM technology. Biomaterials 31, 2176–2183.

[51] Grad, S., Gorna, K., Gogolewski, S. and Alini, M. (2004) Scaffolds for cartilage tissue engineering: effect of pore size. European Cells and Materials 7, 3-3.

[52] Woodfield, T.B.F., Malda, J., Wijn, J. de, Peters, F., Riesle, J. and van Blitterswijk, C.A. (2004) Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique. Biomaterials 25, 4149–4161.

[53] Holland, T.A., Bodde, E.W.H., Cuijpers, V.M.J.I., Baggett, L.S., Tabata, Y., Mikos, A.G. and Jansen, J.A. (2007) Degradable hydrogel scaffolds for in vivo delivery of single and dual growth factors in cartilage repair. Osteoarthritis and Cartilage 15, 187–197.

[54] Holland, T.A., Tabata, Y. and Mikos, A.G. (2005) Dual growth factor delivery from degradable oligo (poly (ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering. Journal of Controlled Release 101, 111–125.

[55] Holland, T.A., Bodde, E.W.H., Baggett, L.S., Tabata, Y., Mikos, A.G. and Jansen, J.A. (2005) Osteochondral repair in the rabbit model utilizing bilayered, degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds. Journal of Biomedical Materials Research Part A 75A, 156–167.

[56] Park, H., Guo, X., Temenoff, J.S., Tabata, Y., Caplan, A.I., Kasper, F.K. and Mikos, A.G. (2009) Effect of swelling ratio of injectable hydrogel composites on chondrogenic differentiation of encapsulated rabbit marrow mesenchymal stem cells in vitro. Biomacromolecules 10, 541–546.

[57] Dadsetan, M., Szatkowski, J.P., Yaszemski, M.J. and Lu, L. (2007) Characterization of photo-cross-linked oligo [poly (ethylene glycol) fumarate] hydrogels for cartilage tissue engineering. Biomacromolecules 8, 1702–1709.

[58] Shin, H., Temenoff, J.S. and Mikos, A.G. (2003) In vitro cytotoxicity of unsaturated oligo [poly (ethylene glycol) fumarate] macromers and their cross-linked hydrogels. Biomacromolecules 4, 552–560.

[59] Shin, H., Quinten Ruhé, P., Mikos, A.G. and Jansen, J.A. (2003) In vivo bone and soft tissue response to injectable, biodegradable oligo (poly (ethylene glycol) fumarate) hydrogels. Biomaterials 24, 3201–3211.

[60] Benya, P.D. and Shaffer, J.D. (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30, 215–224.

[61] Swieszkowski, W., Tuan, B.H.S., Kurzydlowski, K.J. and Hutmacher, D.W. (2007) Repair and regeneration of osteochondral defects in the articular joints. Biomolecular Engineering 24, 489–495.

[62] Martin, I., Miot, S., Barbero, A., Jakob, M. and Wendt, D. (2007) Osteochondral tissue engineering. Journal of Biomechanics 40, 750–765.

[63] van Blitterswijk, C.A., Hendriks, J. and Riesle, J. (2007) Co-culture in cartilage tissue engineering. Journal of Tissue Engineering and Regenerative Medicine 1, 170–178.

[64] Haasper, C., Zeichen, J., Meister, R., Krettek, C. and Jagodzinski, M. (2008) Tissue engineering of osteochondral constructs in vitro using bioreactors. Injury 39, 66–76.

[65] Dirk Schaefer, Ivan Martin, G. Jundt, Joachim Seidel, Michael Heberer, Alan Grodzinsky, Ingrid Bergin, Gordana Vunjak-Novakovic and Lisa E. Freed (2002) Tissue-engineered composites for the repair of large osteochondral defects. Arthritis And Rheumatism 46, 2524–2534.

[66] Gao, J., Dennis, J.E., Solchaga, L.A., Goldberg, V.M. and Caplan, A.I. (2002) Repair of osteochondral defect with tissue-engineered two-phase composite material of injectable calcium phosphate and hyaluronan sponge. Tissue Engineering 8, 827–837.

[67] Shao, X., Goh, J.C.H., Hutmacher, D.W., Lee, E.H. and Zigang, G. (2006) Repair of large articular osteochondral defects using hybrid scaffolds and bone marrow-derived mesenchymal stem cells in a rabbit model. Tissue Engineering 12, 1539–1551.

[68] O'Shea, T.M. and Miao, X. (2008) Bilayered scaffolds for osteochondral tissue engineering. Tissue Engineering Part B 14, 447–464.

[69] Detsch, R., Uhl, F., Deisinger, U. and Ziegler, G. (2008) 3D-Cultivation of bone marrow stromal cells on hydroxyapatite scaffolds fabricated by dispense-plotting and negative mould technique. Journal of Materials Science: Materials in Medicine 19, 1491–1496.

[70] Schek, R.M., Taboas, J.M., Segvich, S.J., Hollister, S.J. and Krebsbach, P.H. (2004) Engineered osteochondral grafts using biphasic composite solid free-form fabricated scaffolds. Tissue Engineering 10, 1376–1385.

[71] Haasper, C., Colditz, M., Kirsch, L., Tschernig, T., Viering, J., Graubner, G., Runtemund, A., Zeichen, J., Meller, R., Glasmacher, B., Windhagen, H., Krettek, C., Hurschler, C. and Jagodzinski, M. (2008) A system for engineering an osteochondral construct in the shape of an articular surface: Preliminary results. Annals of Anatomy - Anatomischer Anzeiger 190, 351–359.

[72] P. Angele, J. U. Yoo, C. Smith, J. Mansour, K. J. Jepsen, M. Nerlich and B. Johnstone (2003) Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro. Journal of Orthopaedic Research 21, 451-457.

[73] Haasper, C., Zeichen, J., Meister, R., Krettek, C. and Jagodzinski, M. (2008) Tissue engineering of osteochondral constructs in vitro using bioreactors. Injury 39, 66–76.

[74] Schnabel, M., Marlovits, S., Eckhoff, G., Fichtel, I., Gotzen, L., Vecsei, V. and Schlegel, J. (2002) Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis and Cartilage 10, 62–70.

[75] Jakob, M., Demarteau, O., Schafer, D., Hintermann, B., Dick, W., Heberer, M. and Martin, I. (2001) Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. Journal of Cellular Biochemistry 81, 368-377.

[76] Mandl, E.W., Jahr, H., Koevoet, J.L.M., van Leeuwen, J.P.T.M., Weinans, H., Verhaar, J.A.N. and van Osch, G.J.V.M. (2004) Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture. Matrix Biology 23, 231–241.

[77] Martin, I., Vunjak-Novakovic, G., Yang, J., Langer, R. and Freed, L.E. (1999) Mammalian chondrocytes expanded in the presence of fibroblast growth factor-2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. Experimental Cell Research 253, 681–688.

[78] Martin, I., Suetterlin, R., Baschong, W., Heberer, M., Vunjak-Novakovic, G. and Freed, L.E. (2001) Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation. Journal of Cellular Biochemistry 83, 121–128.

[79] van Osch, G.J., Marijnissen, W.J., van, d. and Verwoerd-Verhoef, H.L. (2001) The potency of culture-expanded nasal septum chondrocytes for tissue engineering of cartilage. American Journal of Rhinology 15, 187–192.

[80] Kino-oka, M., Maeda, Y., Ota, Y., Yashiki, S., Sugawara, K., Yamamoto, T. and Taya, M. (2005) Process design of chondrocyte cultures with monolayer growth for cell expansion and subsequent three-dimensional growth for production of cultured cartilage. Journal of Bioscience and Bioengineering 100, 67–76.

[81] Lories, R.J.U. and Luyten, F.P. (2005) Bone morphogenetic protein signaling in joint homeostasis and disease. Cytokine & Growth Factor Reviews 16, 287–298.

[82] Storm, E.E., Huynh, T.V., Copeland, N.G., Jenkins, N.A., Kingsley, D.M. and Lee, S.J. (1994) Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature 368, 639–643.

[83] Tsumaki, N., Nakase, T., Miyaji, T., Kakiuchi, M., Kimura, T., Ochi, T. and Yoshikawa, H. (2002) Bone morphogenetic protein signals are required for cartilage formation and

differently regulate joint development during skeletogenesis. Journal of Bone and Mineral Research 17, 898–906.

[84] Francis-West, P.H., Abdelfattah, A., Chen, P., Allen, C., Parish, J., Ladher, R., Allen, S., MacPherson, S., Luyten, F.P. and Archer et al. (1999) Mechanisms of GDF-5 action during skeletal development. Development (Cambridge, England) 126, 1305–1315.

[85] Chang, S.C., Hoang, B., Thomas, J.T., Vukicevic, S., Luyten, F.P., Ryba, N.J.P., Kozak, C.A., Reddi, A.H. and Moos, M., JR. (1994) Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. Journal of Biological Chemistry 269, 28227–28234.

[86] Erlacher, L., Ng, C.K., Ullrich, R., Krieger, S. and Luyten, F.P. (1998) Presence of cartilage-derived morphogenetic proteins in articular cartilage and enhancement of matrix replacement in vitro. Arthritis And Rheumatism 41, 263–273.

[87] Appel, B., Maschke, A., Weiser, B., Sarhan, H., Englert, C., Angele, P., Blunk, T. and Goepferich, A. (2006) Lipidic implants for controlled release of bioactive insulin: Effects on cartilage engineered in vitro. International Journal of Pharmaceutics 314, 170–178.

[88] Appel, B., Baumer, J., Eyrich, D., Sarhan, H., Toso, S., Englert, C., Skodacek, D., Ratzinger, S., Grässel, S., Goepferich, A. and Blunk, T. (2009) Synergistic effects of growth and differentiation factor-5 (GDF-5) and insulin on expanded chondrocytes in a 3-D environment. Osteoarthritis and Cartilage 17, 1503–1512.

[89] Appel, B. (September 2006) Cartilage tissue engineering. Controlled release of growth factors effects of GDF-5, sexual steroid hormones and oxygen. Dissertation. Regensburg.

[90] Tare, R.S., Howard, D., Pound, J.C., Roach, H.I. and Oreffo, R.O.C. (2005) Tissue engineering strategies for cartilage generation - Micromass and three dimensional cultures using human chondrocytes and a continuous cell line. Biochemical and Biophysical Research Communications 333, 609–621.

[91] Tallheden, T., Karlsson, C., Brunner, A., van der Lee, J., Hagg, R., Tommasini, R. and Lindahl, A. (2004) Gene expression during redifferentiation of human articular chondrocytes. Osteoarthritis and Cartilage 12, 525–535.

[92] Takahara, M., Harada, M., Guan, D., Otsuji, M., Naruse, T., Takagi, M. and Ogino, T. (2004) Developmental failure of phalanges in the absence of growth/differentiation factor 5. Bone 35, 1069–1076.

[93] Hotten, G.C., Matsumoto, T., Kimura, M., Bechtold, R.F., Kron, R., Ohara, T., Tanaka, H., Satoh, Y., Okazaki, M. and Shirai et, al. (1996) Recombinant human growth/differentiation factor 5 stimulates mesenchyme aggregation and chondrogenesis responsible for the skeletal development of limbs. Growth Factors 13, 65–74.

[94] Chhabra, A.M.M., Zijerdi, D.M.D., Zhang, J.M.D., Kline, A.M.D., Balian, G.P. and Hurwitz, S.M.D. (2005) BMP-14 deficiency inhibits long bone fracture healing: A biochemical, histologic, and radiographic assessment. Journal of Orthopaedic Trauma 19, 629–634.

[95] Gruber, R., Mayer, C., Bobacz, K., Krauth, M.-T., Graninger, W., Luyten, F.P. and Erlacher, L. (2001) Effects of cartilage-derived morphogenetic proteins and osteogenic protein-1 on osteochondrogenic differentiation of periosteum-derived cells. Endocrinology 142, 2087–2094.

[96] Nikolaou, V.S. and Tsiridis, E. (2007) (i) Pathways and signalling molecules. Current Orthopaedics 21, 249–257.

[97] Simank, H.G., Sergi, C., Jung, M., Adolf, S., Eckhardt, C., Ehemann, V., Ries, R., Lill, C. and Richter, W. (2004) Effects of local application of growth and differentiation factor-5 (GDF-5) in a full-thickness cartilage defect model. Growth Factors 22, 35–43.

[98] Rickert, M., Jung, M., Adiyaman, M., Richter, W. and Simank, H.G. (2001) A growth and differentiation factor-5 (GDF-5)-coated suture stimulates tendon healing in an Achilles tendon model in rats. Growth Factors 19, 115–126.

[99] Chhabra, A., Tsou, D., Clark, R.T., Gaschen, V., Hunziker, E.B. and Mikic, B. (2003) GDF-5 deficiency in mice delays Achilles tendon healing. Journal of Orthopaedic Research: 21, 826–835.

[100] Li, X., Leo, B.M., Beck, G., Balian, G. and Anderson, G.D. (2004) Collagen and proteoglycan abnormalities in the GDF-5-deficient mice and molecular changes when treating disk cells with recombinant growth factor. Spine 29, 2229–2234.

[101] Heidaran, M.A., Daverman, R., Thompson, A., Ng, C.K., Pohl, J., Poser, J.W. and Spiro, R.C. (2000) Extracellular matrix modulation of rhGDF-5-Induced cellular differentiation. The Journal of Regenerative Medicine 1, 121–135.

[102] Bai, X., Xiao, Z., Pan, Y., Hu, J., Pohl, J., Wen, J. and Li, L. (2004) Cartilage-derived morphogenetic protein-1 promotes the differentiation of mesenchymal stem cells into chondrocytes. Biochemical and Biophysical Research Communications 325, 453–460.

[103] Mehlhorn, A.T., Zwingmann, J., Finkenzeller, G., Niemeyer, P., Dauner, M., Stark, B., Südkamp, N.P. and Schmal, H. (2009) Chondrogenesis of adipose-derived adult stem cells in a poly-lactide-co-glycolide scaffold. Tissue Engineering Part A 15, 1159.

[104] Awad, H.A., Quinn Wickham, M., Leddy, H.A., Gimble, J.M. and Guilak, F. (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 25, 3211–3222.

[105] Dragoo J L, Samimi B, Zhu M, Hame S L, Thomas B J, Lieberman J R, Hedrick M H and Benhaim P (2003) Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads. The Journal of Bone and Joint Surgery (Br) 85B, 740-747.

[106] Erickson, G.R., Gimble, J.M., Franklin, D.M., Rice, H.E., Awad, H. and Guilak, F. (2002) Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochemical and Biophysical Research Communications 290, 763–769.

[107] Jung, Y., Chung, Y.-I., Kim, S.H., Tae, G., Kim, Y.H., Rhie, J.W., Kim, S.-H. and Kim, S.H. (2009) In situ chondrogenic differentiation of human adipose tissue-derived stem cells in a TGF-β1 loaded fibrin-poly(lactide-caprolactone) nanoparticulate complex. Biomaterials 30, 4657–4664.

[108] Feng, G., Wan, Y., Balian, G., Laurencin, C.T. and Li, X. (2008) Adenovirus-mediated expression of growth and differentiation factor-5 promotes chondrogenesis of adipose stem cells. Growth Factors 26, 132–142.

[109] Hye-Joung Kim and Gun-Il Im (2009) Chondrogenic differentiation of adipose tissue-derived mesenchymal stem cells: Greater doses of growth factor are necessary. Journal of Orthopaedic Research 27, 612–619.

[110] Hennig, T., Lorenz, H., Thiel, A., Goetzke, K., Dickhut, A., Geiger, F. and Richter, W. (2007) Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGF-β receptor and BMP profile and is overcome by BMP-6. Journal of Cellular Physiology. 211, 682–691.

[111] Nishito, H., Ichijo, H., Kimura, M., Matsumoto, T., Makishima, F., Yamaguchi, A., Yamashita, H. and Enomoto, S.M.K. (1996) Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. The Journal of Biological Chemistry 271, 21345–21352.

[112] Nickel, J., Kotzsch, A., Sebald, W. and Mueller, T.D. (2005) A single residue of GDF-5 defines binding specificity to BMP receptor IB. Journal of Molecular Biology 349, 933–947.

[113] Miyazono, K., Maeda, S. and Imamura, T. (2005) BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine & Growth Factor Reviews 16, 251–263.

[114] Merino, R., Macias, D., Gañan, Y., Economides, A.N., Wang, X., Wu, Q., Stahl, N., Sampath, K.T., Varona, P. and Hurle, J.M. (1999) Expression and function of Gdf-5during digit skeletogenesis in the embryonic chick leg bud. Developmental Biology 206, 33–45.

[115] Phornphutkul, C., Wu, K.-Y. and Gruppuso, P.A. (2006) The role of insulin in chondrogenesis. Molecular and Cellular Endocrinology 249, 107–115.

[116] Watanabe, H., Caestecker, M.P. de and Yamada, Y. (2001) Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta -induced aggrecan gene expression in chondrogenic ATDC5 cells. Journal of Biological Chemistry 276, 14466–14473.

[117] Berry, L., Grant, M.E., McClure, J. and Rooney, P. (1992) Bone-marrow-derived chondrogenesis in vitro. Journal of Cell Science 101, 333–342.

[118] Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M. and Yoo, J.U. (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Experimental Cell Research 238, 265–272.

[119] Mastrogiacomo, M., Cancedda, R. and Quarto, R. (2001) Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells. Osteoarthritis and Cartilage 9, S36-S40.

[120] Arnold I. Caplan (1991) Mesenchymal stem cells. Journal of Orthopaedic Research 9, 641–650.

[121] Marolt, D., Augst, A., Freed, L.E., Vepari, C., Fajardo, R., Patel, N., Gray, M., Farley, M., Kaplan, D. and Vunjak-Novakovic, G. (2006) Bone and cartilage tissue constructs grown using human bone marrow stromal cells, silk scaffolds and rotating bioreactors. Biomaterials 27, 6138–6149.

[122] Barry, F., Boynton, R.E., Liu, B. and Murphy, J.M. (2001) Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Experimental Cell Research 268, 189–200.

[123] Neuhuber, B., Gallo, G., Howard, L., Kostura, L., MacKay, A. and Fischer, I. (2004) Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: Disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. Journal of Neuroscience Research 77, 192–204.

[124] Yoo, J.U., Barthel, T. S., Nishimura, K., Solchaga, L., Caplan, A.I., Goldberg, V.M. and Johnstone, B. (1998) The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. The Journal of Bone and Joint Surgery 80, 1745-57.

[125] Coleman, R.M., Case, N.D. and Guldberg, R.E. (2007) Hydrogel effects on bone marrow stromal cell response to chondrogenic growth factors. Biomaterials 28, 2077–2086.

[126] Mauney, J. and Volloch, V. (2009) Progression of human bone marrow stromal cells into both osteogenic and adipogenic lineages is differentially regulated by structural conformation of collagen I matrix via distinct signaling pathways. Matrix Biology 28, 239–250.

[127] Knoch, F. von, Jaquiery, C., Kowalsky, M., Schaeren, S., Alabre, C., Martin, I., Rubash, H.E. and Shanbhag, A.S. (2005) Effects of bisphosphonates on proliferation and osteoblast differentiation of human bone marrow stromal cells. Biomaterials 26, 6941–6949.

[128] Lijin Zou, Xuenong Zou, Li Chen, Haisheng Li, Tina Mygind, Moustapha Kassem and Cody Bünger (2008) Multilineage differentiation of porcine bone marrow stromal cells associated with specific gene expression pattern. Journal of Orthopaedic Research 26, 56–64.

[129] Fu, L., Tang, T., Miao, Y., Zhang, S., Qu, Z. and Dai, K. (2008) Stimulation of osteogenic differentiation and inhibition of adipogenic differentiation in bone marrow stromal cells by alendronate via ERK and JNK activation. Bone 43, 40–47.

[130] Lee, J.-H. and Kemp, D.M. (2006) Human adipose-derived stem cells display myogenic potential and perturbed function in hypoxic conditions. Biochemical and Biophysical Research Communications 341, 882–888.

[131] Ahn, H.H., Kim, K.S., Lee, J.H., Lee, J.Y., Kim, B.S., Lee, I.W., Chun, H.J., Kim, J.H., Lee, H.B. and Kim, M.S. (2009) In vivo osteogenic differentiation of human adipose-derived sem cells in an injectable in situ-forming gel scaffold. Tissue Engineering Part A 15, 1821-1832.

[132] de Girolamo, L., Sartori, M.F., Albisetti, W. and Brini, A.T. (2007) Osteogenic differentiation of human adipose-derived stem cells: comparison of two different inductive media. Journal of Tissue Engineering and Regenerative Medicine 1, 154–157.

[133 Dhar, S., Yoon, E.S., Kachgal, S. and Evans, G.R.D. (2007) Long-term maintenance of neuronally differentiated human adipose tissue-derived stem cells. Tissue Engineering 13, 2625.

[134] Safford, K.M., Hicok, K.C., Safford, S.D., Halvorsen, Y.-D.C., Wilkison, W.O., Gimble, J.M. and Rice, H.E. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochemical and Biophysical Research Communications 294, 371–379.

[135] Gimble, J.M., Katz, A.J. and Bunnell, B.A. (2007) Adipose-Derived Stem Cells for Regenerative Medicine. Circulation Research 100, 1249–1260.

[136] Im, G., II, Shin, Y.W. and Lee, K.B. (2005) Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis and Cartilage 13, 845–853.

[137] Koga, H., Muneta, T., Nagase, T., Nimura, A., Ju, Y.-J., Mochizuki, T. and Sekiya, I. (2008) Comparison of mesenchymal tissues-derived stem cells for in vivo chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit. Cell and Tissue Research 333, 207–215

[138] Gimble, J.M., Katz, A.J. and Bunnell, B.A. (2007) Adipose-derived sem cells for regenerative medicine. Circulation Research 100, 1249–1260.

[139] Park, J., Gelse, K., Frank, S., von der Mark, K., Aigner, T. and Schneider, H. (2006) Transgene-activated mesenchymal cells for articular cartilage repair: a comparison of primary bone marrow-, perichondrium/periosteum- and fat-derived cells. The Journal of Gene Medicine 8, 112–125.

[140] Peng, L., Jia, Z., Yin, X., Zhang, X., Liu, Y., Chen, P., Ma, K. and Zhou, C. (2008) Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. Stem Cells and Development 17, 761-774.

[141] Rebelatto, C.K., Aguiar, A.M., Moretao, M.P., Senegaglia, A.C., Hansen, P., Barchiki, F., Oliveira, J., Martins, J., Kuligovski, C., Mansur, F., Christofis, A., Amaral, V.F., Brofman, P.S., Goldenberg, S., Nakao, L.S. and Correa, A. (2008) Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Experimental Biology and Medicine 233, 901–913.

[142] Noel, D., Caton, D., Roche, S., Bony, C., Lehmann, S., Casteilla, L., Jorgensen, C. and Cousin, B. (2008) Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. Experimental Cell Research. 314, 1575–1584.

[143] Hui, J.H.P., Li, L., Teo, Y.-H., Ouyang, H.-W. and Lee, E.-H. (2005) Comparative study of the ability of mesenchymal stem cells derived from bone marrow, periosteum, and adipose tissue in treatment of partial growth arrest in rabbit. Tissue Engineering 11, 904–912.

[144] Goessler, U.R., Bugert, P., Bieback, K., Stern-Straeter, J., Bran, G., Hoermann, K. and Riedel, F. (2008) Integrin expression in stem cells from bone marrow and adipose tissue during chondrogenic differentiation. International Journal of Molecular Medicine 21, 271–279.

[145] Kisiday, J.D., Kopesky, P.W., Evans, C.H., Grodzinsky, A.J., McIlwraith, C.W. and Frisbie, D.D. (2008) Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. Journal of Orthopaedic Research 26, 322–331.

[146] Mehlhorn, A.T., Niemeyer, P., Kaiser, S., Finkenzeller, G., Stark, G.B., Suedkamp, N.P. and Schmal, H. (2006) Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. Tissue Engineering 12, 2853-2863.

[147] Vinatier, C., Mrugala, D., Jorgensen, C., Guicheux, J. and Noël, D. (2009) Cartilage engineering: a crucial combination of cells, biomaterials and biofactors. Trends in Biotechnology 27, 307–314.

[148] De Ugarte, D.A., Morizono, K., Elbarbary, A., Alfonso, Z., Zuk, P.A., Zhu, M., Dragoo, J.L., Ashjian, P., Thomas, B., Benhaim, P., Chen, I., Fraser, J. and Hedrick, M.H. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 174, 101-9.

[149] Hattori, H., Sato, M., Masuoka, K., Ishihara, M., Kikuchi, T., Matsui, T., Takase, B., Ishizuka, T., Kikuchi, M., Fujikawa, K. and Ishihara, M. (2004) Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cell source. Cells Tissues Organs 178, 2-12.

[150] Ratanavaraporn, J., Kanokpanont, S., Tabata, Y. and Damrongsakkul, S. (2009) Growth and osteogenic differentiation of adipose-derived and bone marrow-derived stem cells on chitosan and chitooligosaccharide films. Carbohydrate Polymers 78, 873–878.

[151] Monaco, E., Sobreira Lima, A. de, Bionaz, M., Maki, A., Wilson, S.M., Hurley, W.L. and Wheeler, M.B. (2009) Morphological and transcriptomic comparison of adipose and bone marrow derived porcine stem cells. The open Tissue Engineering & Regenerative Medicine Journal 2, 20–33.

[152] Hayashi, O., Katsube, Y., Hirose, M., Ohgushi, H. and Ito, H. (2008) Comparison of Osteogenic Ability of Rat Mesenchymal Stem Cells from Bone Marrow, Periosteum, and Adipose Tissue. Calcified Tissue International 82, 238–247.

[153] Lin, L., Shen, Q., Wei, X., Hou, Y., Xue, T., Fu, X., Duan, X. and Yu, C. (2009) Comparison of osteogenic potentials of BMP4 transduced stem cells from autologous bone marrow and fat tissue in a rabbit model of calvarial defects. Calcified Tissue International 85, 55–65.

[154] Niemeyer, P., Kornacker, M., Mehlhorn, A., Seckinger, A., Vohrer, J., Schmal, H., Kasten, P., Eckstein, V., Suedkamp, N.P. and Krause, U. (2007) Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro. Tissue Engineering 13, 111–121.

[155] Zaminy, A., Kashani, I.R., Barbarestani, M., Hedayatpour, A., Mahmoudi, R. and Nejad, A.F. (2008) Osteogenic differentiation of rat mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells: melatonin as a differentiation factor. Iranian Biomedical Journal 12, 133–141.

[156] Egusa, H., Iida, K., Kobayashi, M., Lin, T.Y., Zhu, M., Zuk, P.A., Wang, C.J., Thakor, D.K., Hedrick, M.H. and Nishimura, I. (2007) Downregulation of extracellular matrix-related gene clusters during osteogenic differentiation of human bone marrow- and adipose tissue-derived stromal cells. Tissue Engineering 13, 2589–2600.

[157] Niemeyer, P., Fechner, K., Milz, S., Richter, W., Suedkamp, N.P., Mehlhorn, A.T., Pearce, S. and Kasten, P. (2010) Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma. Biomaterials 31, 3572-9.

[158] Mauney, J., Nguyen, T., Gillen, K., Kirker-Head, C., Gimble, J.M. and Kaplan, D. (2007) Engineering adipose-like tissue in vitro and in vivo utilizing human bone marroy and adipose-derived mesenchymal stem cells with silk fibroin scaffolds. Biomaterials 28, 5280–5290.

[159] Rada, T., Reis, R.L. and Gomes, M.E. (2009) Adipose tissue-derived stem cells and their application in bone and cartilage tissue. Tissue Engineering, Part B 15, 113–125.

Appendices

List of Abbreviations

ACHMS	atelocollagen honeycomb shaped scaffolds with a membrane seal
ActR	activin receptor
AdBMP	adenovirus expressing bone morphogenetic protein
AdEGFP	adenovirus expressing enhanced green fluorescent protein
ALP	alkaline phophatase
ANOVA	analysis of variance
ASC	adipose tissue-derived stem cells
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BMSC	bone marrow derived stem cells
CAD	computer aided design
CAM	computer aided manufacture
CDHA	calcium-deficient hydroxyapatite
CCM	complete chondrocyte medium
CDMP-1	cartilage derived morphogenetic protein-1 (GDF-5)
COL	collagen (gene)
CT	computer tomography
d	day
2D/ 3D	2-dimensional/ 3-dimensional
p-DAB	p-dimethylaminobenzaldehyde
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamide
DBM	demineralized bone matrix

DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediammintetraacetic acid
EGF	epidermal growth factor
FABP4	fatty acid binding protein-4
FDA	Food and Drug Administration
FGF	fibroblast growth factor
bFGF	basic fibroblast growth factor
GAG	sulphated glycosaminoglycans
GAPDH	glyceraldehydes-3-phosphate dehydrogenase (GPDH)
GDF-5	growth and differentiation factor-5 (CDMP-1)
HuGDF-5	recombinant human growth and differentiation factor-5
GFP	green fluorescent protein
GPDH	glyceraldehyde-3-phosphate dehydrogenase
HA	hydroxyapatite
H & E	haematoxylin & eosin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
IGF-I	insulin-like growth factor-I
ITS	insulin, transferring, selenious acid
LPL	lipoprotein lipase
MCH	polymer by polymerization of polycaprolactone-diol on mannitol, transformation with hexamethyldiisocyanate
MCI	polymer by polymerization of polycaprolactone-diol on mannitol, transformation with polyisophorondiisocyanate
mio	million

MSC	mesenchymal stem cells
MVI	polymer by polymerization of polyvalerolactone on mannitol; transformation with polyisophoronodissocyanate
NEAA	non-essential amino acids
OPF	oligo (poly (ethylene glycol) fumarate
P	passage
PBS	phosphate buffered saline
PCL	poly (ε-caprolactone)
PDGF	platelet-derived growth factor
PGA	poly (glycolic acid)
P-4HB	poly (4-hydroxybutyrate)
PLA	poly (lactic acid)
PLLA	poly (L-lactic acid)
PLGA	poly (lactic-co-glycolic acid)
PLIN	perilipin
PPARγ	peroxisome proliferator activated receptor γ
PU	polyurethane
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rpm	rounds per minute
RT	reverse transcription
Runx2	runt-related transcription factor-2
SD	standard deviation
SEM	scanning electron microscopy
β-TCP	tricalcium phosphate
T-75 culture flask	75 cm^2 cell culture flask

T-150 culture flask	150 cm^2 cell culture flask
VEGF	vascular endothelial growth factor
WW	wet weight

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List of Publications

Publications

[*]Appel, B., [*]Baumer, J., Eyrich, D., Sarhan, S., Toso, S., Englert, C., Scodacek, D., Ratzinger, S., Grässel, S., Göpferich, A., Blunk, T. (2009) Synergistic effects of growth and differentiation factor-5 (GDF-5) and insulin on expanded chondrocytes in a 3-D environment. Osteoarthritis and Cartilage 17 (11), 1503-1512

Englert, C., Blunk, T., Mueller, R., Schulze von Glasser, S., Baumer, J., Fierlbeck, J., Heid, I.M., Nerlich, M., Hammer, J. (2007) Bonding on articular cartilage utilizing a combination of biochemical degradation and surface cross-linking. Arthritis Research & Therapy 9 (3), R47

To be submitted:

Baumer, J., Göpferich, A., Blunk, T. (2010) Differentiation capacity of adipose derived stem cells (ASC) and bone marrow derived stem cells (BMSC) towards fat, bone and cartilage in direct comparison – Review (Chapter 8)

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