**Differentiation of human mesenchymal stem cells into chondrocytes**

**Dissertation**

To obtain a doctorate Department of Biology the Faculty of Mathematics, computer science and natural sciences at the University of Hamburg presented by Alexei Slobodianski from Almaty, Kazakhstan Hamburg 2006

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**III** **List of Abbreviations**

Figure Figure

A. least. distilled water

ACT Autologous chondrocyte transplantation

AP Alkaline phosphatase

ASAP / C Ascorbic acid 2-phosphate + cysteine

bFGF, FGF basic fibroblast growth factor

BMP bone morphogenetic protein

Bp Base pairs

or respectively

approximately approximately

Ca 2 + Calcium ion

CaCl 2 Calcium chloride

CD Cluster of differentiation (CD-molecule)

cDNAs copy deoxyribonucleic acid

Col. Collagen

DAPI 4 ', 6'-diamidino-2-phenylindole

Dex Dexamethasone

DMEM Dulbecco's Modified Eagle Medium

DMMB 1,9-dimethyl-chloride Methlenblau

DMSO Dimethylsulfoxide

DNA dsDNA (Double-stranded) deoxyribonucleic acid

ECM extracellular matrix

EDTA Ethylenediaminetetraacetate

EGF Epidermal growth factor

FACS Fluorescence activated cell sorting

FITC Fluorescein

FCS fetal calf serum

FS Forward scatter

g Acceleration due to gravity

G / D ratio, GAG to DNA - ratio

GAG Glycosaminoglycans

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

hch human Chondrozytenlinie

HD-culture high-density culture

HEPES 2 - [4 - (2-Hydoxyethyl)-1-piperazinyl]-ethanesulfonic

hMSC human mesenchymal stem cells

HS Human serum

IGF-I, IGF-I insulin-like growth factor-I

Ig Immunoglobulin G

JAA Iodoacetamide

K Control

min Minute

MMP Matrix metalloproteinase

**1**

MNC mononuclear cells

mRNA messenger RNA

MW Average

n Number of samples

NaCl Sodium chloride

NaH2PO4 Sodium hydrogen phosphate

O2 Oxygen

p Level of significance

P / S Penicillin / streptomycin

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate Buffered Saline

PCR Reverse transcriptase PCR

PDGF-BB, Platelet-derived growth factor-BB

PE R-phycoerythrin

PG Proteoglycans

PGA poly-glycolic acid

PMSF Phenylmethylsulfonfluorid

mono / poly monoclonal or polyclonal antibodies

pO2 Oxygen partial pressure

qPCR Quantitative reverse transcriptase PCR

R2 Regression coefficient

rpm Rotation Per Minute

RT Room temperature

S. Page

s. see

sec Second

Sox 9 SRY (sex determining region Y)

SS Scatter Site

Standard deviation Standard deviation

STRO-1 stromal cell surface marker

SYBR Green Fluorescent dye for the detection of dsDNA

Table Table

TAE Tris-acetate-EDTA

TBE Tris-borate-EDTA

TBS Tris-buffered saline

TEMED N, N ', N'-tetramethylethylenediamine

TGF-β, TGF, transforming growth factor

Tris Tris (hydroxymethyl)-aminomethane

U Unit

UKE University Hospital Eppendorf

v / v Volume / volume

vs. versus

w / v Mass / volume

WB Western blot

eg for example

**2** Introduction

**First** **Introduction**

**1.1 *Tissue engineering,* new perspectives for the treatment of cartilage defects**

A satisfactory treatment of cartilage defects in the articular surfaces of the human remains one of the unsolved problems of medicine. It is estimated that more than 80 percent suffer from the over-50s under a sometimes very painful cartilage wear (Kowalewski 2005). But not only with increasing age, the cartilage loses its optimum biomechanical properties, more and more active young people are affected. When they are mainly mechanical overload and sports injuries are the cause of damage, not heal spontaneously and more lead in time to the creation of permanent cartilage defects. The result is a progressive degeneration of the painful tissue, which eventually leads to osteoarthritis. The reason for the lack of post-natal regeneration of cartilage tissue (Buckwalter and Mankin, 1998). Without effective therapeutic treatment, such injuries only closed by the formation of mechanically inferior scar tissue. It is estimated the annual costs for the treatment of osteoarthritis in Germany to around 7 billion euros. (Maetzel, 2004). One way to cure such cartilage damage is the replacement of the defective tissue with intact articular surfaces implants, which hopes to establish with the help of *tissue engineering.* Previously applied clinical therapies for cartilage defect repair such as the *shaving* (Messner 1996), subchondral Abrasionsarthoplastik (Johnson, 2001;. Singh et al, 1991), or autologous chondrocyte implantation (. Brittberg et al, 2003) to achieve a mere filling of the defect with no biomechanically competent cartilage . Another surgical procedure is the so-called "mosaicplasty" dar. In this method, small osteochondral cylinder diameter is punched into a mosaic-shaped cartilage defect. This method, however, remains limited to small defects (Hangody et al., 2001). The incomplete defect coverage and the setting of new defects by the removal of uncontaminated place of permanent cartilage are discussion points (cheers, et al, 2002;. Hangody and Fules, 2003). Therefore, the development of a method for the production of one facet implant technicians from autologous cells without damage elsewhere to the central goals of *tissue engineering.*

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**1.2 Structure and function of cartilage**

**1.2.1 Structure of the cartilage and its special characteristics**

There are three different types of cartilage: fibrous, elastic and hyaline cartilage. The fibrocartilage is composed of interwoven collagen fiber bundles, which give it a special strength. Fibrocartilage is found in the menisci, the symphysis and the intervertebral discs. The elastic cartilage that is found in the outer ear canal and the auricle contains, in addition to solid collagen fibers even more special, elastic fiber. The third form of cartilage is hyaline cartilage. It allows for static and dynamic load equally effective shock absorption by distributing the forces acting on a larger area and a virtually friction-free sliding of the articulating joint surfaces. During skeletal formation and growth of hyaline cartilage provides the matrix for the growth of bony skeleton.

As is the production of hyaline cartilage with great hopes for the treatment of articular surface damage just connected, this paper deals only with the addition of this particular type of cartilage. Articular cartilage can be up to 5 mm thick, eg behind the patella (Eckstein et al., 2001). In general, while the more the load is, the thicker the cartilage.

The hyaline cartilage consists mainly of water (about 70-80% share;), extracellular matrix (ECM) and chondrocytes (Mankin and Thrasher, 1975). Compared to other tissues, the proportion of cells in the hyaline cartilage of 1-10% is quite low (Carney and Muir, 1988). Articular cartilage can be constructed to present layered with different functions. In Figure 1 this is shown schematically. Looking at the cross section shown from the top (surface cartilage) to bottom (bone surface), so initially the Tangentialzone, also called the perichondrium, visible, which is cartilage surface. It follows the middle zone (transitional zone), the deep zone (radial zone) and the calcified zone of mineralization, which represents the transition to the subchondral bone (Pullig et al., 2001).

The ECM consists essentially of two main components, namely the layer depending on varying Glykosinaminoglykanen (chondroitin sulfate and keratan sulfate) and collagen type II, IX and XI. The collagen types I and X are, in contrast, only marginal hyaline cartilage. Collagen type I is found only in the uppermost thin layer of cartilage, which is responsible for the particular resistance to the tangential stress (Eyre et al, 1987,.. Mendler et al, 1989). In type X is a Ca + + - binding protein that only the so-called terminally differentiated chondrocytes hyportrophen or near the interface with the

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Bone is formed (Binette et al., 1998). Its share of total collagen in hyaline cartilage is very small, but increases with progressing to osteoarthritis (Stephens et al, 1992,.. Walker et al, 1995).

**SubchondralerKnochenKnorpeloberflächeHypertrophe ChondrozytenKollagen type XSubchondralesKnochenmarkTerritoriumintakte AggrekanperizelluläreRegion VIInterterritoriumnicht intact collagen-AggrekanMineralisierunsgs zoneTangentialzoneKollagen ITransitionalzoneKollagen Type Type Type IIRadialzoneKollagen IIKollagen bone collagen Type II IKollagen ISubchondralerKnochenKnorpeloberflächeHypertrophe I**

**Figure 1: A schematic representation of the vertical longitudinal section of the articular cartilage**

Articular cartilage contains a matrix of collagens and proteoglycans, like other hyaline cartilage, but is not structured homogeneous but composed of different layers. The presentation is adapted from Koopman (Koopman WJ, 2000).

With the depth of the cartilage to change the orientation of the fibers and the cartilage portion of liquid to the proteoglycans and collagen fibers. In the surface zone, the collagen fibrils are oriented tangentially to the cartilage surface (Clark, 1991, Hunziker et al, 2002.) And the collagen content is highest (Poole et al, 1996).. In the middle zone (transitional zone) on the collagen fibrils have no preferred direction, while they radiate into the deep radial zone approximately perpendicular to the calcified zone (Hunziker et al, 2002;. Venn, 1978).

Cartilage tissue in comparison to other tissues shows some peculiarities: it is built from a single cell type, and is neither innervated nor vascularized. The diet made ​​solely by diffusion from the surrounding tissue (Mankin, 1982). The chondrocytes are found in the ECM embedded in special capsules, the so-

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chondrones mentioned, is involved in its construction type VI collagen (P Otte 2001). Involved in what manner the chondrones in the regulation of matrix metabolism are is still not fully understood. A simplified scheme of chondrones is shown in Figure 2. **PcPgTmImC chondrocyte RRRRRKol.** **IXKol.** **VIKol.** **IIKol.** **XI PcPcPgPgTmImC chondrocyte RRRRRKol.** **XI**

**Figure 2: The structure of chondrones.** **The schematic representation**

The chondrocytes are immediately surrounded by a cartilaginous capsule. Together with the name given to the chondrocytes adjacent to the outside Knorpelhof chondrone. - C - chondrocyte; Pg - pericellular glycocalyx; Pc - pericellular capsule, Tm - territorial matrix; In - Inter-territorial matrix, R - receptors (e. g. Anchorin CII or NG-2 receptor), Colossians II - Type II collagen, Col VI - Collagen type VI, IX, Col - collagen type IX, Colossians XI - XI collagen type. The illustration is modified from Poole (Poole et al., 1992)

**1.2.2 Architecture and Biomechanics of cartilage**

The special benefits of biomechanical hyaline cartilage is explained as today according to the principle of the fiber - reinforced material. The fibers are formed by cross-linked collagen fibrils, the material from highly negatively charged glycosaminoglycans (chondroitin sulfate and keratan sulfate) and hyaluronic acid. The latter form of proteoglycans, especially aggrecan, very large complexes, which generate due to their strong tendency to bind water, a very high swelling pressure endosmotic. Without limitation by the three-dimensional collagen fiber - the network would lead to an influx of water five times larger Quellungsvolumen. Swelling pressure of 0.1 to 0.2 MPa is the fibrils under a correspondingly high tensile stress (Katz et al, 1986; Maroudas et al, 1980, Schneiderman et al, 1986...). The collagen fiber network gets its strength from the high cross-linking, where the two types of collagen IX and XI are involved (Diab et al, 1996,.. Mendler et al, 1989). Figure 3 shows the typical structure of a proteoglycan complex is shown schematically.

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**IIFibrilleLinker-SulfatRegionAggrecanAggrecanChondroitin-IIFibrille**

**Figure 3: Molecular structure of the cartilage**

The extracellular matrix (ECM) of the hyaline cartilage consists of a two-component system: long striated fibrils, which consist mainly of collagen type II and the extrafibrillären matrix, composed mainly of proteoglycan aggregates.

**1.2.3 The development of cartilage**

The formation of cartilage, chondrogenesis is a complex process that results in the context of embryogenesis for the development of the skeletal system. It begins with the proliferation and condensation of mesenchymal stem cells (MSCs) (Goldring et al, 2006;. Tacchetti et al, 1992).. The end of each phase is indeed already investigated since 1925 (Fell, 1925), is reproduced in its details not yet complete (Cancedda et al., 2000). It is believed that mesenchymal - epithelial cell interactions play an important role early in the development of cartilage tissue (Tuan, 2004). At this point, initiate morphogenic proteins (BMPs = bone morphogenetic protein), expression of the transcription factor Sox9 (Healy et al., 1999). Sox9 is obviously at a regulatory level is of central importance for the entire sequence of chondrogenesis, because its expression is the condition of the undifferentiated mesenchymal cell observed until the transition to prähypertrophen state (Lefebvre and Smits, 2005). It directly regulates the expression of the genes for type II collagen, type XI and aggrecan (Bridgewater et al, 1998,.. Liu et al, 2000;. Sekiya et al, 2000). After the start of the condensation of mesenchymal cells generated in the first Prächondrozyten center. At the same time the first time the expression of collagen type II was observed (Fig. 4) whereas the expression of markers of undifferentiated cells decreases (Lefebvre and Smits, 2005;. Zhao et al, 1997). The

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Development of Prächondrozyten goes to early chondroblasts associated with an increase in cell proliferation and matrix production. The cells generate more collagen type II and aggrecan. Link protein, collagen type IX and type XI are first detectable (Lefebvre and Smits, 2005). In the next phase, the cells continue to proliferate, eventually auszudifferenzieren to mature chondrocytes. Figure 4 shows an overview of where the marker called the corresponding stages of development are assigned. **MesenchymaleZelle** **Type I (collagen type II) collagen type II, aggrecan, link protein type II collagen, aggrecan, link protein type II collagen, aggrecan, link protein (collagen type X) collagen type X Sox9 Sox9 Sox9Sox9 ZelltypECM regulatory marker marker chondrogenesis MesenchymaleZelle** **Collagen markers regulatory marker chondrogenesis**

**Figure 4: Simplified representation of individual steps of chondrogenesis with the most important marker proteins**

The presentation is adapted from Lefebvre (Lefebvre and Smits, 2005).

While the proliferation of one Avaskularisierung tissue is observed, which is a prerequisite for the formation of cartilage-specific matrix (Yin and Pacifici 2001). The proliferation occurs only during the transition to prähypertrophen chondrocytes to a halt. When walking through the now beginning the development of bone cells and the prähypertrophe hypertrophic stage of development. They initially contain mRNA for aggrecan and collagen type II, but is now increasingly more collagen type X produces. Here take the chondrocytes strongly increased in size and build a matrix for later immigrating osteoblasts and the subsequent mineralization. In the past in the articular surface chondrocytes (articular chondrocytes) the hypertrophy is prevented. They get their differentiated phenotype, which is the prerequisite for the formation of articular cartilage (Goldring et al., 2006). The permanent preservation of the differentiation state of articular chondrocytes is facilitated by several factors. Here avascular tissue that plays in a decreased oxygen content has a role, but in addition some especially **8**

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Growth factors and mechanical stimulation - - mechanotransduction (Szafranski et al, 2004).. Among the growth factors involved in the *Transforming Growth Factor-beta1* (TGF-beta1) and *insulin-like growth factor* I (IGF-I) include (Roark and Greer, 1994;. Wang et al, 1995). IGF-I increases Matrixbiosynthese and increases the mitogenic activity of chondrocytes. At the same time it inhibits matrix degradation. He also has been demonstrated in acute cartilage defects in joints osteoathritischen in higher concentrations (De Ceuninck et al, 2004;. Schmidt et al, 2006;. Trippel, 1995;. Susant van et al, 2000). TGF-ß1 increases the production of large, anionic proteoglycans in the synovial fluid and is up-regulated by immobilized knees (Morales 1991, Nixon et al 1998,.. Okazaki et al 2001). Glucocorticoids have an indirect effect. They neither promote nor the formation of chondrogenesis and preservation of the articular cartilage directly (Li et al., 2006). However, it seems the presence of this hormone in the early stages of differentiation of mesenchymal stem cells to be essential (Sekiya et al., 2002). Is also known that dexamethasone also affects the migration and adhesion of the cells (Frosch et al., 2002), which is reflected both in cell morphology and in growth patterns.

**1.3 *Tissue engineering* of cartilage *in vitro* by aggregation of adult chondrocytes**

The findings on the early chondrogenesis have given rise to the hope of being able to produce autologous cartilage by simulation of chondrogenesis *in vitro* for the treatment of articular surface damage (Solursh, 1988). The variety of approaches can be made ​​in principle be divided into two groups, those who want to stimulate tissue formation by aggregation of adult chondrocytes from the articular surface and those that they want to achieve by the condensation of mesenchymal stem cells. Both methods have their specific advantages and disadvantages.

Method for obtaining primary chondrocytes from biopsy material from the joint surface cartilage of different animal models and man have been long known (Grande et al., 1999). It was shown early on that such chondrocytes still have the special ability to start with intensive cell-cell contacts with the formation of a cartilage-like matrix (Kim et al., 1994). In practice, this was very easy to sedimentation through a so-called pellet culture (micromass culture) achieved. The main disadvantage of the method is needed for that to be a possible therapeutic application of this method is considerably more cells than can be obtained from biopsies. Because so far all attempts have failed, primary chondrocytes while maintaining their differentiated phenotype *in* effectively

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*vitro* to proliferate (Benya and Shaffer, 1982;. Goessler et al, 2005; Yu et al, 2006).. Effective propagation is possible under different conditions, but without the cells simultaneously to de-differentiate. At the molecular level is observed as a consequence of this de-differentiation, the increasing formation of collagen type I, which is different from the collagen type II, not in a position to form three-dimensional collagen fibers with the required biomechanical properties. (. Shakibaei et al, 1995). While they have discussed various mechanisms that could be responsible for the dedifferentiation of chondrocytes in the growth phase, the loss of contact between chondrocytes and matrix (Grundmann et al, 1980 be,. Narrow et al, 2006), cell maturation (Chacko et al., 1969), the aging of cells (Moskalewski et al., 1979) or contamination with fibroblasts ((Norby et al., 1977)). However, methods for re-differentiation as yet only available for a variety of animal models but not in humans (Benya and Shaffer, 1982;. Hauselmann et al, 1992;. Nagel-Heyer et al, 2006).

**1.4 *Tissue engineering* of cartilage *in vitro* by stimulation of embryonic chondrogenesis**

The second approach to the healing of cartilage defects is based on the process of condensation of mesenchymal stem cells during chondrogenesis.

**1.4.1 General properties of stem cells**

In the mesenchymal stem cell, although it is by cell with the ability to divide and in different cell types to mature, but we must distinguish them from the embryonic stem cell, which alone has a maximum potential for self-replication and unlimited differentiation potential. Up to 8-cell stage in humans until about the third day after fertilization, this is referred to as totipotent cells because each cell can be individually develop into a complete individual. Can you can multiply still indefinitely through cell division and educate even any of the approximately 210 cell types of a man, but in contrast to the totipotent cell is not an independent organism more arise from them (Mountford et al, 1998: The cells are called pluripotent. ). The so-called adult stem cells are found in various tissues of adult humans. They are called multipotent, because they also possess the ability to self renew and to develop into specialized cell types of a tissue (Czyz et al, 2003;. Lange et al, 2005;. Storch et al, 2001).

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**1.4 2 properties of mesenchymal stem cells**

Even in the bone marrow of human multipotent stem cells are found, including the *Bone Marrow stromal cells,* which are also known as mesenchymal stem cells (hMSCs). They come when people originally from the mesoderm. Their numbers in the bone marrow is low and decreases with increasing age from approximately exponentially. Can be found in the newborn for about 10 2 10 6 MSCs per nucleated cells, it is the fifties, only about 2 per 10 6 (Caplan, 1994). Because of their large multiplication and differentiation potential, but is more frequently recommended to use these cells in regenerative medicine to the problem of de-differentiation of adult chondrocytes under *in vitro* - to avoid proliferation. Based on the fundamental studies on the differentiation potential of bone marrow (Ashton et al, 1980,.. Friedenstein et al, 1968), the theory of the existence of mesenchymal stem cells has been suggested (Caplan, 1991) Owen, 1985;) and examined their chondrogenic potential (Barry et al ., 2001b; Johnstone et al, 1998).. In addition, it was also demonstrated that MSCs in adipose tissue (Sekiya et al, 2004.) Bennett et al. 1991), bone (Jaiswal et al, 1997) and muscle (Bennett and Adams, 1987,.. Ferrari et al, 1998 can) differentiate. According to recent results is to accept even that mesenchymal stem cells are responsible not only for the musculoskeletal system, but also in cardiomyocytes, endothelial cells and pericytes (Fukuda and Fujita, 2005; Gojo and Umezawa, 2003) and in those able to differentiate with neuronal character (Kopen et . al, 1999; Zhao et al, 2002).

**1.4.3 The heterogeneity of the human bone marrow MSCs occurring**

Even after removal of red blood cells and hematopoietic stem cells obtained from a bone marrow aspirate still not a homogeneous cell population. Rather, one must reckon with the stem cells of different origin and function (Colter et al., 2001). This is shown in Figure 5 illustrate schematic model this heterogeneity. From the undifferentiated hMSCs, so-called RS *(self renew)* cells (Figure 5 in the red box) are shown by asymmetric division of different daughter cells. This results in either re-identical stem cells or progenitor cells of the differentiated non-identical target cells. The progenitor cells have only a limited differentiation potential. They divide symmetrically and produce tri-and bipotent cells, the multipotent hMSCs, although morphologically similar, but already have a different gene expression profile (Baksh et al., 2004). In a given bone marrow so it will only contain the stem cells and the various intermediate stages on the **11**

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branching paths to find the target cells (Figure 5 in the yellow box) (Baksh et al, 2004;. Gronthos et al, 2003).. The proportion of progenitor cells from each individual condition of the donor organism depends on the time of removal from the aspirate (Phinney et al., 1999). As reported, on average, only about one third of human MSCs tripotentes (chondro-, osteo-and adipogenic) has development potential (Zuk et al., 2002) was). This heterogeneity opens the one hand, the possibility for tissue engineering, but makes it clear that the other problem with the use of hMSCs to detach from the bone marrow. Because for the production of a hyaline cartilage has a very homogeneous population of differentiated chondrocytes are available.

**Differentiated cells, progenitor cells OsteoblastenAdipozytenChondrozytenMyozytenSehnenzellen**

**MSC (multipotent)**

**Proliferation**

**Differentiation**

***self-renew***

**Progenitor**

**(Multi-Potent)**

**Progenitor**

**(Bipotent)**

**Progenitor**

**(Tripotent)**

**Progenitor**

**(Unipotent)**

**Genetic modifications Phenotypic modifications**

***Stem Cell Compartment Cell Compartment Committed***

**Isolation**

**A**

**S**

**S**

**S**

**Cells**

**Osteoblasts**

**Adipocytes**

**Chondrocytes**

**Myocytes**

**Tendon cells**

**Progenitor cells**

***renewself-Figure* 5: A model of the development of adult MSC**

The multipotent, undifferentiated MSCs divide asymmetrically. This creates an identical stem cell (in red frame) and an identical progenitor cell. Then go through the MSC genetic modifications and generate progenitor cells with similar phenotype and self-renewal potential. A - Asymmetric cell division, S - symmetric cell division. The different states of differentiation will not only go through one after another, but exist in a bone marrow aspirate and always occur together (in the yellow frame). The presentation is adapted from The Bacchae (Bacchae et al., 2004).

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**1.4.4 Methods for obtaining a suitable for *in vitro* chondrogenesis of bone marrow cell population**

The heterogeneity encountered in a bone marrow cell populations makes an effective selection of appropriate cells for chondrogenic differentiation is necessary. Successful attempts to isolate chondrocytes directly from the mixture of different cell types have not previously been described, probably because the proportion of cells with differentiated phenotype is too low. The approach to capture all the cells that are already on the path of chondrogenic differentiation (blue circles in Figure 5), falls short. On the one hand, should this procedure, specific markers for each stage of differentiation are available, which is not currently the case (Bianco and Gehron Robey, 2000; Conget and Minguell, 1999. Yoo et al, 2005), on the other hand, can be fully and partially differentiated cells proliferate *in vitro* significantly more difficult to obtain the differentiation status as undifferentiated. This is based on the phenomenon that the proliferation potential decreases with increasing degree of differentiation, which should be indicated with the blue and red wedge in Figure 5.

Especially the last argument in favor of the alternative that proliferate preferentially as the undifferentiated stem cells. In this case, the chance to be used, the relative proportion decreases to less divisive, partially differentiated cells with the number of passages. This idea proved to be especially so far as successful if the cells were seeded at the same time to proliferate in very high dilution (Prockop et al., 2001). It is likely that the differentiation-promoting paracrine factors in a higher initial cell density accumulate faster, which then leads to the throttling of the proliferation rate. The result would be not only a lower Endzellzahl, but also an increase in the heterogeneity of the cell population with regard to the differentiation status of the individual cells and not a degradation.

The proliferation capacity of a population of mesenchymal stem cells in cell culture depends on both the age and condition of the bone marrow donor as well as from the time of removal from (Caplan, 1994;. Digirolamo et al, 1999). Therefore, in addition also tried the mitogenic effects of various growth factors such as FGF2 (fibroblast growth factor 2), PDGF (Platelet-Derived Growth Factor) and EGF (Epidermal Growth Factor) to use to increase the proliferation rate maximum (Bianchi et al. 2003;. Gruber et al, 2004; Yao et al, 2003).. However, for the objective of the selective enrichment of undifferentiated cells is left strictly as proliferation-stimulating growth factors often also affect the differentiation potential. Whether this is desirable or undesirable is manifested as a pre-selection effect in the sense of the objective must be checked in each case. **13**

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In addition to the maximum rate of proliferation, other criteria have been proposed for the enrichment of cells with specific differentiation potential. Thus, the application of a mechanical cell-sieve appears for selecting cells with certain size to be as easy as successful (Hung et al., 2002). In another case, an attempt was made ​​to separate by sedimentation of the cells over a Ficollgrandienten unwanted progenitor cells (Lange et al., 2005).

**1.4.5 Methods for identification of different populations of hMSCs**

The ability to reproducibly enrichment or isolation of certain mesenchymal cell types including progenitor cell makes a powerful method for identification is required. Despite an intensive search in recent years it has not yet been able to find unique high specific surface antigens for this question. For this reason, it is also not yet been possible to use cell sorter to isolate a defined mesenchymal cell types. Currently can only be used on hematopoietic stem cells and leukocyte antigens CD34 and CD45 occurring to demonstrate the absence of these cell types. Only in combination with some positive markers are used during routine inspections. These include CD105 (endoglin), a receptor for the growth factor TGF-β1, CD90 (Thy1), a thymocyte antigen and the antigens STRO1 and CD106 (VCAM1) (Barry et al, 1999,.. Gronthos et al, 2003 ; Simmons and Torok-Storb, 1991;. Tepliashin et al, 2005). Specific antibodies directed against these antigens can indeed be replaced as part of FACS analysis of important information, but can not elaborate identification of cell populations on their ability to differentiate into the various target cells (Pittenger et al., 1999) .

**1.4.6 The pellet culture as a differentiation model**

A major advance for the potential use of *in vitro* chondrogenesis were studies that demonstrated that the suspected proliferation capacity can be used practically (Yoo et al., 1998). Varied according to the studies on the possibility of MSCs *in vitro* into osteoblasts (Jaiswal et al., 1997) and chondrocytes (Mackay et al., 1998), found the underlying differentiation model of pellet culture, a rapid acceptance (Johnstone et al. , 1998, Yoo et al, 1998).. It has been demonstrated in various ways, how effective is the system of 3D cultures for the production of cartilage repair tissues for articular cartilage defects (Adkisson et al, 2001;. Angele et al, 1999,.. Solchaga et al, 1999). In most cases, cell-cell contact to initiate a differentiation process, lasting 2-3 weeks

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Introduction

used, which is usually also supported by various differentiation factors. Barry was able to show that the different TGF-β isoforms-β1 β3,-have a strong chondrogenic effect (Barry et al., 2001b). The two factors bind to endoglin (CD 105), which is in the control of chondrogenic differentiation of MSCs and their interaction with hematopoietic stem cells are important (Barry et al., 2001a). It is known that the factors TGF-β1-β3 to be formed in embryonic cartilage, where they are ascribed an important role in the chondrogenic transformation in the context of cell condensations (Hall and Miyake, 2000). Another important factor with strong anabolic properties of IGF-I, which has been used successfully for cartilage formation in animal models (.. Hardingham et al, 1992; Yaeger et al, 1997). The frequent use of dexamethasone to support the differentiation is based on the observation that it stimulates the formation of the transcription factor Sox9 (Sekiya et al., 2002).

**1.4.7 limits the pellet culture as a differentiation model**

In spite of its successes in the use of various forms of pellet culture but also the limitations of this differentiation model for use in *tissue engineering* visible. The repeated together with the expression of collagen type II shown increase in the formation of collagen type I and type X leads to the conjecture that the technique of pellet culture all chondrogenic differentiation stages of mesenchymal cell are passed through to terminal differentiation (Parsch et al ., 2004). Be a prerequisite for the use of *in vitro* differentiated cells for the formation of hyaline cartilage, however, the permanent arrest of chondrocytes in Differenzierunkstatus commitment for which the expression of collagen type II, but not the type of X is characteristic.

**1.5 An alternative model for differentiation of mesenchymal stem cells**

The development of an alternative differentiation model, which has the use of mesenchymal stem cells for *in vitro* production of hyaline cartilage to the target, one must consider the causes that are potentially responsible for the failure of the pellet culture model in its present form (Lawrence et al., 2005 ,. Parsch et al, 2004). Here there are different starting points.

The first in the pellet culture model unintended consequence of terminal differentiation could be very successful in simulating the *in vitro* chondrogenesis of

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Introduction

His growth plate, which also includes the hypertrophy of the chondrocytes. In this case would be examined, checked in how far this process *in vitro,* such as use of appropriate growth factors, or may be interrupted.

Second alternative can also be assumed that the pellet culture is an inadequate model for chondrogenesis. While *in vivo* of chondrogenesis to proceed at a number of processes in topologically and temporally ordered manner, which are supported in the various phases by different factors, is in the pellet culture is applied over several weeks, held constant culture condition. It is assumed that the conditions that promote the differentiation of mesenchymal stem cells is still very immature at first, later, an inhibitory effect on already differentiated cells that have already begun with the formation matrix. This could possibly be a changed composition of the culture period of added growth factors provide a remedy. It is known that the most frequently used to promote the differentiation factors used, TGF-ß3 and dexamethasone, the matrix formation activity (De Luca, 2006;. Fujita et al, 2004;. Goessler et al, 2006; Ohno et al, 2005. ). This could actually have been the omission of these factors have a positive influence. However, appropriate protocols are not yet known.

Third is still conceivable that exist regarding the use of the differentiation status of cells from heterogeneous cell population at the beginning of asynchronous conditions in the pellet culture. While some of the cells by the presence of differentiating factors offered benefits, the other part is inhibited at the matrix formation. In this case, it would in addition to seasonal adjustments of the growth factor profile and the matrix formation are delayed until all cells have the same differentiation state.

**1.5.1 The three-phase model**

Which could in the last section under 3 conditions required to be checked in a so-called three-phase model. It could be the three of chondrogenesis in running processes - proliferation, differentiation and matrix formation of separate periods are assigned so that the culture conditions can be optimized separately for each phase. As illustrated by the diagram in Figure 6, the pellet culture as a differentiation model is compared to only a two-phase model dar. The idea of different stages of development to be separated from each other is not fundamentally new. That the separation of the tissue formation of the proliferation benefits, was recognized earlier (Strehl et al., 2002). In the current case has changed for the first and third stage is unlikely: The proliferation may

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in conventional monolayer - Technology and matrix formation in pellet culture are carried out. For the second phase of the embedding in alginate gel offers good conditions.

**Phase culture culture culture phase three-phase model two-phase model pellet**

**Figure 6: Two-phase and three phase model for chondrogenic differentiation of hMSC**

In a two-phase model (direct pellet culture) the cells after isolation from bone marrow aspirate selectively increased sedimentation in the proliferative phase (first phase), a pellet culture (second stage) and cultured for three weeks. , In which two different processes (differentiation and matrix formation) and interfere with each other. The additional phase in the alginate culture separates the temporal differentiation of the cells and matrix formation (three-phase model).

**1.5.2 The technology Alginatkultur**

Alginate consists of linear polymers derived from brown algae. Similar to agarose gel, it forms a large pore, but not during cooling of dilute solution, but in the presence of Ca + + ions (M & M 2.4). This represents a major advantage. For a brief treatment with EDTA as a chelator for Ca + + ions in the alginate gel can depolymerize again. In alginate embedded cells can be released so again. In particular, the compatibility with chondrocytes has been demonstrated extensively (Hauselmann et al., 1992). Also, the embedding of chondrocytes in alginate for redifferentiation already described by chondrocytes (Chubinskaya et al, 2001,.. Heyland et al, 2006;. Mok et al, 1994). Some authors have described the synthesis of cartilage - specific markers, such as the collagen types II, IX and X described in alginate (Bonaventure et al, 1994; Petit et al, 1996..). In various animal models, it is also managed from the alginate gel and then isolated differentiated cartilage chondrocytes *in vitro* to breed (Heyland et al., 2006). The example of embedding in an agarose gel has been the possibility

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the differentiation of mesenchymal stem cells in the presence of TGF-ß3 and dexamethasone demonstrated (Huang et al, 2004;. Shyy and Chien, 1997). Other authors have alginate used for this purpose and demonstrated the expression of collagen type II and the formation of glycosaminoglycans (Kavalkovich et al, 2002,.. Mehlhorn et al, 2006).

The particular suitability of Alginatkultur technique for three-phase model is that the matrix for the formation of essential cell-cell contacts can be effectively prevented during the differentiation phase. The schematic comparison of the two - - phase model with the three - phase - the model is illustrated in Figure 6.

It is also important that can be recovered during the several weeks of culturing differentiated cells again, or use it to analyze using the technique of pellet culture to be able to matrix formation. This is one planned for use for *tissue engineering* very important aspect.

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**1.6 The aim of this work**

This work was begun with the intent to use the large multiplication and differentiation of mesenchymal stem cells potential to improve the conditions for *in vitro* production of cartilage implants for the treatment of joint surface defects. According to present knowledge, the numerous studies on differentiation model of pellet culture, but very important knowledge for the pursuit of this objective are delivered, while also highlighting its limitations. With the Alginatkultur is building on an alternative differentiation model designed it, built into a three-phase model, which could enable *in vitro* production of hyaline cartilage cells from the bone marrow. For the experimental verification of this thesis is presented concretely, the following four tasks:

First development of conditions for the enrichment or isolation of mesenchymal cells from human bone marrow, which are suitable for chondrogenic differentiation

Second Quantitative differentiation of human mesenchymal cells into chondrocytes with differentiated phenotype in the Alginatkultur

Third analysis of the differentiated cells

4th Functional verification of the differentiated cells by their ability to form cartilage tissue of human

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Materials and methods

**Second Material and Methods**

This chapter describes the experiments used in the implementation of the materials and methods. The outline of this section is carried out in the order of the tests. First, the cell culture methods and isolation of the sample material under investigation are described. Following the presentation of the different methods of analysis and information is for statistical analysis. All the methods described in this part of the standard methods. Possible deviations are noted in the results section.

**Chemicals and solutions:**

All chemicals were, unless otherwise indicated, in purity "analysis" is used and by the following companies related: Amersham Pharmacia Biotech (Freiburg, Germany), Merck KGaA (Darmstadt, Germany), Roth (Karlsruhe, Germany), Roche Molecular Biochemicals ( Mannheim, Germany), Serva (Heidelberg, Germany), Sigma (Munich, Germany). The solutions were diluted with deionized water (Milli-Q Water Purification System, Bedford, USA) was prepared and autoclaved as needed (20 min, 120 ° C, 2x105 Pa) or sterile filtered (pore diameter 0.25 microns, Sartorius Minisaart).

**2.1 Cell culture**

Material:

• sterile workbench Hera safe (Heraeus, Hanau)

• CO2 / O 2 incubator (Heraeus Instruments GmbH, Hanua)

• CASY ® Cell Counter + Analyser System Model TT (Scharfe System,

• Reutlingen)

• Buffer CASY (Scharfe System, Reutlingen)

• Zentriguge Hettich Rotanta / TRC (Hettich centrifuge, Tuttlingen)

• microscope (Olympus CK2, Olympus Germany GmbH, Hamburg, Germany)

• GFL water bath (Lab GmbH Burgwedel)

• Set of 5 pipettes (Eppendorf, Hamburg)

• Mettler PC180 balance (Mettler-Toledo GmbH, Giessen) battery cell culture pipette for Accu-Jet (fire Gm

• bH, Wertheim) • PBS (Gibco 14190-994, Invitrogen)

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Materials and methods

• Trypsin-EDTA solution (Gibco 25300-054, Invitrogen)

5-025, Invitrogen)

in USA)

All the e bathe),

**.1.1 Extraction and isolation of stem cells and chondrocytes**

• DMEM +1000 mg / L glucose + Glutamax I (GIBCO 2188

• DMEM +4500 mg / L glucose + Glutamax I (GIBCO 31966-021, Invitrogen)

• penicillin-streptomycin 10 000 units / mL (GIBCO 15140-122, Invitrogen)

• Antibiotic / antimycotic solution (100x) (GIBCO 15240-062, Invitrogen)

• Fetal calf serum (FCS) (Cat No.: DE14-801F 8 South American Orig

• growth factors: bFGF, TGF-ß3, IGF-I, PDGF-BB (R & D Systems Inc., Minneapolis, USA), dexamethasone (Sigma, Munich)

Vrbrauchsmaterialien for cell culture derived from the company Nunc (Wies

Sarstedt (USA) and Falcon (Becton Dickinson Labware, USA)

**2**

**Human stem cells**

Material:

• Culture medium: DMEM 1000 mg / L glucose, 10% FCS, 1% penicillin / streptomycin

Major fü

• Ficoll 1.077 g / ml (Sigma, Munich)

chhrung:

The experiments in this work were carried out with human stem cells from

the bone marrow transplant department at the University Hospital Eppendorf, were made ​​available. The (2-3 ml) by aspiration from the iliac bone marrow was first extracted in 20 ml DMEM LG, resuspended supplemented with 10% FCS. In a next step, the nucleated cells are separated from the other cell types. This was done using a Ficoll - gradient. For this purpose were overlaid 20 ml Ficoll in a 50 ml Falcon tube with 20 ml of sample carefully. This was done very slowly to the forming border, Ficoll - phase and cell suspension - stage to be able to recognize. Finally, the tubes for 20 minutes at 800 \* g were centrifuged (without brakes). After centrifugation a ring about 0.5 to 1 cm thick in the middle phase of the tubes visible (Fig. 7) was. **21**

Materials and methods

**0.5-1 cm stage of MNC0 ,5-MNC**

**Figure 7: Schematic representation of the Ficoll gradient separation**

The phase (yellow) of the mononuclear cells (MNC) after separation in Ficoll gradient.

This phase was removed very carefully with a pipette. Next were added to 2 to 3 ml of removed cell suspension 10 ml of serum-containing medium. The tube containing the cell suspension was centrifuged (5 min at 400 \*) and the supernatant was discarded. The resulting pellet was resuspended in 5 ml of fresh medium was added and the resulting cells counted using the CASY device. Then the cells were in a range of 6 to 20 million cells per T 25 or T 75 culture flask for 72 hours were plated

**Human chondrocytes**

Material:

• 4% formalin in PBS, pH 7.4 (Merck, Darmstadt)

• Petri dish

• Scalpels

• RNAlater stabilization solution (Qiagen GmbH, Hilden)

Implementation:

The source of the human cartilage was the Department of Forensic Medicine, University Hospital Eppendorf. The results obtained from the coroner's cartilage pieces were washed, cleaned of blood and tissue debris and crushed in a Petri dish with scalpels. For the histology of the cartilage pieces in formalin and for PCR analysis in RNAlater stabilization solution were kept.

**2.1.2 cell preparations**

It was performed a total of 34 isolations of hMSC from bone marrow aspirate from the pool. The age of healthy donors ranged 2-42 years (20 men and 14 women). After isolation, the first human stem cells should be **22**

Materials and methods

increasingly be to provide a sufficient quantity for the target trials. Depending on the number of these isolated cells were seeded and used after Ficoll gradient separation either in one, two or three culture flasks. The cells were cultured under different conditions were considered as different cell preparations.

**2.1.3 Cell Counting**

Material:

• CASY Cell Counter (Scharfe System GmbH, Reutlingen, Germany)

• Buffer CASY (Scharfe System, Reutlingen)

• Neubauer hemocytometer (Brand GmbH, Wertheim)

• Trypan blue solution: 0.5 g trypan blue (Sigma, Munich), 0.9 g NaCl per 100 ml of distilled water. Water

Implementation:

The number and viability of cells was usually using the CASY cell counter, determined. The measured values ​​could be printed for further analysis. If necessary, an additional cell counting in a Neubauer hemocytometer after trypan blue staining was carried out. Trypan blue is a dye that is retained by the membrane of living cells, where the dead cells with permeable porous membrane for the dye and are stained blue. The cell suspension and trypan blue solution were mixed in a 1:1 ratio, placed in a Neubauer chamber and counted the cells rapidly. The vitality was derived from the ratio of viable cell number (unstained cells) to the total number of cells (unstained and blue stained cells).

**2.2 Proliferation of cells**

Material:

• Culture medium: DMEM 1000 mg / L glucose, 10% FCS, 1% penicillin / streptomycin

Implementation:

The cultivation of the cells was carried out as follows. Fig.8

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**Cells after separation imFicoll GradientenPassage-0 (up to approximately 80% confluence).. N.. 3 days after removal of the non adhäriertenZellenHeterogene cell population (hämatopoetitischeZellen and hMSC) hMSCPassage n 1 (up to approximately 80% confluence) passage cells 1 +1**

**Figure 8: Schematic representation of the cell culture of hMSC**

The cells were seeded in culture flasks and supplemented in DMEM with 10% FCS, proliferating 1% penicillin / streptomycin up to 80% confluence. Here, the medium two to three times per week was changed. Before trypsinization of these cells were washed twice with PBS. Depending on the content of the culture bottle was an appropriate volume of trypsin / EDTA solution was added (T25 flask: 2.5 ml, T-75 flask: 4.5 ml, T-175-bottle: 7 ml) and the detachment of the cells checked under a microscope. If necessary, the remaining cells were detached by tapping, and then inactivates the trypsin by adding serum-containing medium. The number of cells was determined and the cells were centrifuged at 400 g for 5 minutes. The cells were then resumed in serum-containing medium and plated according to appropriate use in seeding density. In general, the cells were used after passage 1 for Alginatkultur, the pellet culture or analysis. Depending on use, the cells were supplemented with different factors. To the factors used and their concentrations are listed below.

Growth factor

Concentration [ng / mL]

bFGF

10 ng / mL

TGF

10 ng / mL

PDGF

1 ng / mL

**2.2.1 Growth behavior**

For the growth kinetics of hMSC were in a given concentration

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in culture flasks with an area of 75 cm ² (T75) exposed and cultured under certain conditions. Every 14 days the cells were trypsinized, counted and will continue under the respective cell culture conditions. The multiplication factor was calculated as follows:

VF = cell count after passage s **/** cell number after passing n-1

VF-multiplication factor

n - Passage number

For P0 after adherence VF was not determined because the initial number of hMSC after Ficoll gradient separation was not known. The creation of the growth curve of a cell population (proliferation rate) was determined by extrapolating the number of cells of a bottle. As a starting point for the growth curve used the number of cells after passage 0th

**2.3 Differentiation of cells *in vitro***

Material:

• Culture medium: DMEM 4500 mg / L glucose, 10% FCS, 1% penicillin / streptomycin

• ITS + (BD Biosciences, Heidelberg)

• sodium pyruvate (Sigma, Munich)

• ascorbic acid 2-phosphate (Sigma, Munich)

• proline (Sigma, Munich)

• dexamethasone (Sigma, Munich)

• TGF-ß3 (R & D Systems Inc., Minneapolis, USA)

• IGF-I (R & D Systems Inc., Minneapolis, USA)

• ITS + (BD Biosciences, Heidelberg)

**2.3.1 Two-phase model of chondrogenesis**

**Direct Chondrogenesis**

Implementation:

For direct chondrogenesis, cells were first trypsinized as described above, washed with PBS and counted. The cells were then centrifuged again, and as used in one volume differed from the recorded medium. The medium was supplemented with the following substances:

Substance

Final concentration of dexamethasone 0.39 μ g / ml

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Sodium pyruvate

100 μ g / ml

Ascorbic acid 2-phosphate

)

Anach the cell suspension into several 15 ml tube was divided so that each

**.3.2 Chondrogenesis three-phase model**

50 μ g / ml

Proline

40 μ g / ml

IS +

1:100 (v / v

D

Tubes received 300,000 cells. The preparation of pellets by centrifugation (5 min at 200 g \*). Subsequently, the pellets in polypropylene - tubes with semi-open lid for better gas exchange in an incubator at 37 ° C, incubated for 5% CO 2 and 20% O 2. After 2 days, the pellets were molded into pellets at this time were (one pellet per well with 2 ml of medium) in a 12-well plate to. The medium was exchanged three times per week until the end of the experiment after 21 days.

**2**

**Production of "alginate beads"**

Material:

• 2.4% alginate alginate in 1-fold security buffer 150 mM NaCl (Sigma, Steinheim)

Major fü

• 1-way security buffer (150 mM NaCl, pH 7.4)

• calcium buffer (100 mM CaCl 2)

chhrung:

The differentiation of stem cells was carried out in the alginate culture. For this purpose

The cells were embedded in alginate gel. Therefore, the cells were centrifuged after trypsinization and cell counting (5 min, 400 g) and then washed 1-fold washing buffer. After another centrifugation (5 min, 400 g), the cells were taken in washing buffer and mixed with 2.4% alginate in a ratio of 1:1. The final concentration of alginate was thus 1.2%. To the extent not otherwise specified, the differentiation of the cells occurred in alginate at a cell density of 2 x 10 6 cells / ml. For the production of Alginatkultur (alginate *"beads"),* the cell suspension was dropped into a petri dish with calcium buffer. When dropped into the calcium alginate polymerization buffer, so as to form alginate beads *(beads)* (Fig. 2).

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Materials and methods

**Ca2 + Ca2 + Ca2 + Ca2 + Ca2 + Ca2 +-alginate GelhMSCMonolayer cell-culture-GelSuspensionZell GelSuspensionvor contact with Ca 2 + ion-cell GelSuspensionnach contact with Ca 2 + + Ca2 + Ca2 + IonenNaNaNaNaNaNaNaCa2 Ca2 + Ca2 +**

**A: Preparation of alginate culture B: Molecular structure of the alginate culture**

**A1: A2: A3: B1: B2:**

**IonenNaNaNaNaNaNaNaNaNaNaNaNaNaNaCa2 + Ca2 + Figure 9: Schematic representation of the alginate culture of hMSC**

1), the hMSC of the

Bottom of the cell culture bottle replaced enzymatically and the alginate gel solution is prepared. In the second

For the curing of these so-called alginate - "beads" for about 10 minutes ranged

once with 1-fold

The alginate culture is created in three steps (A): In the first step (A

Step (A2) two components are mixed together in a defined ratio and mixed. In the third step (A3.), this cell-gel suspension using a pipette dropwise into a CaCl 2 solution, and remains there until the polymerization. Molekülare the structure of the alginate-cultured with hMSC (B) prior to polymerization (B1) and after (B2).

Calcium buffer. Of a milliliter of cell suspension resulted in approximately 40 to 45 alginate beads, each containing about 40,000 to 50,000 cells. After 10-minute hardening for 10 minutes, the *beads*

Wash buffer and then further washed once with supplemented medium. Finally, the cells were cultured in medium with added supplements and transferred for further cultivation in sterile flasks.

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Materials and methods

**Alginate culture**

Implementation:

The cells in alginate - "beads" were in culture medium (see above) for one to 3 weeks

but

Phosphate

)

uring the cultivation in alginate was formed around the embedded cells

**cher cells from the alginate and further processing**

37 ° C, 5% CO 2 and 10% O 2 cultured. The culture medium (five times in volume) were, if not otherwise specify, the following factors added: Substance final concentration

Dexamethasone

0.39 μ g / ml

Sodium pyruvate

100 μ g / ml

Ascorbic acid-2

50 μ g / ml

Proline

40 μ g / ml

IS +

1:100 (v / v

W

pericellular matrix was formed hofförmig. This court is called, in analogy to the chondrones as Pseudochondron.

**Is**

Material:

• ED • 1-way security buffer (150 mM

TA buffer (40 mM EDTA in 150 mM NaCl, pH 7.4)

NaCl, pH 7.4)

Major fü

chhrung:

The isolation

the Pseudochondrone from alginate was carried out in several steps.

**Ellet after alginate culture**

After washing in 1-fold washing buffer, the cells were incubated in an EDTA buffer of at least five times the volume of the *beads* were incubated for about 5 -10 below shaking. After the complete dissolution of the alginate gel Pseudochondrone were 5 min at 200 \* g centrifuged, resuspended in wash buffer and finally counted. For the analysis required Pseudochondronen were removed at this step. The remaining Pseudochondrone were again centrifuged and vary depending on the experiment in supplemented medium and used for further processing in pellet culture (see pellet - after alginate culture).

**P**

Implementation:

The preparation of the pellets after Alginatkultur followed the same principle as for

the direct method described chondrogenesis, but with a difference. The quantification of the cells to the pellet culture after alginate was performed by counting the

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Alginate - "beads" (approx. 50 000 cells per bead). Finally, place the Pseudochondrone centrifugation at 200 \* g for 10 min. By default, the alginate pellets were after the same factors as for direct pellet - added culture. Variations in the factors are added to the description of the experiments indicated.

**2.3.3 adipogenic differentiation**

Material:

• maintenance medium: DMEM 4500 mg / L glucose, 10% FCS, 1%

KS, 1%

Durchfü

Penicillin / streptomycin • adipogenesis inducing medium: DMEM 4500 mg / L glucose, 10% F

Penicillin / streptomycin, 1 micron dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, 2 microM insulin. Currency:

To carry out the adipogenic differentiation, the cells at a density of

**.3.4 Osteogenic Differentiation**

5x10 3 cells / cm 2 in 6-well plates and cultured with the respective culture medium for 5-7 days at 37 ° C and 5% CO 2. Thereafter, the culture (day 0) was stimulated for three days, with an adipogenesis-inducing medium (Pittenger et al., 1999). The medium was then removed and the cells further cultured for two days in adipogenesis maintenance medium. This change of medium-cycle of three days and two days induction medium was repeated three times with maintenance medium and the cultures were then further cultured for a week in maintenance medium. Even non-stimulated controls were treated the same, except that, instead of the adipogenesis-inducing medium, the medium-sustaining. The proof of adipogenesis was histologically (see Oil Red O staining).

**2**

Material:

• Basal medium: DMEM 4500 mg / L glucose, 10% FCS, 1% penicillin / streptomycin

Durchfü

• osteogenesis-inducing medium: DMEM 4500 mg / L glucose, 10% FCS, 1% penicillin / streptomycin, 0.1 mu.M dexamethasone, 10 mM β-glycerophosphate, 0.05

mM ascorbic acid-2-phosphate. Currency:

The osteogenic differentiation of mesenchymal stem cells was done according to the

Differentiation protocol by Jaiswal (Jaiswal et al. 1997). For osteogenic differentiation, cells were seeded at a density of 5x10 3 cells / cm 2 in 6-well plate and cultured at 37 ° C and 5% CO 2 for 5-7 days with basic osteogenic medium and then induced (day 0). The stimulation was carried out over a period of 21 days **29**

Materials and methods

in osteogenesis-inducing medium. Unstimulated controls were treated the same, except that instead of the induction medium was basic medium used. Evidence of the formation of mineralized matrix was histologically (see von Kossa staining). **2.4 Molecular biological analysis**

Material:

• 5415C tabletop centrifuge (Eppendorf, Hamburg)

)

**.4.1 RNA isolation**

• vortexer VF2 (Janke & KunkelI KA Laboratory Technology

• PicoFuge (Stratagene Heidelberg)

**2**

Material:

• RNeasy kit ink. QIAshredder homogenization kit and DNA digestion with the kit

Sigma, Munich)

Major fü

RNase-free DNase I (Qiagen GmbH, Hilden) • Mortar and gun cartilage pieces

• cells, pellets, or

• Liquid Nitrogen

• Ethanol 70% 2-mercaptoethanol (

• chhrung:

A contamination

To avoid contamination with RNases, were all working with RNA

**onzentrationbestimmung of RNA**

performed with gloves. Total RNA isolation was performed under standardized conditions, using the RNeasy kit. For total RNA isolation, cells were used after trypsinization and washing with PBS. For extraction of RNA from the pellet culture and native cartilage were the first biological sample in a mortar using the Pistel crush (for cartilage using liquid nitrogen) and then worked up according to the manufacturer's instructions with the kit. The RNA was stored as a total RNA in RNase-free water and sterilized 1.5-ml tubes as aliquots of 15μl at -80 ° C approx. Working with the RNA and thawing of the aliquots were performed on ice.

**K**

Implementation:

The RNA concentration of a solution in the photometer (Eppendorf GmbH, Hamburg)

using the optical density determined. Is the measurement at 260 nm while the RNAGehalt

at at 280 nm of the protein content of the sample is shown. The ratio between

**30**

Materials and methods

260 nm and 280 nm is a measure of the purity of the sample. This was in all the tests in normal range 1.7 to 2.0. The samples were used for the measurement of H 2 O in the ratio 1:50 (v / v). The RNA concentration was calculated using the following formula: **concentration in mg / mu.l = absorbance × dilution × 0.04 2.4.2 cDNA Synt**

**hese**

Marerial:

• cDNA synthesis kit: Cloned AMV First-beach cDNA Synthesis Kit (Invitrogen,

plex (Eppendorf, Hamburg)

tion:

Netherlands)

• ThermomiDurchführ

Kits for the D

t or the cDNA isol - synthesis, the RNAie

measure described above, so that you too for the comparative

• 10 mM dNTP - Mix 1 mu.l

• DEPC. H

CNA was used RNA synthesis solution, previously with the RNeasy

ierworden (see RNA isolation) was. V

Concentration w

Could always use the same amount of RNA samples. For the synthesis of cDNA molecules that are complementary to the mRNA molecules, the cDNA synthesis kit was used. As a primer oligo (dT) primer were used. The composition of the complete reaction mix (per sample) was: • oligo (dT) primer 1 mu.l • ng RNA in RNase-free water 100

Mu.l 2 O at 13

• 5x First beach mu.l of buffer 4

• RNAseOUT (40 U / uL) 1 mu.l

• 0.1 M DTT, 1 mL

• Reverse transcriptase (AMV) 1 mu.l

• The total mu.l 20 ng) and then 60 minutes at 50 ° C

e eve ranskriptase incubated for 5 min at 85 ° C

iv ea r further use at -80 ° C.

Asatz was 5 minutes at 60 ° C (Annealin

birt. Subsequently, the T Rrse

d

ktiert and the cDNA synthesis product to

stored for **determining the concentration of DNA.** Implementer: The concentration of DNA was determined by two different methods

carried out:

**31**

Materials and methods

First measurement of optical density by absorption spectrophotometry using the

ppendorf GmbH, Hamburg). The DNA concentration is photometrically

s used, the wavelengths of 260-280 nm. The samples were used for

retical fractionation may

Photometer (E

by measuring the optical density of DNA solutions are determined. For the measurements

the measurement with H 2 O in the ratio 1:50 (v / v). Second, the estimation of concentration by agarose gel electrophoresis. To determine the concentration of DNA samples that could not be determined photometrically, was a reference DNA (DNA standard, Invitrogen) of known concentration and size applied to the gel. After elektroph

Concentration of the sample are compared to the estimated reference DNA **2.4.3 DNA separation in agarose gel** material - DNA: • Agarose powder (Biorad, Laboratories GmbH, Munich).

• electrophoresis with accessories (New England Biolab, Frankfurt aM)

EDTA, pH 8.0 (acetic acid)

H, Karlsruhe)

idiumbromid solution (10mg/ml)

ot G3 and software

Major fü

• 1x TAE buffer (40 mM Tris-acetate, 1 mM • 6xDNA sample buffer Roti-Load DNA (RED

• Eth

• Gel Documentation BDA Digital Camera Canon Powershot Sh

BioDoc Analyze (Biometra GmbH, Göttingen)

• Standard size 100 bp DNA ladder (ROTH, Karlsruhe)

chhrung: For e horizontal agarose gels were used.

Dab r, the

ments occurred were 1.0% and 2.0%-owned

Agarose gel in 1 × has been

Tues separation of DNA fragments

the concentration of agarose gels according to the size of eiichtet Fragmenteennt should be. Depending on the size of the fragments

Agarose

e used gels in 1 × TAE buffer. To prepare the

TAE buffer, boiled. The addition of 0.5 mg / ml ethidium bromide was carried out after cooling the gel solution to about 50 ° C. Then the gel was poured into the gel tray. As running buffer 1x TAE buffer was used. Depending on the thickness and density of the gel, different voltages (from 50 to 120 V) were applied. For all samples was in front of each gel run 6xDNA sample buffer in the ratio 1:6, where (v / v). The visualization of DNA fragments in the gels was ethidiumbromidhaltigen under UV light and documented photographically. The determination of the size of DNA fragments in agarose gels was carried out by the added size standard. **32**

Materials and methods

**2.4.4 Polymerase chain reaction (PCR)**

aterial:

M

• Platinum ® Taq DNA Polymerase kit (Invitrogen, Netherlands)

• TGradient PCR machine (Biometra, Göttingen)

• Primers: **Primers for PCR**

**G**

**s sequence**

**Product**

**Size (bp)**

**Tm**

**° C**

GAPDH

forward - ACCACAGTCCATGCCATCAC

reverse - TA TTCAC

CACCCTGTTGCTG

455 55

Collagen Type I

(Col1a

1)

forward - TGACGAGACCAAGAACTG

reverse - CCATCCAAACCACTGAAACC

660

55

Collagen type II

(COL2A1) forward - GGCAATAGCAGGTTCACGTACA

reverse - CGATAACAGTCTTGCCCCACTT

614 62

Osteocalcin

forward - CATGAGAGCCCTCACA

reverse - AGAGCGACACCCTAGAC

310 52

ALP

forward - ACGTGGCTAAGAATGTCATC

reverse - CTGGTAGGCGATGTCCTTA 475 53

**Table 1: a primer**

hführung:

**ür PCR**

Durc

For a PCR reaction mix was used per reaction, 100 ng of template cDNA.

De pipetted in a volume of 25μl as follows.

Component volume final concentration

mu.l 1X

s reaction was

PCR buffer 2.5

Mu.l of 10 mM dNTP mix 0.5 mM 0.2 mM MgCl 2 0.75 50 mu.l 1.5 mM

Primer Mix

Mu.l ≥ 1

mu.M each 0.5

CDNA template

Mu.l ≥ 1

approximately 100 ng

Polymerase

Mu.l 0.2

2 Units

H 2 O

add 25 ml

**33**

Materials and methods

The PCR reaction was em na program:

tep process Tm (° C) time

Denaturation 95 ° C, 2 min. -

Denaturation 95 ° C, 30 sec.

see primer table

sec.

n k.

Repeat steps 2 to 4 34

Repetitions

The analysis of the RT-PCR was performed in a 1-2% agarose gel (see DNA separation in

arosegel).

Following ch

S

1

2

Primer annealing 3: Depending on primer 30

4

Elongation

72 ° C;

30 se

5

Elongation

72 ° C;

3 min.

6

Store

4 ° C;

~

Ag

**2.4.5 Quantitative PCR (qPCR)**

Materials: • LightCycler II (Roche GmbH • Master SYBR Green I Ki

Mannheim)

t (Roche Diagnostics GmbH, Mannheim)

• LightCycler capillaries (Roche Diagnostics GmbH, Mannheim)

• Primers: **Primers for qPCR**

**Gene**

**Sequence**

**Product**

**Size (bp)**

**Tm**

**° C**

**Reference**

CAP H

D forward - GAGTCAACGGATTTGGTCGT

reverse - TTGATTTTGGAGGGATCTCG

238 57 NM\_002046

Collagen

Type I

(Co

l1a1) forward - AGGGCCAAGA

CGAAGACATC

reverse - TGTTGTGCGATGACGTGATCT

137

NM\_000088 57

Collagen

Type II

(COL2A1)

forward - CTCCTGGAGCATCTGGAGAC

reverse - ACCACGATCACCCTTGACTC

152

61

NM\_001844Kollagen

Type X

forward - CAGGCATAAAAGGCCCACTA

reverse - AGGACTTCCGTAGCCTGGTT

179

57

NM\_000493

Sox 9

forward - ACACACAGCTCACTCGACCTTG

reverse - GGAATTCTGGTTGGTCCTCTCTT

102 61 NM\_000346

**Table 2:**

**Primers for qPCR**

**34**

Materials and methods

IMPLEMENTA

ng:

For quantification of mRNA from cells was carried out as follows. And RNA was

2.4.1 better in isolation, as described in 2.4.2 in

cDNA and transcribed using the Light Cycler technology, according to the

He r eason was pipetted for each sample, the following approach:

chrieben from cells or cell aggregates

REATING quantified. Hie

• 10x master mix SYBRGreen 1.5 mu.l of 25 mM MgCl 2 1.8 • • mu.l Primer mix 0.2 mu.l cDNA template • ≥ 1 mu.l (100 ng) • H 2 O to 15 ul

The i of 15μl was 400 nM (per primer).

For an R o mmer pipetted ne template cDNA. The

Rea io amplicon were as follows

Pro.

ling: Depending on primer

see primer table

5 sec.

n k.

44

Repetitions

° C;

C;

Detektio szenz vo R Green took place during the synthesis phase in

° C. After Gram always a melting analysis of the obtained

Products made. This product specificity was checked. Were analyzed

Samples, only one d elzku alyse

Prmerpaarkonzentration in the reaction volume

Oh i was Kntrolle eaktionsansatz

ktn reaction mixtures containing at least two per

conducted program

Step process, Tm (° C) first time Denaturation 95 ° C, 10 min. - 2 denaturation 95 ° C, 1 sec. Third PrimeranneaSchritt 2-4

4th

Elongation

72 ° C;

20 se

6th

Melting curve

of 65

to 95 °

-

7th

Store

4 ° C;

~

The

n the fluors

n SYB

72

End of each

ms was

The

ie in the Schm

rvenan

n distinct peak, ie a product

ufwiesen. Been made ​​in establishing the method for each PCR run, the

LightCycler software data exported to Excel format, the initial concentration of

a

control PCR products obtained in both by-products as well as to their identity (size of the product) in an agarose gel. The analysis of the PCR data was performed using the Roche LightCycler Software Ver.3. The absolute quantification was performed using the program LinRegPCR Ver 7.5 in 2004 (Amsterdam, Netherlands). The program allows, on the basis of the

**35**

Materials and methods

Zielgenmolekülen in the solution used to calculate cDNA (Ramakers et al., 2003). The relative quantification of target gene was performed using the *housekeeping* gene

GAPDH, whose expression in the cell is assumed to be constant (Barber et al., 2005). Of each cDNA as a reference value, the concentration of GAPDH was determined and normalized to the cDNA concentration (Kuhne and Oschmann, 2002). The expression values ​​were analyzed with the spreadsheet program Excel 2003 and represented graphically **analyzes 2.5.1 Histological sections 2.5 protein** material. • Tissue Processor (Citadel, ThermoShandon)

• microtome (SM 200R Schlittenmikrotrom, Leica)

Auda-Königshofen)

inthe ED 53

l)

itrogen)

absolute anol (Merck KGaA, Darmstadt)

• Slides Histo Bond (Paul Marienfeld GmbH & Co.KG • L incubator WTB B

• GFL water bath (Lab GmbH Burgwede • PBS (Gibco 14190-994, inv

• Eth

• Formalin (4% in PBS, pH 7.4, Merck)

• Antibodies (see Table 3)

Primary antibodies

Per n

tei host / producer isotype dilution comments

CD 45

Mouse/IgG1 Acris 1:100 mono / FITC / F

CD 90

Mouse IgG1 1:100 Acris mono / FITC / F

CD 5

10 Mouse IgG1 1:100 Acris mono / FITC / F

CD 106 Mouse IgG1 1:100 Acris mono / FITC / F

STRO-I Mouse IgM

RD System 1:200 mono / F

Isotype Control

Mouse IgG

Acris

1:100

FITC / mono / F

Collagen Type I

Mouse IgG2

Acris

1:100

Mono / C

Collagen Type I

Mouse IgG1

Medicare Corp.

1:100

mono / PS

Collagen Type I

Goat / IgG

Southern

Biotechnology

Associates

1:1000

poly / WB

Collagen type II

Mouse IgG2

Acris

1:100

Mono / C

Collagen type II

Mouse IgG1

Medicare Corp.

1:100

mono / PS

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Materials and methods

Collagen type II

Goat / IgG

Southern

Biotechnology

Associates

1:1000

poly / WB

Collagen type VI Mouse IgG2

Medi Corp. 1:100 mono / PS / C

**Secondary antibodies**

Goat anti-rabbit

IgG

Southern

Biotechnology

Associates

1:4000

WB

Goat anti-Mouse ...

IgG

Southern

Biotechnology

Associates 1:200 (H + L) Biotin

Goat anti-Mouse

IgG

Chemicon

1:200

(H + L) Biotin

Goat anti-Mouse ...

/ IgG

Invitrogen 1:500 PE / C

...

Goat anti-Mouse

/ IgG

Chemicon 1:200 FITC / C

...

Goat anti-Mouse

/ IgM

Acris

1:1000

FITC / F

**Table 3: Antibodies**

expand abbreviations in CC-par lease, WB blot Fdurchflusszytometri

.

**he**

Abbreviation

the table: -

ytospins, PS

affinschn

West-

cal analysis

Implementation:

After the pellet culture for at least 24 h, the pellets were fixed in formalin. Anus

washing in PBS, the samples through an ascending alcohol series (70% -

100%) dehydrated. For embedding a Tissue Processor was used. The

solvent contained in the samples was carried at 56 ° C in paraffin machines

first

s and incubated overnight at 37 ° C

ow. With the help of a microtome tissue sections were prepared from 5 microns in thickness.

Thereafter, the sections were mounted on slides

**2.5.2 Immunostaining** dried material. • hyaluronidase 0.5 mg ml -1 in 0.1 M phosphate buffer, pH 5.5 (Sigma, Munich) • PBS (Gibco 14190-994, Invitrogen, Karlsruhe)

• coverslips (Paul Marienfeld GmbH & Co.KG, Lauda Königshofen)

**37**

Materials and methods

• Alkaline phosphatase - reagent (Vestastain, Vector Laboratories, USA) • hemalum solution (Merck KGaA, Darmstadt)

• Ultra Mount (Dako, Hamburg)

2)

stratlösung:

Major fü

• Antibodies (s Tab

• Sub

150 ml Tris / NaCl buffer pH 8:24 300 mg sodium nitrite

Mu.l Neufuchsinlösung 300 (5% in 2 M HCl)

20-30 naphthol AS-BI phosphate in 750 mg mu.l of dimethylformamide

chhrung: The hi munfärbung first with xylene

developed a growing alcohol series (100%, 96%, 90%,

70% distilled water.) Rehydrated. Subsequently, the epitopes of hyaluronidase

unmasked at 37 ° C in ends. Then the sections were

with 10% F to block the nonspecific binding for 30 min

dealt with. mmer

histological sections needed for the

parffiniert and connectable

from one end by

a moist chamber for 3 Stu

CS in PBS at 37 ° C z

The incubation with the primary antibody

rn was performed in a humid Kai 4 ° C. Then the sections were washed twice in PBS

overnight be

washed. The incubation with the secondary antibodies was performed for an hour in a moist chamber at room temperature. At the next step, the alkaline phosphatase reagent was applied to the sections and incubated for 30 minutes. After three washes with PBS, followed by incubation with substrate solution for 30 min in the dark. Thereafter, the sections were washed with tap water and in *A.* *least.* transferred. Counterstaining of cell nuclei with 1:5 (v / v) *with* distilled *water.* Hemalum solution was diluted for 30 sec in Coplin jars. Finally, the sections were incubated for 10 min. blued in tap water. Eindeckeln cuts to the ultra-Mount and coverslips were used **2.5.3 Semi-quantitative determination of collagen (PAGE, Western blot).** Material: 6 M guanidine • in 0.2 M Tris pH 8.5 (Sigma, Steinheim) • dithiothreitol (Serva, Heidelberg) • 0.6% and 10% acetic acid (Merck KGaA, Darmsta • JAA solution: 0.1 M iodoacetamide in DMSO (Sigma, Steinheim)

dt)

• dialysis solution: 250 ml of 6 M urea (Serva, Heidelberg), 2.5 ml of 0.1 M PMSF

• Methyl red solution: 2% methyl red in 0.6% acetic acid (Merck KGaA, Darmstadt)

10% separating gel for gels: 10 M urea (60 ml), distilled water. (17.5 ml), glacial acetic acid (7

ml), 14 ml 50% acrylamide solution (Biorad, Laboratories GmbH, Munich), APS -

MED -

• (Hoffmann-La Roche Ltd., Basel, Switzerland) in DMSO, 1 ml of solution JAA

• 7.5

Mu.l 10% ammonium 750 (Merck KGaA, Darmstadt), TE

**38**

Materials and methods

N, N ', N'-tetramethylethylenediamine mu.l 750; (Biorad, Laboratories GmbH,

00 mu.l glacial acetic acid, 4

•

(

hlor-3-

Major fü

Munich)

• 4% stacking gels for 10: 31.6 ml of urea, 13.4 ml of distilled water, 3 ml of acrylamide, 350 and 350 mu.l APS mu.l of TEMED.

PBS (10-fold: 0.58 M NaHPO, 0.17 M NaHPO, 0.68 M NaCl)

• 2424

• least 20 s in PBS, 0.02% sodium azide (Merck KGaA, Darmstadt) in A.. Wash buffer: 0.2% casein (I-block, Tropix, Bedford, USA) / 0, 1% Twe

• Blocking buffer: 0.5% casein / 0.1% Tween 20 (Serva Heidelberg) in distilled PBS and 0.02% sodium azide in A.. Streptavidin-alkaline phosphatase (AP) (Invitrogen Netherlands) 1:4000 inWaschpuffer

• AP substrate buffer: 0.1 M diethanolamine (Tropix, Bedford, USA) / HCl, pH 10, 1 mM magnesium chloride (Merck, Darmstadt), 0.02% sodium azide substrate solution for AP: 66 mu.l p-Nitrotetrazolium

• Blue-Sigma Steinheim, 50 mg / ml in 70% dimethylformamide, Merck KGaA, Darmstadt), 33 mu.l of 5-bromo-4-C

Indolylphosphate-toluidine salt (Sigma Steinheim, 50 mg / ml in 100% dimethylformamide) in 10 ml of substrate buffer.

• Blot membrane: PVDF membrane (Biorad, Laboratories GmbH, Munich)

chhrung: n I e Koll and collage

lets initially using

in 500 t

not ae 30

Mu.l 50 minutes were up 10% acetic acid

by 1 and 0 -

100 ul of sample removed. As a dialysis solution was added 250 ml of 6 M

agen II can be isolated by gel electrophoresis auftgetrennt (PAGE) demonstrated undWesternblot. For this purpose, Pel

mu.l guanidine hydrochloride long 2 hours at 60 ° C. (meisuf solve it yourself!). After the addition of 50 mL dithiothreitol, each sample was further incubated at 60 ° C. To the reduced Pr

Mu.l 5 JAA solution was added. Then, the guanidine hydrochloride

hour of dialysis

Urea, 2.5 ml PMSF and 1 ml JAA solution in 0.6% acetic acid applied. Finally, the total sample volume of 10-methyl mu.l solution was added. Then came the electrophoretic separation, which was performed in 7.5% acrylamide gels in the presence of acetic acid and urea. For the production of the separation gels were used for gel solution of ammonium peroxodisulfate (APS) and N, N ', N'-tetramethylethylenediamine (TEMED) were added for polymerization. The gel was then with 500 mu.l 0.6% acetic acid, and covered with a layer could polymerize for one hour. Thereafter, the stacking gel (4%) were poured and allowed to stand overnight to polymerize. The cleaning of the gels was performed in the electrophoresis in 0.6%

Acetic acid at 100 V for one hour. The collagen - collagen type I were standard (100 ng, beef) and collagen type II (50 ng, beef) from Southern Biotechnology Associates, applied in each case in the first two bags. Of each dialyzed sample was added in the other pockets each 10 mu.l. Was then applied to the gels have a voltage of 100 V for 30 min and 200 V for another 150 minutes.

**39**

Materials and methods

The blotting was carried out in 0.6% acetic acid at 80 V for one hour. Thereafter, the blot membrane to develop first for 5 minutes in PBS and then incubated for one hour at room temperature in blocking buffer. The incubation with primary antibodies (see Table 1) was carried out overnight. Then the membrane was washed twice with wash buffer and then incubated for two hours with the secondary antibody (see Table) at RT. Min after a fresh wash of 5 in the gels wash buffer for 30 minutes with alkaline phosphatase at RT were treated.

This was followed by three washes for 5 minutes in wash buffer and two washes of 5 minutes each in substrate buffer for AP. After a final incubation in substrate solution for AP in the dark at RT for 15 minutes, five minutes twice in A. Blot membranes were washed and dried least **2.5.4 Creation of cytospins** material. Cyto chamber top centrifuge (Hettich, look at yourself in the lab) PBS (Gibco 14190-994, Invitrogen) Histo Bond slides (Paul Marienfeld GmbH & Co.KG, Lauda Königshofen)

Acetone (Merck KGaA, Darmstadt

Implementation:

The knowledge gained from the Alginatkultur Pseudochondrone were using the

centrifuged

Cyto chamber with rubber sealing ring by a

hrough the

surface of cells of 0.85 cm 2. Per slide were

a. 10 000 Pseudochondrone from the alginate culture in a volume of 200 mu.l PBS

ch removal of the liquid, the slides were from the

Cyto chamber and centrifuge essay on specially coated slides

(Min 200 g \* 10). It was the

Clip to slide down and placed in an essay. D

Centrifugation resulted in a circle

c

is used. Well

Solved cyto chamber and dried. For storage at -80 C, the cytospins frozen and thawed as needed later lying. The obtained cytospins were fixed with acetone for one minute and immunostaining performed **Pseudochondronen 2.5.5 fluorescent labeling of** material. Stock solution of DAPI (5 mg / ml stock solution 0.1 mg / ml in methanol)

Methanol (Merck KGaA, Darmstadt)

Fluoromount-G (Biozol Sales GmbH)

**40**

Materials and methods

Coverslips (Paul Marienfeld GmbH & Co.KG, Lauda Königshofen) Procedure:

The fluorescent staining of the cytospins was performed as described above for paraffin sections,

ronidase lasted only 30

min. In addition, the cytospins with fluorescently labeled secondary antibodies

incubated in the dark. Was then washed 3 times with PBS and

and using Fluoromount-G with coverslips

nkeln (max. 24 hours)

but with some differences: the unmasking of hyaluronic

(See Table 1)

DAPI for 15 min in the dark at room temperature. Finally, the

Cytospins washed 3 times with PBS

coverslipped. They were stored at 4 ° C in You

**2.6 Other techniques 2.6.1 Determination of DNA** material: (Victor 1420 multilabel counter, Wallac Distribution GmbH, Freiburg) fluorometer • • Thermomixer 5436 (Eppendorf, Hamburg) • Papain solution: 500 mu.l of 0.1 M NaH2PO4 / 0.005 mM EDTA, pH 6 ; 5 mu.l Mercaptoethanol, 2.5 mu.l papain solution (10 mg / ml, F. Hoffmann-La Roche Ltd.,

Basel, Switzerland)

• DNA dilution buffer (2.5 M NaCl / 17.5 mM sodium citrate, pH 7)

5 mM sodium citrate, pH 7)

s DNA, F. Hoffmann-La Roche Ltd., Basel,

• bisbenzimide solution (stock solution 2 mg / ml bisbenzimide in DNA-buffer approach,

rva Feinbiochemica GmbH & Co., Heidelberg

Major fü

• Approach buffer (2 M NaCl, 1 • DNA standard (25 micrograms / ml Kalbsthymu

Switzerland).

Se

chhrung: One is by qg

Application of GAG and DNA - analysis allows.

Rix on each cell

give e

For an e

60 °

Eppend 50μl of the solution obtained with

NA-dilution buffer 1:5 (v / v). As a standard used was a 1:5 diluted DNA

For the determination of DNA in all wells of the microtiter plate was

further diluted. This resulted in a dilution series for each sample. Subsequently

uantitative s statement about the cartilage-specific Matrixbildun The GAG / DNA values ​​were

statements about how much glycosaminoglycan and thus how much Mat

ildt was

Bstimmung the DNA content of the samples overnight (16 hours) b

C/800rpm in a Thermomixer in 500μl with papain-digested solution filled orfgefäßen. For the determination

D

Standard solution.

Mu.l buffer 100 presented approach. In the first line in two wells 100 may mu.l of the diluted sample and then 1:2 (v / v) into the underlying wells ever

**41**

Materials and methods

mu.l were added to each 100 dye solution. To determine the DNA content of each sample was duplicated in 96-well microtiter plates measured fluorometrically. The

Measurement of the plates was done at an excitation of 360 nm and an emission of 460 nm in the computer-based fluorometer. The measured values ​​were issued in the format of Excel files and using a regression analysis (Excel Macro; Adamietz) evaluated **2.6.2 GAG determination.** Material: • Photometer (counter Victor 1420 Multilabel, Wallac Distribution GmbH, Freiburg) • Thermomixer 5436 ( Eppendorf, Hamburg) • Papain solution: 500 mu.l of 0.1 M NaH2PO4 / 0.005 mM EDTA, pH 6, 5 mu.l Mercaptoethanol, 2.5 mu.l papain solution (10 mg / ml, F. Hoffmann-La Roche Ltd., Basel, Switzerland ) • dilution buffer (2 M NaCl / 15 mM sodium citrate, pH 7) • Standard GAG: chondroitin sulfate C from shark 10 micrograms / ml (Sigma, Steinheim) • 1.9-Dimethylmethylenblauchlorid solution: 27.2 mg / l 1, 9 -

Dimethylmethylenblauchlorid (Serva, Heidelberg), 5168 g / l glycine, 4.03 g / l NaCl,

32.3 ml / l of 1 M HCl, absorbance at 525 nm 0.530

Implementation:

To determine the GAG content was previously developed for the analysis of DNA

Papainpuffer made

Sta-us a chondroitin sulfate-C produced. The

Me n ow the DNABestim

be

e ellt train. Thereafter, in

s all customer

eter at a wavelength at u

of 53 r

sis.

e Matrixverdaulösung reused. To create a GAG

ndrdkurve advance a stock solution was a

ssug was well into 96-well microtiter plates wmung performed. In all 100 wells were mu.l least a.. submitted, 100 l of each digested

egben and a dilution series as described above until

mu.l WLLs 150 1.9-Dimethylmethylenblauchlorid solution was added and 30 seconds

Ramtemperatur incubated. The measurement was performed in Photom0 nm was also evaluated as a means of determining the DNA

Regression

**2.6.3-dimethylmethylene blue dye (DMMB)** Material: • Cooking Oils solution: 78 mM Dimethylmethylenblauchlorid 1.9 (Merck Darmstadt) at least a. • PBS (Gibco 14190-994, Invitrogen). Implementer: For qualitative detection of sulfated glycosaminoglycans which are a crucial component of hyaline cartilage, which were isolated from the alginate cell

**42**

Materials and methods

mixed solution - with the dimethylmethylene blue. After 5 min. Incubation, the cells were washed with PBS and thoroughly documented photographically.

**ian - blueness**

Ma ia

**2.6.4 Alc**

rrrr: 10% formalin (10 ml of t he • 37% solution to 27 ml of PBS)

) 50 mg of Alcian blue-Lösung8 GX (Sigma Munich) in 50 ml 0.9%

• Alcian Blue solution:

a

NaCl plus 6 ml of conc. Acetic acid, pH 1.5 b) 4 g MgCl 2 (Fluka No. 63 063) in 50 ml 0.9% NaCl mix a and b after 3 days through a 0.45 micron filter filter • 2% acetic acid (2 ml of conc. Acetic mix with 98 ml ad) Implementation:

With the Alcian blue staining of histological evidence of acidic Proteolykanen done.

And that made ​​the first Alginatsbeads *in toto* for an hour in 10% formalin

wash in 2% acetic acid and overnight at RT

u-colored solution. Subsequently, the first Alginatsbeads 3 x in 2%

Ess ä te the microscopic

Evaluation and photographic documentation.

**2.6.5 Oil Red**

Material:

g

fixed at RT. Then for 5 min

Alcian-Bla

igsure least 3x and then into A.. washed. After this successful

**O staining (Adipozytenfärbung)**

Oil d

pA

Re O stock solution

0.5 g Oil Red O in 100 ml of isopropanol

Oil Red O staining solution solution stock solution / H 2 O, (6v/4v) let stand 24 hours and then filter implementation: Oil Red O is a dye that accumulates in the lipid content of tissues. The vital staining with Oil Red O neutral fats appear red. Thus one can

Cytoplasma stored

sen was the aspiration of the culture medium after 30min. with the staining solution

incubated. After several washes with PBS could be in

n recognize. The data were photographically

increasing number as well as the widening of the Z

Fat droplets seen on the culture period.

The Zellra

overlaid and at RT

Red colored light microscope Fetttröpfche

documented.

**43**

Materials and methods

**2.6.6 Von Kossa staining (detection of osteogenic mineralization)**

Material:

• 5% silver nitrate solution: 100 mg of silver nitrate in 2 ml of Aqua solve Bidistilled

• 1% pyrogallol: 20 mg pyrogallol in 2 ml double-distilled water dissolve • 5% thiosulfate: 100 mg of sodium in 2 ml double-distilled water • solve formalin (10% in PBS without Ca, pH 7.4, Merck) Procedure:

With the von Kossa staining, the formation of a mineralized matrix is demonstrated. The carbonates and phosphates in the matrix-bound calcium is exchanged unresolved by silver ions and reduced to metallic silver. Calcium-containing zones are stained brown-black and so clearly reveal the degree of mineralization.

For the von Kossa staining, cells were fixed for 10 min in 10% formalin. Anus

x with A.

ash. Then the cells were covered with 2-3 min and 1% pyrogallol

dan h for 5 minutes

with s and air dried.

**sscytometrische analysis**

ät

r

ight (SC) and recorded as a measure of the granularity of the cells (scattering

. hen

Incubation (30 min) were dissolved in 5% silver nitrate solution in daylight for 2 cells

least weight

ac several times with distilled A.. washed. The cells were then

disabi sodium thiosulfate solution, fixed 2 x A. least. washed

**2.6.7 flow-**

By flow cytometry, both the cell size and granularity

the cells as well as fluorescently labeled antibodies to various surface markers and intracellular molecules and examine evidence. The flow cytometric analysis of this work were performed on the device Cytomics FC 500 (Beckman Coulter). For this purpose the cells with fluorochrome-labeled monoclonal antibodies (see Table 3) were stained and analyzed by light scattering and fluorescence intensity. The light scattering in the forward direction (180 ° - Forward Scatter) is a measure of the size of the cells (small cells scatter less light). The 90 degree wide angle scattered light, a small portion of the light is, as side scatter

L

Membrane folding of the cells affected) regarded

Materials: • cytometer Cytomics FC 500 (Beckman Coulter) • tabletop centrifuge 5415C (Eppendorf, Hamburg) • vortexer VF2 (Janke & KunkelI KA Labortechnik) • Buffer (Beckman Coulter) • wash buffer (PBS containing 1% FCS and 0.1% NaN) • Fixierpuffer ( PBS with 1% paraformaldehyde) • Antibodies (see table 3) 3

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Materials and methods

Procedure: The cells to be analyzed were first washed with Dulbecco's PBS pH 7.4, and fixed for 10 minutes in ethanol. After centrifugation at 8000 rpm for 5 min, cells were taken in washing buffer with FCS, non-specific binding sites to

. lockie Thereafter, the cells in 1.5 ml tubes according to the number of

divided. For FACs analysis were at least 100,000 cells per assay

different n 5 min, 1,000 rpm, 4 ° C)

Xt 2 min, 1,000 rpm, 4 ° C). Cells with 5 -

Resuspend able 2 10 d) and 60

Min at times with Wash Buffer

what ep were rn the cells in 500 ml

Fixi For non - conjugated antibodies, cells were treated with

usgetesteten antibody concentrations of secondary antibodies

ondronen was an important measure of the

hlten area was measured and

signed the macro would

Number of green or red areas (FITC and PE for the matrix) with the number of

b

Attempts

wedet: 1 each - Centrifuge 5 x 10 5 cells in 1.5 ml tube (

Wed 100 - 150 mu.l wash cold washing buffer (5

mu.l he been tested antibody concentrations (see T

. The cells were then ren. 2 e

Dark at 4 ° C. Incubate

. chn In directly with FITC-labeled antibody

Date erungspuffer.

5-10μl of a

resuspended, incubated for 30 min in the dark at 4 ° C and finally also included in fixing buffer. The fluorescently-labeled cells were kept on ice until measurement in the dark. The flow cytometric measurement was performed at Cytomics FC 500 (Beckman Coulter). The evaluation of the percentage of labeled cells was performed using the software (Beckman Coulter CXP) to unlabeled cells (for analysis directly after Ficoll gradient) or against CD 45 (in the analysis of cells after adherence). Due to the possible autofluorescence of cells, the samples were incubated for control with a control antibody and analyzed (isotype control or secondary antibody in the indirect labeling). **2.6.8 Quantitative analysis of stained cytospins** The quantitative evaluation of the number of Ch

Differentiation status of cells. For this purpose, the cartilage-specific collagen II Pseudochondrone against collagen VI and collagen I were stained and compared. After recording the images of the cytospins using the fluorescence microscope using the program Lucia G (Nikon GmbH, Düsseldorf) were evaluated. This software worked with to RGB (Red Blue Gün) which defines for each color intensity range from 0-255. This was the program, the colored areas (eg FITC or DAPI) both recognize and count their number and their area. The area of the AusgewÃ

acted as a mask. After the program for the GE

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Materials and methods

blue areas (DAPI for cell number) are compared in this screen. The blue dots (nuclei) that have a green or red region (matrix) were considered as labeled cells. In addition, the surfaces of the stained areas could be measured. As a result was able to not only the number of stained cells, but also the relative area of the stained areas (green and red

**DNA calibration curve**

He was number of cells in pellet culture indirectly by measuring the DNARegressionsanalyse

of experimentally

DNA obtained initial values ​​used. Therefore, a calibration curve formed

Are color-field area by the number of blue points determined). In the Appendix an example *screenshots* demonstrating the method are presented. **2.7 Analysis 2.7.1 GAG / DNA analysis (G / D ratio)** As the number of cells depending on culture conditions varied in the experiments were not always the absolute GAG levels to be compared directly be. For this reason, for the statistical analysis of the GAG / DNA ratio formed (G / D ratio). For this purpose, the GAG / DNA - ratio formed (GAG content by the DNA - content). The resulting value is the ratio of the number of cells cultured on matrix. This analysis allowed both comparison and the statistical evaluation of the various tests.

The determination of d

Salary. For the evaluation, a

To verify the accuracy of cell counting, the values ​​obtained were compared with literature values ​​retrieved from DNA content (Baserga, 1989). To construct the calibration curve of the third passage cells were used. The cells were adjusted to the respective cell number (Table 2), immediately centrifuged and digested. Cell count 12 500

0 250000 500000 750000 100000

0

125 000

0

15 000

00

175 000

00

20000000

Number of samples 5 6 5 5 5 4 3 5 4

**Table 4:. The cell number and number of samples taken for DNA calibration curve,** the measured values ​​of the samples differed for the calibration curve is not significant (R 2 = 0.9987, p <0.001) from the expected values ​​(Fig. 10).

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**0**

**2000**

**8000**

**10 000**

**12 000**

**16 000**

**1x105 5x105 1x106 2x106 1.5 X106**

**14 000**

**4000 DN**

**6000 A**

**Amount [ng]**

**Cell number**

**Figure 10: DNA calibration curve - comparison of the regression of the measured and expected**

**Values**

The expected regression line is indicated by the dotted line. The means and

Standard deviations are shown.

**2.7.2 Statistical analysis and bioinformatics**

For the determination of qualitative data (eg blots, or dyes), the

Minimum of two samples analyzed for each experimental conditions. For the

statistical analysis of the experiments were used at least three samples. The

Statistical analysis of experimental results was carried out using Microsoft Excel 2003.

The respective mean values ​​with standard deviation and the regression line

specified. The analysis indicated significance of their results were

de

Were design and database search - for the sequence analysis, the primers

following

• National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov

• UCSC Genome Bioinformatics http://genome.ucsc.edu/

Er3): http://frodo.wi.mit.edu/

Identified **4000 DN 6000 A 14000** m t-Student test.

following online tools used:

• Whitehead Institute for Biomedical Research (Prim • OligoPerfect Invitrogen ™ Designer: http://www.invitrogen.com/

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**3.Ergebnisse**

The aim of experimental investigations of this work was to develop a method for chondrogenic differentiation of human bone marrow mesenchymal stem cells occurring. The results section is divided into the following four parts:

• extraction of cells

• proliferation *(in vitro* multiplication)

• Differentiation in two - or three - phase - Model

• matrix formation

Since the isolation and proliferation of cells *in vitro* methods used to influence the outcome of differentiation very much, these steps are presented in separate chapters. The following is a comparison of advantages and disadvantages of the two differentiation models. In the last section, the ability of differentiated cells to form cartilage after aggregation is investigated. Additional records can be found in the appendix.

The following diagram (Fig. 11) shows all the essential steps of the work, whose results are described below in detail. **Proliferative 3 -4 weeks of alginate culture 1 -3 weeks 3 WochenGewinnungder pellet culture cells DEX ± ± ± TGF IGFAdhärenzAussaatdichte ± bFGF PDGF ± ± ± O2 ± TGFZelldichteDauer DEX ± ± TGF-IGFErstellen derAlginat KulturSedimentieren proliferation of cells ± IGF IGF ± 3 cells**

**Figure 11: Schematic representation of the sequence of trials**

Unless otherwise indicated, all experiments were performed under standard conditions, which are listed in the "Materials and Methods." Changes to this are always shown in the legend to the figures.

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**3.1 Separation of cells on the Ficoll gradient**

To isolate a suitable population of human mesenchymal stem cells (hMSCs) were caused by aspiration from human bone marrow-derived cells first subjected to centrifugation on a Ficoll gradient (s 2.1.1) to separate mononuclear cells from erythrocytes and granulocytes. In Figure 12 is indicated, extracted at the position (yellow marking) nucleated cells. **PlasmaFicoll** were **(granulocytes) ErythrozytenGranulozytenMononukleäreZellenLymphozyten, MonozytenStammzellenPlasmaFicoll (MonozytenStammzellen**

**Figure 12: The separation of the cell suspension on Ficoll gradient**

The phase boundary (yellow) between plasma and Ficoll-phase contains the mononuclear cells (MNC).

The yield of cells depending on the age of the donor was between 6 million to 20 million cells from older to younger donors. Major constituent of the cells thus obtained were, however, hematopoietic cells. The proportion of undifferentiated mesenchymal stem cells in the total mononuclear cell fraction is estimated by various authors to about 1:100,000 (Caplan *et al.* 1994).

**3.1.1 Separation of hematopoietic cells**

The large excess of hematopoietic cells can be using a very simple and now widely used method to reduce very effectively. As described in the Methods section, adhere to the cells of mesenchymal origin highly selectively on surfaces of conventional plastic cell culture vessels. After 24-72 h adhesion can be the biggest part of the hematopoietic cells removed by thorough washing. The success of the application of this technique was routinely monitored using flow cytometric analysis.

This is a representative result of the selective effect on the adhesion of hMSC cell culture bottles in the bottom of Figure 13 is shown. It draws on the

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hematopoietic cells for flow cytometry selectively fluorescently labeled using specific against the surface markers CD 45 antibodies directed. The analysis shows the cell frequencies as a function of the strength of the fluorescence signal, **the number of cells number of cells fluorescence intensity fluorescence intensity A:. B: cells from Ficoll-GradientenTrennungdirektnach Adhäsionadhärieren number Adhäsionadhärierenadhärieren**

**Figure 13: Successful removal of CD45-positive cells after separation on Ficoll - gradient and adhesion**

The figure shows the presence of CD45-positive (dashed line), hematopoietic cells before and after the adhesion. As a control, unlabeled cells (gray underlay) are shown.

A: Flow cytometric analysis of CD45-positive (dashed line), hematopoietic cells in a freshly isolated cell population.

B: Flow cytometric analysis of CD45-positive cells (dashed line) after its removal by adherence (passage 0).

To better understand the distribution is also not labeled CD 45 selected cells with gray backing. When comparing the two images is striking that virtually hematopoietic cells with fluorescence above the noise after the Adhäsionphase no longer detectable.

In the graphs 14A and 14B, the parameters Forward Scatter (FS) was applied for the size and side scatter (SS) for the granularity or roughness of the surface against another.

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**FSSS A: SSRSMSCFS B cells from Ficoll-GradientenTrennungdirektnach Adhäsionadhärieren FSSS A: AdhäsionadhärierenZellen Adhäsionadhärierenadhärieren**

**Figure 14: Different morphology of the cells directly after Ficoll gradient separation as compared to the cells that adhered to the surface of culture bottles**

The cells were trypsinized for passage 0 (B) and with cells from the Ficoll - compared gradient fractionation (A) using flow cytometric analysis. The adhesion of cells leads to the reduction of the heterogeneity of the cell population. Abbreviations: FS = scale size of the cells. SS = scale granularity of the cells. MSC = hMSC, RS self-renew cells (a subpopulation of hMSC).

When comparing the two graphs can be seen already, a reduction of the heterogeneity of the cell population used by the Zelladhärierung. While visible in front of adhesion at least four fractions are found after essentially two populations, a smaller one with small, agranular cells (RS) and a larger one with large, granular cells (MSC), which agrees well with the data of other authors ( Prockop et al., 1997, Lange et al., 2005).

**3.1.2 The heterogeneity that after Ficoll gradient separation derived cell population of hMSC**

The use of flow cytometry with fluorescently labeled antibodies against specific surface markers of various mesenchymal cell types confirms the expected heterogeneity of the mononuclear cell population (MNCs). As can be seen from the breakdown in Figure 15, only a small fraction of CD45-negative cells with antibodies against four different surface marker is detected.

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**02468CD 105CD106STRO 1Fluoreszenzfarbstoff 90CD-coupled antibody proportion of labeled cells [%]**

**Figure 15: Analysis of different surface antigens to characterize the heterogeneity of MNC directly after separation on Ficoll gradient**

The figure summarizes the results of quantitative analysis by flow cytometry. Four different fluorescently labeled antibodies were used to analyze the gradient centrifugation through Ficoll-derived cells. In addition to CD 90 - and CD 105-positive undifferentiated MSCs are very few chondrogenic, CD 106-positive progenitor cells and found significantly more osteogenic I STRO-positive progenitor cells (n = 3).

Already, the proportion of these cells with the surface markers of mesenchymal cells such as CD 90 (1.82%) and CD 105 (0.90%) is low. Even cells with the marker CD 106 (0.40%), which can be found on chondrocytes and their progenitor cells are barely detectable. A slightly higher proportion of cells carrying the marker STRO-1 (5.73%), which indicates the presence of osteogenic precursor cells. Overall, flow cytometric analysis confirmed that isolated from the bone marrow cells contain only a very small fraction of hMSC that qualify as candidates for the chondrogenic differentiation in question. Even after the successful removal of hematopoietic cells using Adhesion of the cell population remains very mixed.

Supplemented and confirmed this impression by the results of gene expression analysis by RT-PCR technique. To determine how large proportion of osteogenic or chondrogenic differentiated cells in the fraction is isolated from the bone marrow mononuclear cells, primers were used for those genes whose expression increased according to the differentiated cells is characteristic. To demonstrate osteogenic differentiated cells, the mRNA of osteocalcin and alkaline phosphatase and was the detection of chondrogenic differentiated cells, the mRNA of collagen type II, and compared the mRNA of collagen type I as a marker of stem cells with the concentration of GAPDH mRNA. Figure 16 shows the electrophoretic separation of amplified by PCR cDNAs of these marker genes on an agarose gel. One can see an expression of markers of osteogenic differentiation, whereas it is for the chondrogenic differentiation-specific genes collagen II is not evidence. The expression of

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Collagen type I, which applies to non-specific as chondrogenesis, was also demonstrated. **IOsteocalcinGAPDHALPKollagen marker collagen IIKontrolle500 bpMarkerKollagen bp400 BP300 bp**

**Figure 16: PCR analysis of some differentiation markers directly after isolation**

A representative result of gel electrophoresis of PCR amplified cDNAs shown by using the designated genes. A basal differentiation can be seen only for the osteogenic markers osteocalcin (weak band), alkaline phosphatase (ALP) and stem cell markers, type I collagen. Serves as a positive control, the housekeeping gene GAPDH, as a negative control a PCR reaction without template (control).

**3.1.3 multipotency of cells obtained**

As a direct proof of chondrocytes or their progenitor cells from bone marrow in the isolated population of mesenchymal cells either using flow cytometry or by using the RT-PCR was possible attempt was made ​​to determine the chondrogenic differentiation potential. These were enriched on the Ficoll gradient centrifugation and adhesion to plastic of hematopoietic cells liberated cells to provide a sufficient number of cells initially subjected to a further passaging (passage 1). To check the chondrogenic, osteogenic and adipogenic differentiation potential were on cell aliquots as described in "Materials and Methods" specific differentiation protocols applied. After three weeks under conditions of adipogenic differentiation (see 2.3.3) could be formed in the cells of the typical fat-containing vesicles with the dye Red O staining (see 2.6.5) (Fig. 17B). Similarly succeeded after three weeks under osteogenic differentiation conditions (see 2.3.4) with the von Kossa staining (see 2.6.6), the detection of calcium deposits as an indication for the formation of osteoblasts (Fig. 17 D).

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**B: redo FärbungD: vonKossa-FärbungF: collagen I staining C: redo color: vonKossa-FärbungG: collagen II staining A: Proliferation Passage 1AdipogeneseOsteogeneseChondrogeneseMonolayerkulturPelletkultur** + - **B differentiation: differentiation**

**Figure 17: Tripotenz the cells after one passage**

The Tripotenz the cells after passage 1 without addition of growth factors in the proliferative phase with a seeding density of 1000 cells per cm 2 was confirmed by differentiation assays. Scale bar: 200 microns.

A: morphology of the cells prior to differentiation assays.

B: The Red O staining shows fat deposits in the cells after 3 weeks of adipogenic differentiation.

C: control of adipogenic differentiation.

D: Osteogenic differentiation could be using the von Kossa staining demonstrated after 3 weeks by the presence of insoluble calcium salts.

E: control of osteogenic differentiation.

F and G: For chondrogenesis, cells were aggregated and 3 weeks in the presence of TGF-ß3 and dexamethasone as well as other standard additives (see 2.3.1) cultivated. The proof of chondrogenic differentiation was made ​​by a immunohistological staining against collagen I and II as well as a GAG determination (G / D ratio = 1.7 [ng / ng]).

To examine the chondrogenic differentiation potential of the cells for the initiation of intensive cell-cell contacts were centrifuged into pellets and cultured for 3 weeks in the presence of appropriate growth factors such as TGF-ß3 and dexamethasone. In paraffin sections obtained from the pellets could be using Immuno Although small amounts of collagen type I, but not detected by collagen type II, which serves as chondrogenic markers. In addition, the preparations were not typical of hyaline cartilage morphology, but were more like a cell aggregation without significant extracellular matrix components. This is also confirmed by the results of the quantitative determination of glycosaminoglycans formed. They showed only a very small increase in GAG / DNA ratio (G / D ratio of 0.0 to 1.7 [ng / ng]).

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This results in the overall impression that the population-derived hMSC chondrogenic progenitor cells is likely to contain too little to get it under the conditions generally recommended for *tissue engineering* of cartilage sufficient number of chondrocytes.

**3.2 proliferative phase - *in vitro* propagation**

**3.2.1 sowing density and propagation factor**

One way to gain from the hMSC population of bone marrow, a sufficient number of cells that differentiated into chondrogenic, the selective enrichment of rapidly proliferating, less differentiated stem cells (Prockop et al., 2001). This can be achieved by maximal stimulation of proliferation of hMSCs over several passages, which also leads to slower relative decline and unwanted, partially differentiated cells. To achieve this goal, two methods were used:

a) A reduced seeding density of cells

b) use of specific mitogenic growth factors

Typical for primary chondrocytes optimal sowing densities are approximately 5000 cells per cm 2. If these conditions are also used for propagation of stem cells, the results are unsatisfactory. However, the seeding density by a factor of 10 or more reduced, an increase in cell proliferation is observed that the losses resulting from the initial dilution is not only compensated but exceeds. In order to verify these relationships hMSC were isolated after removal of the adherent cells (passage 0) with different sowing densities (500, 1000 and 5000 cells per cm 2) but otherwise identical conditions proliferated. The cells were trypsinized and seeded in each case after 2 weeks again. The cell number was determined after each passage. The result is shown in Figure 18.

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Results

**10.0001.000.000100.000.00010.000.000.00050010005000Relative increase in cell number sowing density [cells / cm 2] 5Passage0123410.0001.000.000100.000.00010.000.000.00050010005000Relative 5Passage01234**

**Figure 18: Effect of sowing density on the proliferation rate of cells**

The cells were proliferating without WF during the first six passages with different sowing densities. After each passage, the cells were counted. Seeding densities: 500 cells per cm 2; 1000 cells per cm 2; 5000 cells per cm 2. The cells with a low seeding density proliferate faster than cells with a higher seeding density. Represented the mean values ​​(1 <n <4). Representation of the ordinate in logarithmic scale.

It was noted that the multiplication factor of the cells is inversely correlated with the seeding density. Beside it was also not overlook that the proliferation rate decreases with the number of passages, and this effect is much stronger at higher seeding densities. Experiments with sowing densities well below 500 cells per cm 2 failed.

**3.2.2 Depending on the morphology of the cells of the seeding density**

In addition to a significant increase in proliferation rate, the application of such a low seeding density apparently had other effects on the cells. It shall first have a different morphology of the cells was observed (Fig. 19 AC). Figure 19 shows representative phase contrast images of cells are shown, which were passaged with sowing densities 500-5000 cells per cm 2. It can be seen only at the low seeding densities, elongated, spindle-shaped cells. In comparison, the cells were larger and showed a chopped-wide elongated shape when they were seeded at a seeding density of 5000 cells per cm 2.

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**5001000 sowing density [cells / cm 2] 5000 A: B: C:**

**Figure 19: Dependence of the morphology of the cells of their seeding density**

The proliferation of the cells without WF, but with different planting densities influenced the morphology of the cells. After the first passage were sown at a density of 500 cells per cm 2 elongated, spindle-shaped cells observed. A similar morphology was, though not so much to see in a pronounced form, even at a seeding density of 1000 cells per cm 2. In contrast, the cells were seeded at a seeding density of 5000 cells per cm 2, and showed a higher cut-up, wide-stretched form (light micrograph, phase contrast). Scale bar: 100 microns. More pictures can be seen in the appendix.

Were increased with different sowing densities of cells using flow cytometry comparing the visual impression could be confirmed by the morphological differences (Fig. 20) **seeding density [cells / cm 2] 10005005000FSFSFSFSSSSSSSSSSSSSFSFSPassage 1Passage 6 A:. B: C: D: E: F:**

**10005005000FSFSFSFSFSFSSSSSSSSSSSSSSSSSSSSSSSSSFSFSFSFSPassage Figure 20: Sowing density and passage number affect the morphology of the cells**

Morphological differences in size and shape of the cells, which were caused by differences in seeding density over several passages, with the flow cytometry - analysis confirmed. Cm at seeding densities of 500 cells cm 2 and 1000 2 cells, the cells after passage 1 and passage 6, essentially the same division into two subpopulations. In contrast, the heterogeneity of the cells in shape and size at a seeding density of 5000 cells per cm 2 is significantly larger. These differences are even more pronounced after passage 6. FS = scale size of the cells. SS = scale granularity of the cells.

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Figure 20 shows the FS / SS plots of cells that have proliferated with different sowing densities. Cells with low seeding densities (500 and 1000 cells per cm 2) do have a broad size distribution, but overall have a low granularity (Fig. 20 A and B). The result will not change significantly when the cells were seeded at 5000 cells per cm 2. Now, more cells could be observed with increased granularity, which is typical of more differentiated cells. Furthermore, only the cells with low seeding densities, a separate population of very small cells with very low granularity simultaneously detected (Fig. 20 A and 20 B vs. Fig. 20 C). This fraction was already observed by other authors and referred to as RS cells. The lower three images (Fig. 20 D - 20 F) show the same analysis, however, cells that were previously proliferated for six passages. In comparison to the result after only one passage has increased the granularity at all seeding densities and shows a wider distribution. The cells with lower seeding densities have indeed retained the unity, have so-called RS cells (cells *self renew)* decreased in number.

**3.2.3 Influence of mitogenic growth factors on the proliferation rate**

It is known that the presence of various mitogenic growth factors can influence in the culture medium not only the rate of proliferation, but also the potential for differentiation. Generally, growth factors are desirable to prevent a spontaneous differentiation during proliferation, as this will receive a maximum of pluripotency. On the other hand, in this particular case, growth factors are required, the permit or facilitate predifferentiation towards chondrocytes. Therefore, the criterion for the selection of appropriate growth factors alone is not the optimal stimulation of the proliferation rate but also the extent to which later differentiate into chondrocytes is favored. With this aim, some growth factors were examined, including some combinations for their ability to support the proliferation and differentiation of chondrocytes. These growth factors or their combinations have proved to be chondrogenic positive effect on the proliferation of human chondrocytes. Figure 21 shows the first effects are shown on the proliferation rate of cells.

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**10.0001.000.000100.000.00010.000.000.000ohne ​​WF (n = 4), bFGF (n = 4), bFGF, TGF, PDGF (n = 1), bFGF, TGF (n = 1), TGF, PDGF (n = 1) increase the Passage012345Relative cell numbers by adding WF10.0001.000.000100.000.00010.000.000.000ohne ​​WF**

**Figure 21: Influence of mitogenic growth factors on proliferation of cells**

The addition of different WF leads at the same seeding density of 500 cells per cm 2 to differences in the proliferation rate of cells. A higher rate of proliferation is achieved by adding a combination of bFGF and bFGF, TGF and PDGF. A combination of bFGF and TGF shows worse results than the control without added growth factors. The lowest rate of proliferation was observed upon addition of PDGF and TFG. Shown are the mean values. Representation of the ordinate in logarithmic scale.

The highest rate of proliferation is achieved in the presence of bFGF. Only slightly worse cuts off a combination of the three growth factors bFGF, TGF-ß3 and PDGF. However, it seems that this combination while simultaneously stimulate the proliferation to a significant extent the formation of extracellular matrix. The apparent matrix secreted by the cells could not be removed simply by trypsin treatment of the culture. A solubilizing and resuspension of the cells was only after treatment of the sedimented cell pellets after Zellzentrifugation but not with collagenase hyaluronidase, which was interpreted as indirect evidence for the formation of a matrix of collagen content. The remaining combinations of growth factors showed poorer results in terms of cell proliferation rate compared to the control that was cultivated without external growth factors. Therefore, bFGF was applied in addition to the proliferation of the cells by default.

**3.2.4 Flow cytometric analysis of cells after the proliferation phase**

An important note for the possibility that mitogenic growth factors may also influence the differentiation potential of stem cells comes from cytometric analysis of undifferentiated hMSCs using the specific surface markers CD 90 and CD 105th Figure 22 shows the results of a comparative analysis of cells are shown to proliferate with or without bFGF

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were. It is seen that the proportion of cells with these markers is significantly smaller when the proliferation of cells stimulated by bFGF. It remains open whether this effect for the purpose of supporting the chondrogenic differentiation, is not useful or **020406080100CD 105Fluoreszenzfarbstoff 90CD-coupled antibody portion of the labeled cells [%] + bFGF. -**

**Figure 22: Influence of the addition of FGF to the presence of differentiation markers CD 90 and CD 105 during the proliferation of cells**

A change in the state of differentiation of the cells after one passage can be followed using flow cytometry. The proportion of CD 90 - and CD 105 - labeled cells is shown. The concentration of hMSCs for characteristic markers CD 90 and CD 105 takes visible from a passage in the presence of FGF. Shown are the averages of three independent experiments.

**3.2.5 Effect of mitogenic growth factors on the synthesis of glycosaminoglycans in pellet culture with and without differentiation**

Based on the results of flow cytometric analysis has been suggested that in the presence of mitogenic growth factors show proliferating cells an altered differentiation potential. To check whether any of the already to study the proliferation rates of the factors employed may support chondrogenic differentiation, as well as the ability to GAG synthesis in pellet cultures as a function of the added in the proliferative phase of mitogenic factors was determined (Fig. 23).

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**02468G / D**

**Ratio [**

**ng / ng] bFGFTGFPDGF ----- +++-++++-+**

**Figure 23: Effect of various growth factors on GAG synthesis in pellet cultures after stimulation of proliferation**

The cells were proliferated in the presence of various growth factors and with a seeding density of 500 cells per cm 2 and then sedimented to pellets. The GAG production was calculated in proportion to the amount of DNA (s 2.7.1) and the influence of the applied in the proliferative phase of growth factors on GAG production in the pellet culture is shown. Of all the controls without growth factors, a mean value is indicated (n = 4).

The pellet cultures were prepared with an identical number of cells and other matching culture conditions in order to subsequently determine the rate of synthesis of glycosaminoglycans. The only difference was the conditions of the previous proliferation of the cells used. As a comparison, in this case were used pellet cultures of cells that had proliferated without added growth factors. In Figure 23 we see a significantly increased GAG production in the pellet culture if the cells had proliferated with the addition of bFGF.

**3.2.6 The morphology of the cells that were used for chondrogenic differentiation**

The results of the experiments described so far support the idea that conditions are maximal stimulation of the proliferation rate as low seeding density and use of bFGF is not in contradiction to the maintenance of chondrogenic differentiation potential of cells. Therefore, cells were always below under the optimal conditions for cell proliferation (seeding density of 500 cells / cm 2, addition of bFGF) supplemented when it came to the optimal maintenance of the chondrogenic differentiation potential. Here were helpful observations on the morphology of the cells, which proved to be highly dependent on the current rate of proliferation of the cells. Were so small, colony-forming cells to be seen only at high proliferation rates (Fig. 43). Appeared more frequently large, ovoid or triangular - shaped cells, this was always an indication of a low proliferation rate and probably

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chondrogenic differentiation potential are considered inadequate, as it turned out in subsequent experiments for differentiation.

**3.3 embedded in alginate gel as hMSCs differentiation model**

The embedding of cells in an alginate gel - hereinafter referred to as alginate culture - its purpose, the cells spatially separated. This should in particular cell-cell contacts can be avoided without access to the cells of soluble factors limiting noticeable. It is discussed that the cell-cell contacts with mesenchymal stem cells, presumably at the initiation of differentiation into chondrocytes are involved, as they trigger in the native chondrocyte matrix synthesis. Both processes run sequentially in so-called pellet cultures in which hMSCs centrifuged to a pellet and then cultured for up to three weeks with a suitable medium. The success can be quantified by the main products of the formed cartilage matrix - type II collagen and glycosaminoglycans (GAG) - to be confirmed. Alternatively, the pellet culture in the adjacent part of the processes of differentiation and matrix synthesis are separated in time, if you prevent the essential for chondrogenesis cell-cell contacts during differentiation in alginate culture. To assess the effectiveness of alginate culture as differentiation model, the success by determining the matrix synthesis in a subsequent pellet culture are compared with the matrix synthesis, which is in pellet cultures observed not previously separately differentiated hMSCs (direct pellet culture).

**3.3.1 GAG synthesis in pellet cultures with and without differentiation in alginate culture**

To the GAG synthesis in pellet cultures with and without prior differentiation in alginate culture: compare (alginate culture standard conditions, see 2.3.2), cells were incubated for proliferation of the first to the third passage under identical conditions with respect to the medium composition and the addition of growth factors (proliferation: the addition of bFGF, seeding density 500 cells / cm 2; pellet culture: standard conditions, see 2.3.1) were cultured and analyzed. In Figure 24 A, the cellular DNA related to the GAG values ​​are compared. One can see that in the pellet cultures after differentiation in alginate culture 3-5 times more GAG is formed as a pellet after direct culture. It seems that the differences with the decline in proliferation rate in successive passages to correlate (Fig. 24 B). This supports the hypothesis that proliferation and preservation of the (chondrogenic) differentiation potential are positively correlated.

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**05101520123G / D**

**Ratio [**

**ng / ng] PassageGAG / DNA analysis of Pelletkulturenin relation to the cell passage number used for the correlation factors increase with the number of Passagen0102030123VermehrungsfaktorPassageR2 = 0.97970102001530 VermehrungsfaktorG / D**

**correlation factors with the relative increase of GAG formation rate of A: B: C: pellet Kulturnach Alginatdirekte Pellet-KulturVermehrungsfaktor05101520123G/KulturVermehrungsfaktor**

**Figure 24: Comparison of GAG / DNA ratio for direct pellet culture and pellet culture after differentiation of alginate as a function of passage number and proliferation rate**

A: The formation rate of GAG in alginate - depending on the pellet culture passage number. It decreases continuously during the first three passages, whereas with direct chondrogenesis remains practically unchanged.

B: decrease of the propagation factor with increasing passage number.

C: Positive correlation between G / D ratio and multiplication factor. A positive correlation between GAG - Education and proliferation rate is confirmed by the regression.

**3.3.2 Optimization of conditions for the differentiation in alginate culture**

To optimize the differentiation of hMSCs in the alginate matrix synthesis in culture was followed by a pellet as a function of culture

First the duration

Second of cell density

Third of the oxygen partial

is measured.

The culture conditions for each phase were:

Proliferation: Sowing density: 500 cells per cm 2, with bFGF

• Alginate Culture: IGF-1, TGF-ß3, dexamethasone, with additives (s. 2.3.2).

• pellet culture after alginate: IGF-1, TGF ß3, dexamethasone, with additives (s 2.3.1).

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The results are shown in Figures 25-27.

**Influence of the duration of the alginate culture on the success of the differentiation**

The matrix formation in the pellet culture after differentiation is dependent on the duration of phase differentiation in alginate culture. Be seen from the Figure 25 A, the matrix formation significantly in the first two weeks of increases. The cell number, measured by DNA content decreases, while slightly after three weeks in alginate (Fig. 25 B).

**Duration of the alginate culture [day] 0100020003000DNA content [**

**ng] 71 421 B: 01020G / D**

**Ratio [**

**ng / ng] 21Dauer the alginate culture [days] 714 A:**

**Figure 25: The effect of duration of culture in alginate matrix formation in the pellet culture after differentiation in alginate**

The cells were pelleted after every 7, 14 or 21 days in alginate culture under standard conditions to the pellet for 3 weeks and stimulates matrix formation by adding Chondrogenesemedium. Finally, a GAG / DNA analysis was performed (n = 4).

A: The G / D ratio increases with increase in the duration of phase differentiation in alginate.

B: The DNA content shows a slight decrease in the number of cells in the pellet culture after 3 weeks of differentiation in alginate culture.

It was shown the largest G / D ratio in the pellet culture when the cells were three weeks in alginate culture. This could be attributed to the decrease in DNA content after three weeks in alginate. Since the cells, the pericellular matrix form, no longer able to proliferate, a small drop in DNA content after 3 weeks in alginate culture was positive. Also showed that the DNA content based on GAG production in the third week, no major fluctuations. Therefore, a three-week differentiation period was considered as the optimal culture condition.

**The cell density in alginate gel and its effect on the pellet culture**

An important parameter for the success of the alginate culture may also be embedded in the initial density of the alginate gel cells. There is not excluded that the cells secreted paracrine factors to the success of

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Say in differentiation, the effect of different cell densities on the GAG synthesis in the subsequent pellet culture was determined.

The results shown in Figure 26 show significant differences in the use of one, two and four million cells / ml. **01000020000300001 million2 million4 Mio.GAG [ng] 01000200030001 million2 million4 Mio.DNA [ng] 048 121 million .2 million4 Mio.G / D ratio [ng / ng] A: B: C: cell count [cells / ml] cell count [cells / ml] cell count [cells / ml]**

**Figure 26: Dependence of the effectiveness of differentiation in alginate gel used by the initial cell density (n = 3)**

A: The GAG and DNA analysis of the pellet after alginate culture shows that the optimal cell density for a differentiation of the cells in alginate culture 2 million cells per ml alginate.

In the application of other cell densities during the Alginatphase a suboptimal differentiation state is reached and as a consequence be less matrix in the pellet culture produced.

B: The DNA analysis also shows that the optimum is for the cells at 2 million cells per ml alginate.

C: The G / D ratio confirmed that the cells reach a density of 2 million cells per ml of alginate gel in the following pellet culture after the highest GAG formation rate per cell.

The GAG production achieved at a cell density of 2 million cells / ml at a maximum. Its strong, over-proportional increase of 1 million with the increase in cell number at 2 million cells indicates a support point of paracrine factors during differentiation.

The decrease in G / D ratio by about a factor of four at 4 million cells / ml can not be explained by the loss of cells because the DNA content is only a factor of two. In terms of the related to the DNA content of GAG production is the mean cell density (2 million cells / ml) is optimal.

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Results

**Dependence of differentiation of hMSCs in alginate gel on oxygen partial pressure in the gas phase of the incubator**

It has long been known that hyaline cartilage has a lower oxygen pressure than most other tissues, including bone. It was therefore also examined whether the differentiation in alginate gel by reducing the partial pressure of oxygen is transported into the area. As can be seen from Figure 27, is already having a reduction to 10% O 2 positive effect on the G / D ratio. Therefore, this observation in the determination of the optimal conditions for differentiation was considered. **0102051020G / D**

**Ratio [**

**ng / ng] oxygen content in the gas phase [%]**

**Figure 27: Influence of oxygen partial pressure in the alginate culture on GAG synthesis in the subsequent pellet culture**

The differentiation potential of cells is influenced by the oxygen partial pressure in the Alginatphase. The reduction of oxygen content in the gas phase of the cell incubator during alginate culture influences the differentiation status of cells and as a consequence, the matrix formation in the subsequent pellet culture. Shown are the averages of five independent cell preparations.

**3.3.3 Identification of the alginate-chondrocyte culture formed**

As a criterion for the optimization of culture conditions on chondrogenic differentiation in alginate culture, the ability to form glycosaminoglycans in a subsequent pellet culture was used. This highly complex process was necessary because all attempts to establish a quantitative correlation between those already in the Differenzierunsphase in alginate culture formed cartilage components such as collagen type II and glycosaminoglycans, and the progress of differentiation have failed. It could be won only qualitative evidence presented in the figure 28. Figure 28 A, the existence of a pericellular GAG-containing matrix directly by staining the cells embedded in the gel as well as from cells after their isolation has been shown from the alginate.

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Results

**A: cells in alginate B: cells after isolation from Alginat100 microns A: micron**

**Figure 28: Direct Detection of GAG formation in the alginate culture**

May GAG formation begins during the differentiation phase in the alginate culture, what we recognize a mihilfe Alcian blue staining of alginate beads with the embedded cells: A. Each cell forms an intensely stained pericellular yard. This court notes the existence of the so-called chondrones.

B: The farm is also after the release of cells from the alginate gel to see. The isolated cells from the alginate gel was GAGs with 1,9 - dimethylmethylene blue (DMMB)

stained.

Because of the higher specificity of immunohistochemical studies were also performed. They provide valuable direct and reproducible conclusions about the success of the differentiation of hMSCs in alginate culture. Crucial to the success of the differentiation is in fact not the amount of gebildenten matrix components, but the proportion of cells in the hMSC population employed in the course of treatment in the alginate culture the ability to form cartilage-specific matrix components wins. The results described below are based on the highly specific for differentiated chondrocytes property of forming within the tissue formation of so-called chondrones that can be imagined as a kind of shell surrounding the chondrocytes. For immunohistochemical analysis of cells that were differentiated under conditions optimized in the alginate culture from the hMSCs, these were sedimented after solubilization from the gel onto glass slides (cytospins) and stained using antibodies against collagen type I, II and VI with fluorescent dye (see 2.5.5). To identify the position of the cell nuclei were also stained with the DNA-specific fluorescent dye DAPI. An overview of the rubbed cells that were cultured under standard conditions is shown in Figure 29. It can be seen that the cells are stained specifically with all three antibodies, while in the control without primary antibody only the DNA of the cell nuclei as a result of simultaneous DAPI staining is visible. Considering further that the pericellular staining is strongest, which in the enlarged view in Figure 30 is even clearer condenses the assumption that in the alginate gel formed pericellular chondrones real shells are very similar.

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Results

**A: I + DAPIB collagen: collagen II + DAPIC: collagen VI + DAPID: without primary antibody + Dapia: DAPI**

**Figure 29: Direct Detection of collagen formation in the alginate culture**

In the alginate culture cells were differentiated using a treatment with citrate released from the alginate gel and then centrifuged for immunological analysis on slides. In the overview images can be seen that the cells next to the pericellular collagen types I and II and type VI collagen have expressed. Bottom right is a control without primary antibody, in which one detects only the cell nuclei stained with DAPI. All cells can be identified by the formed collagen as differentiated chondrocytes. Scale bar: 300 microns.

These are the hMSCs differentiated from the pseudo-chondrones in Figure 30, A and C juxtaposed for comparison. In accordance with findings of other authors, the Collagen Type VI cell appears in both cases, something closer in distance to surround as collagen type II, which is found in a slightly greater distance. As is further known that collagen type VI in hyaline cartilage pericellular exclusively, occurs in the chondrones ie, proof of this collagen type, a suitable tool with very high specificity for the identification of differentiated chondrocytes.

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Results

**A: B: C: D: Native cartilage collagen II μm50 50 microns 50 microns μm50 collagen-VIPseudo ChondronA: μm50 μm50 μm50 μm50 chondrone**

**Figure 30: Comparison of the structure of native and pseudo-chondrones chondrones from the alginate gel**

The two left images (images A and C) show the typical topology of collagen II and VI colors of the chondrones from native cartilage (immunostaining of Parafinschnitten). The images B and D show the staining of cytospins right after isolation from the alginate gel. The staining of collagen type II in both cases shows a radially outgoing structure (A and B). In contrast, type VI collagen staining is localized more compact and closer to the cell surface (C and D).

Because of all cell types arising from hMSCs differentiated chondrocytes exclusively to the formation of such chondrones are capable of this detection system can be regarded as specific for the formation of differentiated chondrocytes in primary culture cells.

**3.3.4 The quantification of alginate in the culture formed chondrones**

For the potential use of the chondrocytes *in vitro* production of hyaline cartilage for therapeutic tick the provision of a homogeneous population of differentiated cells is an essential condition. Therefore, the quantitative differentiation, that is the most complete transformation of hMSCs used in differentiated chondrocytes, as important as the one shown above qualitative identification of chondrones. To determine, had formed what proportion of the released from the alginate gel cells during the differentiation phase chondrones, were immunologically stained cytospins of the software Lucia G (Nikon Japan) using evaluated (see 2.6.8), which defines the number of cells allows a minimal fluorescence signal. As shown in Figure 31 A, showed the mean of four independent analysis accounts for nearly 98% of cells stained with DAPI at the same time next to a nucleus exhibited a type VI collagen-containing pericellular sheath.

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**0255075100Kollagen IIKollagen IKollagen VIAnteil the chondrones [%] 015304560Kollagen IKollagen IIKollagen VIRelative amount of matrix formed [stained area /**

**Cell count] A: B:**

**Figure 31: Quantitative analysis of the alginate gel formed Pseudochondrone**

Isolated from the alginate Pseudochondrone were compared using collagen I, II and VI, marked directed, fluorescently labeled antibodies and quantitatively analyzed (n = 4).

A: Virtually all cells have a pericellular court of collagen type VI. Collagen I and II were also detected in the majority of the cells.

B: All cells from the alginate gel have a production of the three different types of collagen.

The relative amount of the resulting collage shows less collagen VI as collagen I and II

The proportion of cells and collagen type I and type II was contained somewhat lower. It is assumed that this is the result of experimental manipulation. During the preparation of cytospins, a partial loss of the mechanically sensitive Chondronhülle is not to be avoided completely. Since the stain with collagen types I and II parts are further out, they are more influenced by mechanical losses.

**3.3.5 Effect of dexamethasone in the differentiation of hMSCs used in the alginate culture**

The presence of glucocorticoids (dexamethasone) is consistently described by many authors to be very conducive to the success of chondrogenic differentiation. Therefore, all previously described experiments with the addition of these supposedly beneficial conditions were performed. In order to verify whether this is actually used for the alginate culture system, in addition some comparative measurements were performed with and without the addition of these two factors.

The absence of dexamethasone were in differentiation medium formed chondrocytes to collagen type I, II and VI, marked with fluorescence, and the results obtained are summarized in Figure 32. It can be seen first in Figure 32 A, the proportion drops of chondrone-forming cells from 100% to around half, if we dispense with the addition of dexamethasone. At the same time lowers the stained area (matrix volume) to about 10-30%, probably not caused by a reduced secretion of the three collagen types I, II and VI (data

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shown). Even more dramatic is the effect that if the expression of collagen II gene using qPCR is investigated. Here is found by differentiation in the presence of dexamethasone approximately 13 times more mRNA in the cells. By contrast, the omission of dexamethasone on the expression of collagen X as a marker for terminally differentiated chondrocytes and Sox9, an earlier marker for chondrogenesis, only a weak effect (Fig. 32 B). **VIAnteil 9Relative gene expression (normalized to GAPDH [factor ]) Versuch12-DEX, TGF + + IGF + DEX + TGF, IGF + A: B: C:**

**0510152025G / D**

**Ratio [**

**ng / ng] in pellet Kultur0255075100Kollagen IIKollagen I**

**The collagen chondrones [%] 051015Kollagen IIKollagen IKollagen XSox IGF - Figure 32: Effect of the addition of dexamethasone on the differentiation of cells in alginate culture**

The important role of dexamethasone in the differentiation of the cells was determined using computer-assisted quantitative analysis of Immunofluoreszenzmarkierung, qPCR and GAG / DNA analysis indicated (data from two independent cell preparations).

A: The percentage of fluorescently labeled antibody detected using the chondrones is based on the DAPI-positive cells shown. The omission of dexamethasone in the differentiation phase has a negative effect on the Chondronbildung.

B: The relative expression of genes of various cartilage marker is shown. Here it is set in the absence of dexamethasone achieved result = 1.

C: In this illustration, the production of GAG relative to DNA content in the pellet culture shown as a function of whether the differentiation was performed in alginate in the presence or absence of dexamethasone (in duplicate).

The late effects of dexamethasone in the alginate culture on the GAG formation in the pellet culture can be found in Figure 32 C. The omission of dexamethasone in the alginate culture appeared in two independent experiments a negative effect on matrix formation in the subsequent pellet culture.

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**3.3.6 Effect of TGF-ß3 on the differentiation of hMSCs used in the alginate culture**

The effect of omission of TGF-ß3 in the differentiation medium in the alginate culture was studied. Regarding the formation of chondrones is the result shown in Figure 33 are not uniform **ratio [(normalized to GAPDH [VIAnteil the chondrones [%] + DEX, TGF-, IGF + + DEX + TGF, IGFVersuch12 + A:. B: C:**

**010203040G / D**

**ng / ng] in pellet Kultur051015Kollagen IIKollagen IKollagen XSox 9Relative factor gene expression]) 0255075100Kollagen IIKollagen I collagen Figure 33: Effect of the addition of TGF-ß3 on the differentiation of cells in alginate culture**

The important role of TGF-ß3 for the differentiation of the cells was determined using computer-assisted quantitative analysis of Immunofluoreszenzmarkierung, qPCR and GAG / DNA analysis showed.

A: The proportion of detected chondrones is based on the DAPI-positive cells shown. The omission of TGF-ß3 in the differentiation phase has a negative effect on the Chondronbildung.

B: The relative expression of cartilage marker genes in different cells from the alginate culture. This is achieved in the absence of TGF-ß3 result = 1.

C: The production of GAG is based on the DNA content in the pellet culture shown as a function of whether the differentiation was performed in alginate in the presence or absence of TGF-ß3 (in duplicate).

So it seems the proportion of type VI collagen with chondrones and collagen type II, not decrease, when TGF-ß3 is omitted from the differentiation medium. Significantly more important it is the omission of TGF-ß3 but from the transcription of the collagen II gene. In contrast to collagen type II to type I collagen matching losses with respect to the mRNA and protein synthesis as a result of

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Waiver to the addition of TGF-ß3 found to differentiation medium. The changes in the expression of collagen type X and Sox9 are minimal.

Also, the GAG / DNA analysis confirms that the matrix formation decreases in the pellet culture in the omission of TGF-ß3 in the alginate culture.

**3.3.7 Effect of IGF-I on the differentiation of hMSCs used in the alginate culture**

The effect of the omission of IGF-I was out of the alginate culture during the differentiation phase also examined using three different analysis. Figure 34 A shows the result shown that IGF-I is a very effective stimulator of the differentiation of cells in alginate gel in terms of the capacity for matrix formation in the subsequent pellet culture. **051015G / D**

**Ratio [**

**ng / ng] in pellet culture 50 microns μm50 0235Kollagen IIKollagen IKollagen XSox 9Relative gene expression (normalized to GAPDH [factor]) + DEX + TGF, IGF + DEX + TGF, IGF + + DEX + TGF, IGF + DEX + TGF, IGF + A: B: C: + DEX + TGF, IGF D + DEX + TGF, 051015G + IGF / IGF μm50 μm50**

**Figure 34: Effect of IGF-I on the differentiation of cells in alginate culture**

The role of IGF-I for the differentiation of the cells using the GAG / DNA analysis, and qPCR Immunofluoreszenzmarkierung was shown.

A: The matrix formation in the pellet culture after differentiation in alginate gel as a function of IGF-I during differentiation, as measured by GAG / DNA ratio (n = 5).

B: The expression of some genes of cartilage markers in the alginate culture in response to IGF-I. The expression in the absence of IGF-I has been set = 1.

C and D, immuno-staining of collagen type VI in cytospins after differentiation in alginate gel with and without addition of IGF-I.

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This finding was confirmed by the qPCR method in practice (Fig. 34 B). The expression of collagen type I and II, and is of Sox9 by addition of IGF-I stimulates in the alginate culture. At the same time the amount of mRNA of collagen type X unchanged.

Figure 34 shows representative images of C and D with antibodies against collagen type VI marked chondrones obtained after alginate culture with and without addition of IGF-I. One recognizes here a strong effect of IGF-I on the Chondronbildung in the differentiation phase. The quantitative evaluation of the visualization was augrund dispensable.

**3.4 The matrix formation in pellet cultures with and without differentiation of hMSCs in alginate culture**

The aim of the differentiation of hMSCs used in the alginate culture of chondrocytes with providing the ability to let themselves be stimulated by cell-cell contacts for the formation of cartilage, similar to what we had observed in freshly isolated from *adult* articular chondrocytes. To evaluate the acquired properties in the alginate culture is the behavior of hMSCs are compared to those of cells that were not subject to a separate differentiation.

**3.4.1 Matrix form**

The result of the determination of the educated during 3 weeks of pellet cultures glycosaminoglycans (GAGs) with and without prior differentiation in alginate culture is shown in Figure 35. The regulations pertaining to the DNA content of GAG levels are on average more than threefold higher when the hMSCs were previously subject to a separate phase of differentiation. For better comparability were used in this comparison of identical standard culture conditions, including the selection and concentration of growth factors.

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**0102030G / Culture 0102030G / D**

**Ratio [**

**ng / ng] p = 0.0028 direct pellet culture pellet Kulturnach alginate culture**

**Figure 35: GAG / DNA analysis of matrix formation in pellet culture with and without alginate phase**

The comparison of the GAG formation was carried out in direct pellet cultures (two-phase model) and in pellet cultures after alginate culture (3-phase model). The additional involvement of the alginate culture to differentiation will generate a significant increase in GAG production of cells in the pellet culture with them. The statistical significance using Student's t tests determined. (Averages of six independent experiments of different cell preparations).

To assess the separate effect of differentiation on collagen synthesis, Western blot analysis was taken (see 2.5.3) **with AlginatM1M2DimerMonomerDimerMonomerohne AlginatA: IB collagen: collagen II IImit AlginatM1M2DimerMonomerDimerMonomerDimerMonomerDimerMonomerohne**

**Figure 36: Collagen formation in pellet culture with and without alginate phase**

The result of a representative Western blot analysis: Immunological identification of collagen I and collagen II after extraction from pellet cultures with and without additional differentiation in alginate culture. Equal amounts of protein on the gel were applied. Marker: M1 standard of collagen type I, M2 standard of collagen type II

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In Figure 36, a slightly stronger form of collagen type II can be seen, though not as pronounced as on the GAG formation. Since type II collagen cross-linked to the biosynthesis, the results can be affected differently by effective extraction of protein from the tissue.

Therefore, additional paraffin sections were prepared and subjected to immunohistological staining. In Figure 37 it can be seen that were stained in the pellet culture separate without differentiating (direct pellet culture), collagen type I and type II collagen is relatively weak. Contrast, we find an effect of the additional differentiation (pellet culture after alginate) a visibly weaker staining of collagen type I and a stronger staining for collagen type II compared to collagen type I. **A: B: C: D: direct pellet culture (2 -phase model) AlginatPelletkultur (3-phase model) IKollagen collagen II A: II**

**Figure 37: Immunohistology of the pellet formed in cultures of cartilage tissue with and without additional differentiation in alginate-pellet culture**

Histological sections of pellet cultures without (A and B) and with a separate differentiation in alginate gel (C and D) were stained immunologically with antibodies against collagen type I and type II collagen. After additional differentiation in alginate gel, the formation of collagen type II is significantly stronger than that of collagen type I. Scale bar: 100 microns.

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**3.4.2 The influence of chondrogenic differentiation in alginate culture on gene expression in the pellet culture**

To determine to what extent reflected the differentiation of hMSCs in alginate culture on chondrogenic tissue-specific gene expression, the concentrations of the mRNAs of some genes were examined, whose activation is characteristic for differentiated chondrocytes. Figure 38 shows the amount of detected by qPCR cDNAs of collagen types I, II and X, as well as Sox9, which were formed without (gray bars) and with (blue bars, two cell preparations) prior differentiation. **0255075Kollagen IIKollagen IKollagen XSox 9Relative gene expression (normalized to GAPDH [factor]) direct pellet pellet KulturV1 Kulturnach AlginatV1Pellet-Kulturnach AlginatV20255075Kollagen AlginatV2direkte AlginatV2**

**Figure 38: Target gene expression of some genes in pellet culture with and without additional differentiation in alginate culture**

Shown is the normalized to GAPDH quantity of mRNA molecules (overridden in cDNA) of the identified genes in different pellet cultures with and without additional differentiation in alginate culture. The direct expression in pellet culture was set = 1. The data showed two independent cell preparations (V1 and V2). Culture pellet and alginate culture were carried out under standard conditions.

One can see that is expressed under the specific conditions of measurement, the functionally important for differentiated chondrocytes of type II collagen gene in very different degrees. Thus, in pellet cultures as a result of the additional differentiation in alginate culture 30-70 times higher levels of mRNA measured in question. Noteworthy in this context, the strong fluctuations of expression that were observed in two different cell populations. For the genes of collagen types I and X, and for Sox9 in comparison to only minor stimulation of gene expression as a result of the additional differentiation observed.

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**3.5 The optimization of culture conditions for the formation of matrix in the pellet culture**

The temporal separation between differentiation and matrix synthesis in a three-phase model offers the possibility to optimize both processes separately. The conditions described in the literature regarding the medium composition and the addition of growth factors corresponded to the experience with pellet cultures of undifferentiated hMSCs. Since the differentiation in alginate culture was aimed at differentiating the used hMSCs to before the start of the pellet culture chondrocytes, the conditions for the optimal matrix synthesis in the pellet culture in the three-phase model should be less on recommendations for the optimal differentiation based, but rather take into account experience with the matrix formation in adult chondrocytes.

**3.5.1 Effects of dexamethasone, TGF-ß3 and IGF-I in the pellet culture matrix synthesis**

Primary chondrocytes, as they are isolated from the articular surface of cartilage, have a great potential for the formation of hyaline cartilage, while they their differentiated phenotype, for example in the context of cell proliferation in monolayer culture, have not yet lost (dedifferentiation). Differentiated cells in pellet culture through intensive cell-cell contacts are stimulated to matrix formation, affect factors such as dexamethasone and TGF-ß is not a stimulant, but inhibitory. To check whether behavior differentiated under the terms of the alginate-cultured cells in the pellet culture more like differentiated chondrocytes or remain as undifferentiated hMSCs, the matrix formation in the pellet culture was determined if dexamethasone, TGF-ß3 and IGF -I in the culture medium were missing.

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**0204060G / D**

**Ratio of [**

**ng / ng] TGFDex ++++--- IGF ++++--- -**

**p = 0.0041 p = 0.0783 p = 0.0190 p = 0.0050**

**Figure 39: Effect of omission of dexamethasone, TGF-ß3 and IGF-I from the culture medium of the pellet culture on the formation of GAG**

After differentiation in alginate in the presence of three factors dexamethasone, TGF-ß3 and IGF-I pellet cultures were incubated in the absence of one or more factors conducted from the culture medium. The statistical significance using Student's t tests determined and based on the G / D ratio of the experiments with DEX, TGF and IGF in the pellet culture. Shown are the averages of four independent cell preparations (n = 4).

Figure 39 is the result of the determination of GAG synthesis in pellet cultures represented, in which one or more of the missing factors used in the differentiation phase. It is clearly seen that the GAG synthesis is benefiting from the absence of two of the three factors. Is dispensed simultaneously to TGF-ß3 and dexamethasone, resulting at least in terms of GAG synthesis, however, no additive effect. The omission of IGF-I acts as opposed to locking, as described by differentiated chondrocytes.

The results of GAG analysis are corroborated by immunohistochemical analysis of collagen formation. Figure 40 in paraffin sections from different pellet samples are shown which were stained with antibodies against each collagen type I and II.

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**Collagen in pellet culture IKollagen IIDEXTGFIGF +++-+++-+--+--- A: B: C: D: E:**

**pellet culture collagen DEXTGFIGF + + + Figure 40: The effects of DEX, TGF-ß3 and IGF-I on the biosynthesis of collagen type I and II in the pellet culture after differentiation in alginate culture**

Made after the differentiation phase (standard conditions) from the alginate gel released cells cultured with or without single or multiple growth factors in the pellet culture for 3 weeks. Subsequently, the formed pellets were embedded in paraffin, sectioned, and stained for collagen type I or II. Scale bar: 100 microns.

Figure 40A shows the result of a pellet culture after the Alginatphase, when the hMSCs using all three factors (Dex, TGF-ß3 and IGF-I) for differentiation and matrix synthesis were stimulated simultaneously. Besides the formation of collagen type I collagen type II can be seen. A shift in the collagen type II / I ratio toward collagen type II can be seen already. Even better is the result in terms of collagen type II / I ratio, however, when dexamethasone or TGF-ß3 alone or

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together during matrix synthesis were omitted in the pellet culture (Fig. 40 B, C and D). There seems to be a significant additive effect with regard to the formation of collagen type II to type. On the other hand, increases in this case in the absence of TGF-ß3 also the level of collagen type I bit. The omission of all three factors was a strong staining and collagen II to collagen I somewhat weaker staining was observed (Fig. 40 E). This representative result is confirmed by further pictures in the Appendix.

**3.5.2 Effects of dexamethasone, TGF-ß3 and IGF-1 in the pellet culture, gene expression**

The immunohistochemical studies were supplemented by qPCR analysis. In Figure 41, the data from the qPCR analysis of three independent experiments are shown with different cell preparations. Susceptible to this was that the preparations were obtained with a high collagen type II / type I collagen ratio and low collagen type X values ​​from cultures with high multiplication factor in the proliferative phase. **Sox9Kollagen XKollagen I + DEX + TGF + IGF19, 30,180,090,110,3 S33 W \* 8 , 40,071,055,272,6 S2418, S3219 50,250,030,330,24, 30,190,070,260,28 S33VF \* I collagen IIVersuchSox9Kollagen IIVersuchSox9Kollagen XKollagen**

**DEX + TGF-+ IGF**

**S 24 14.78 16.41 2.74 0.29 8.4**

**S32 3.05 0.50 0.54 0.37 18.5**

**S33 7.02 3.57 2.96 0.14 19.3**

**Experiment II collagen collagen collagen X Sox9 VF \***

**Collagen I collagen X Sox9**

**+ DEX + IGF**

**S 24 16.86 12.47 5.70 0.36 8.4**

**S32 4.40 0.21 0.64 0.44 18.5**

**S33 3.80 1.36 1.81 0.34 19.3**

**TGF-**

**Experimental collagen II VF \***

**Collagen I collagen X Sox9**

**- DEX - TGF + IGF**

**V24Y 5.04 8.59 4.08 0.25 8.4**

**Experimental collagen II VF \***

**Figure 41: Effect of omission of various growth factors on gene expression of important markers during cartilage matrix formation in the pellet culture**

The mRNAs isolated from pellets were overwritten in cDNAs and analyzed using the qPCR method. The table shows the normalized to GAPDH, the results are summarized as factors of 3 independent experiments (= different cell preparations). All pellets were after the three-phase model won. The various approaches differ in the presence of the indicated growth factors in the pellet culture. \* Attempt S33W shows qPCR data after repeated transfer of mRNA from experiment S33 cDNA as a control for the experiment. Same cell preparations as in Figure 30 VF: multiplication factor (see 2.2.1)

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To help the review process, are also in Figure 42 faced by each of the three growth factors caused changes in the example of two cell preparations again. **012345Kollagen IIKollagen IKollagen XSox (012345Kollagen IIKollagen IKollagen XSox 9Relative gene expression (norm to GAPDH [factor]) 0246810Kollagen IIKollagen IKollagen XSox 9Relative gene expression (GAPDH norm on [factor]) + DEX, TGF-, IGF + (B)-DEX, TGF-+ IGF (C:)-DEX + TGF, IGF + (A:) + DEX , + TGF, IGF + (AC) A: B: C:**

**9Relative norm to GAPDH gene expression [factor]) Figure 42: Effect of TGF-ß3, dexamethasone and IGF-I gene expression important to the cartilage markers**

The results of the S33 and S24 cell preparations from Figure 41 are classified by growth factors in three separate graphs shown.

Quite analogous to the observed effects on the G / D ratio (Fig. 39) by selective omission of dexamethasone and TGF-ß3 from the culture medium of the alginate pellet culture after the transcription of the gene of type II collagen is stimulated. The amounts of mRNAs isolated from the pellets of the collagen genes of type I, II and X, and the Sox9 gene were shown in Figure 42 as a multiple of the expressed GAPDH mRNA.

The changes found show that the gene of collagen type II as a result of the omission of dexamethasone and TGF-ß3 in the culture medium of the pellet culture after alginate was activated obviously irrelevant. Parallel were observed for the collagen type X is also an activation by a comparable factor, but at a much lower level. In contrast, for the expression of genes of collagen type I and the transcription factor Sox9 is detected no significant changes as a result of the omission of dexamethasone or TGF-ß3 in the culture medium.

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The simultaneous omission of TGF-ß3 and dexamethasone has no apparent additive effect on gene expression. This result corresponds to the G / D increase in the cultivation without dexamethasone and TGF-ß3 in pellet culture after alginate (Fig. 39). The expression of genes of collagen type I and X is without the addition of two factors, however, increased (Fig. 42 C). The expression of Sox9 in these experiments shows no major changes.

Overall, these results agree well with the immunohistochemical data shown and described the effects on GAG synthesis in line. As a common outcome of this analysis can therefore be said that matrix synthesis is strongly inhibited in the pellet culture in the presence of dexamethasone and TGF-ß3, if the cells used were previously differentiated into chondrocytes in alginate culture. This also means that the two work factors examined dexamethasone and TGF-ß3 opposed to the differentiation in alginate culture or matrix synthesis in the subsequent pellet culture.

**3.5.3 Depending on the potential of increased chondrogenesis *in* hMSCs in *vitro* multiplication factor of the proliferative phase**

In the determination of gene expression to compare the effects of dexamethasone and TGF-in pellet culture was noted that the multiplication factor during the proliferation of the cells had an effect on chondrogenesis. As already shown in Figure 24, requires a high multiplication factor of a good G / D ratio in pellet culture after alginate. During the culturing of the cells is a connection between the morphology and the multiplication factor was determined. Figure 43 shows the basis of representative examples of typical morphological features of hMSCs that were observed in the early proliferative phase. Cells, which was long before the separation of hematopoietic cell adhesion were given the opportunity to, on average much larger and flatter. In addition, frequently oval and triangular cells were observed (Fig. 43 B). The cells was only 1-3 days given the opportunity to adhere before the cell supernatant was removed (see 2.2), showed that instead often a colony-forming morphology (Fig. 43 A). In large sections could be determined that the colony-forming cells had a significantly higher VF.

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Results

**Adherence 1-3 TageAdhärenz 5-7 days during the passage of replication factor -12.66 -2.13 1Vermehrungsfaktor at passage 1A: Colony bildeneZellenB "egg" or "triangular" cells after Ficoll-passage 0Zellen GradientenAuftrennungAdhärenz GradientenAuftrennung**

**Figure 43: The relationship between cell morphology on the one hand and Adhärenzzeit and proliferation rate of cells on the other hand,**

All pictures were taken 7 days after isolation. The proliferation medium contained bFGF. The multiplication factor was calculated according to one passage at a seeding density of 500 cells / cm 2 and the addition of bFGF. Scale bar: 100 microns (light micrograph, phase contrast).

In the course of the research conducted on the labor provisions of chondrogenesis potential showed that the various parameters such as GAG / DNA ratio, immunohistological collagen staining and gene expression of cartilage markers reproduce well made, if they were performed with the same cell preparation. Increased fluctuations occurred, however, if the results obtained with different cell preparations were compared. When searching for the causes noticed that cells with the morphology shown in Figure 43 A showed a higher and those with the morphology shown in Figure 43 B is usually a lower potential for chondrogenesis. One possible explanation for the variation and an indication of a link between the quality of the cells and the observed chondrogenesis provides the potential shown in Figure 44 Correlation of GAG / DNA levels reached during the proliferation and the multiplication factor. It is seen that in all cases where a conducive for the formation of matrix combination of growth factors in the pellet culture was chosen, even

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Results

a significant positive correlation between G / D ratio and proliferation rate is. **0.124202040600102030 R2 = G / D**

**Verhältis DEXTGFIGF DEXTGFIGF + + + + + + = 0.94130204060010203 VFR2 G / D**

**VehältnisVF - DEXTGFIGF + + - + + DEXTGFIGF**

**0**

**R2 = 0.269102040600102030 G / D**

**VehältnisVF + - + DEXTGFIGF + - + R2 = 0.75970204060010203 DEXTGFIGF G / D VehältnisVF - DEXTGFIGF + - + DEXTGFIGF A: B: C: D:**

**0**

**Figure 44: The relationship of GAG formation in the pellet and the culture reached in the proliferative phase of multiplication factors**

The GAG / DNA ratio as a measure for the three-phase model matrix formation observed in the pellet culture in each case for different growth factor combinations with the achieved in the proliferative phase proliferation rate (VF = multiplication factor as a measure of the rate of proliferation) correlated.

**3.5.4 Comparison of product formed in the pellet culture with native cartilage, human cartilage**

**Analysis of gene expression**

Figure 45 shows results normalized to GAPDH mRNA, a qPCR analysis of some key cartilage markers. Compared to the native human cartilage in the observed values ​​with those obtained with cell preparations with high proliferation rates and with different combinations of various growth factors in a three-phase model.

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Results

**0369Kollagen IIKollagen IKollagen XSox9Relative gene expression (normalized to GAPDH [factor]) native cartilage Pelletkulturnach alginate + DEX + TGF + DEX + IGFPelletkulturnach alginate, TGF-, + IGFPelletkulturnach alginate DEX + TGF + DEX IGFPelletkulturnach alginate, TGF-, + IGF IGF0369Kollagen IGFNativer**

**Figure 45: Comparison of the more important in pellet cultures after three-phase model obtained with the marker gene expression in native cartilage, human cartilage**

Which was in pellet cultures in the presence of various growth factors on the three-phase model formed mRNA by qPCR analysis with the level of mRNA compared to native cartilage. Presented the results from pellet culture after alginate (standard conditions) of two cell preparations with a multiplication factor of at least 9.2.

The Figure 45 shows that the absence of dexamethasone and / or TGF-ß3 to a very strong induction of the expression of collagen type II leads, which exceeds that in native cartilage by a factor of 20 to 30. However, the simultaneous absence of both factors caused a significant gene-activation of collagen types I and X. Since the activation of these two genes for the formation of hyaline cartilage is not desirable, the absence is only brings one of the two factors optimal results. It is for type X collagen expression reached the same level as in the native cartilage. These conditions are also optimal for the expression of Sox9, which can be increased compared to native cartilage nor easy.

**Immunohistochemical analysis**

To assess the extent to which the technique of pellet culture is, from the hyaline in the alginate culture differentiated chondrocytes, cartilage tissue for medical use to breed, were the results of immuno-histologically stained sections from the pellet samples with such from virgin, human cartilage from the articular surface compared. Figure 46 shows for both cartilage samples from each staining with collagen type I, type II and type VI antibodies directed. It can be seen in the tissue produced *in vitro,* the different localization of two antigens. While collagen type VI is found preferably pericellular, collagen type II is found throughout the extracellular matrix. When compared with the native

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Results

Samples is mainly that the intercellular spaces in the *in vitro* test are not yet as large as in the natural tissue. The lower intensity of staining of the collagen type II antibodies in native cartilage and not in the indicated *in vitro* culture suggest that the epitopes are subject to the effective cross-linking of this collagen type, and thus less accessible to the antibody molecules.

[Alexei S1] **native cartilage collagen VI IKollagen IIKollagen 50μm50μm50μm50μm50μm50μm native cartilage *in vitro* VI 50μm50μm50μm50μm50μm50μm50μm50μm50μm50μm50μm50μm *in vitro* cartilage**

**Figure 46: Comparison of immunologically stained histological sections of pellet cultures after three-phase model with the sections of native human cartilage**

The immunostaining of paraffin sections were performed in native cartilage and pellet cultures of the three-phase model with antibodies against collagen types I, II and VI. The conditions for pellet culture were: proliferation in the presence of bFGF at a seeding density of 500 cells per cm 2; alginate culture with TGF-ß3, dexamethasone and IGF-I; pellet culture without dexamethasone, however, TGF-ß3 for the first 10 days and IGF-1 for 21 days.

In the short period of only three weeks *in vitro* cultured samples was the opportunity for strong cross-linking not been obvious. The minimum

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Results

Staining with collagen type I antibodies and the different collagen type II and type VI made ​​visible distributions are typical of hyaline cartilage.

In this chapter, the culture conditions were examined by a three-week pellet culture phase after differentiation in alginate. There were the effects of some growth factors are shown in the matrix formation phase. In summary reads itself concludes that the use of three-phase model results show significant advantages of this method in terms of quality and *in vitro* cultured cartilage compared with the results of the two-phase model better results.

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Discussion

**4th Talk**

**4.1 The extraction of cells**

In this study, the chondrogenic differentiation of human mesenchymal stem cells *in vitro* was investigated. Requisite for the treatment of this question, the isolation and characterization of a suitable cell population. As shown in the present work, can human mesenchymal stem cells from bone marrow aspirates of adult donors are isolated and expanded *in vitro.* A generally accepted, standardized method for the isolation of hMSCs from bone marrow, there is not currently (Haynesworth et al, 1996;. Pittenger et al, 1999).. By other authors has already been shown that the type and quantity of recovered cells are affected by the isolation method (Lange et al., 2005). The cells used in this study were obtained with the most commonly used method: the separation on a Ficoll gradient followed to select those cells by adherence to the plastic surface of conventional cell culture vessels. To control these first steps to characterize the cells using flow cytometric analysis were examined. Primarily those already described in the literature, morphological and immunological parameters were used (Colter et al, 2001,.. Prockop et al, 2001).

The figure 14 to be seen on the graph on the size and granularity reflects the already observed by various authors heterogeneity of the cells again (Muraglia et al., 2000). The adherent cells (passage 0), followed by thorough rinsing significantly reduces the heterogeneity of the cell population. The effectiveness of this is mainly for the separation of the cells serving hämopotoetischen action demonstrated by the use of CD45 as a marker.

As a positive immunocytochemical marker for multipotent mesenchymal stem cells were the only two in the literature are standard markers, CD 90 and CD 105, used (Jones et al., 2002). In addition, STRO-1 were used as markers for osteogenic and CD 106 as a marker for chondrogenic progenitor cells (Dennis et al, 2002,.. Diaz-Romero et al, 2005; Gronthos et al, 1994;. Oyajobi et al, 1999). . It turned out, in accordance with statements of other authors, that the proportion of mesenchymal stem cells in freshly isolated cells is very low. With STRO-1, the presence of a small amount of osteogenic progenitor cells can be detected, which was also confirmed by RT-PCR analysis by detection of mRNAs of type I collagen, osteocalcin, and alkaline Phosphatatse. In contrast, neither using flow cytometry (CD 106) or RT-PCR (collagen

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Discussion

Type II) progenitor cells with chondrogenic predifferentiation be found, which corresponded to the expectations from the literature (De Ugarte et al., 2003).

For further confirmation of the multipotent character of the isolated cells were standard protocols used (Jaiswal et al, 1997;. Johnstone and Yoo, 1999;. Pittenger et al, 1999) to determine the potential to differentiate into adipocytes, osteoblasts and chondrocytes. In this case, histochemical and immuno-histochemical staining was used. The results were used as quality control for all the further investigations of this work routinely from 2-3 ml of bone marrow aspirate produced hMSC populations.

**4.2.** **Proliferation**

**4.2.1 The heterogeneity of cell population**

An essential prerequisite for the planned experiments was the possibility for the *in vitro* proliferation of hMSCs. Here was the problem of heterogeneity of cell population in the center of what has been already addressed by various authors (Reyes and Verfaillie, 2001;. Yoo et al, 2005;. Zohar et al, 1997). Since the early experimental work there is no generally accepted method for the separation of undifferentiated hMSCs was available, had initially based on the experience of other authors, a convenient procedure to be designed and established.

Was already the results of flow cytometry to make clear that the various cell preparations have a highly variable composition (Smith et al., 2004). They are always a mixture of mesenchymal cells with different proliferation and differentiation capacity dar. it, similar to Colter et al. (2001), in addition to a large, relatively heterogeneous cell population of hMSCs, whose properties are not characterized in detail, a small subpopulation labeled RS cells are shown. The proportion of these cells *in vitro* and is probably also *in vivo* is always very small and is generally regarded as a potential supply for every need. According to some authors, these cells have only a maximum proliferation potential and are minimally differentiated (Colter et al., 2000). Others think these RS cells not only for the undifferentiated cells in the isolated population (Sekiya et al, 2002a,.. Smith et al, 2004). Some of them are even rather unsuitable for the chondrogenesis seen *in vitro* (Hung et al., 2002). For the above reasons, the interest was in regard to the planned experiments for the quantitative differentiation equal to the greater fraction of heterogeneous composition. In the literature, various methods have been described, which was trying to achieve a greater homogeneity of the cell population of mesenchymal stem cells (Baksh et al, 2003;. Curran

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Discussion

. et al, 2005; Mareschi et al, 2001).. Thus, by Hung et al. proposed the use of a sieve to separate the hMSCs with high proliferative and Differenzierungspotezial based on their size (Hung et al., 2002). Others have tried to find a clear marker for the undifferentiated hMSCs appropriate in order to sort out using a cell sorter (Van Vlasselaer et al, 1994;. Waller et al, 1995,.. Zohar et al, 1997). To this end, more than 200 different antibodies were tested by surface molecules, yet to achieve but without clear success. Another concept is the idea of selective enrichment of undifferentiated hMSCs from (Prockop et al. Al., 2001). According to the principle that proliferation potential and differentiation potential are inversely correlated, should the higher proliferative potential of undifferentiated stem cells can be used to separate them by maximal proliferation of pre-differentiated effectively already. How Prockop et al. have shown, this procedure could be used advantageously for the enrichment of the cells when the property is taken into account that undifferentiated hMSCs can proliferate at extremely low seeding density is particularly effective. As confirmed by other authors it is actually the proportion of undifferentiated cells enriched (Sekiya et al., 2002a). The explanation can be assumed that paracrine factors that stimulate the differentiation are undesirable, the less the impact. By a lower seeding density was actually a much higher proliferation rate can be achieved. If so, the proportion of undifferentiated cells would be increased, it could also increase the likelihood of being able to use the cells later exclusively for chondrogenic differentiation. As a measure of the success of a chondrogenic differentiation factor could increase the correlation of a measure of the proliferation rate and the amount of produced glycosaminoglycans seen. It was shown that the cell populations that were propagated with greater proliferation rate produce, after chondrogenic differentiation more GAG in the pellet culture. This is interpreted to mean that cell populations that were as a result of low seeding density increases with higher proliferation rate, a higher proportion - can contain cells that differentiate successful chondrogenic later - probably undifferentiated. This assumption is supported by analysis of cell morphology using flow cytometry. With high proliferation rates, with sowing density of 500 to 1000 cells per cm 2 increased cell populations less heterogeneity in size and light transmission when compared to those that have been propagated at a seeding density of 5000 cells per cm 2. It is not so much increased the share of so-called RS cells, but rather the proportion of daughter cells, although according to the theory have been indeed a lower proliferative potential, but still a reasonably broad differentiation potential according exhibit.

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Discussion

As another attractive way to increase the proliferation rate in addition to lowering the seeding density, the use of mitogenic growth factors was examined. Was about the influence of various growth factors on proliferation of hMSCs already reported in several studies (Chiou et al, 2006;. Marcopoulou et al, 2003;. Sundberg et al, 2006).. According to these results in this study particularly the effect of bFGF, TGF and PDGF was tested. Be very beneficial to increasing the proliferation rate has subsequently of in accordance with data of other authors (Bianchi et al, 2003; Trippel, 1995.; Chiou et al, 2006.) Proved the use of bFGF. This is also reflected in the results of GAG / DNA analysis. Although it is known, that can affect some growth factors in addition to the proliferation and differentiation of cells (Chiou et al., 2006). The effect can be explained by the idea that bFGF acts in the sense of an increase in the broad differentiation potential. To determine possible changes in the differentiation potential of cells as a consequence of the result of bFGF, flow cytometric analysis were performed. Case of propagation in the presence of bFGF, the cells showed a reduced presence of CD 90 and CD 105 as a marker of undifferentiated hMSCs. This can be interpreted as evidence that bFGF reduces the differentiation potential of cells, or in other words, a predifferentiation in a certain direction steers. The simultaneously observed positive effect on the GAG production may mean that while chondrogenic differentiation miteingeschlossen is very likely. A chondrogenic effect of bFGF was observed even *in* the *in vitro* proliferation of adult chondrocytes (Trippel, 1995). In relation to the objectives of this work, a method for differentiation of hMSCs to develop into chondrocytes, this supportive effect of bFGF was used at the chance.

Another interesting finding was used: During the investigation of optimal Adhärierungszeit for the separation of the hematopoietic cells was observed that cells adhere to proliferate faster, faster. Therefore, the cells in terms of achieving maximum proliferation rates only 24 to 72 hours was given the opportunity to adherent. As more and more slowly proliferating cells had adhered well to the typical changes in morphology, the visual impression could be used for quality control in Phasenkontrastmikrokop.

**4.2.2 The importance of a high proliferation rate in the enrichment of hMSCs**

The isolated during the course of three years from bone marrow aspirates derived hMSCs from different donors and were only gradually, with full

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Discussion

Consideration of the above results in terms of achieving a maximum rate of proliferation dissected. To have to demonstrate again how great the impact of the proliferation rate on chondrogenic differentiation, were the results of GAG production (GAG / DNA values) in the pellet culture in the context of the three-phase model as a function of the rate of proliferation with the cells grown *in vitro* were compiled. Figure 44 shows four different correlations for four different growth factor combinations. It is apparent that measured in all cases be achieved at higher proliferation rates than multiplication factor, higher GAG / DNA values, the slopes of the regression lines are very very different. The result allows two conclusions: firstly, the importance of the high proliferation rate demonstrated as a basis for a successful differentiation. On the other hand, it reflects the different properties of the cell preparations used in this study. Despite the large number of measurements, it is therefore not likely to win by averaging results from different cell preparations with different multiplication factors greater statistical reliability of the statements. When comparing the results obtained with different multiplication factors must be taken into account instead. Therefore, the results of qPCR analysis also illustrated together with the other analytical data. Also in this case in accordance with the assessment of the graphs in Figure 44 the best result when cells were used with high multiplication factors. Experimentally has also been shown to assess the morphology of the cells during proliferation as very helpful. The colonies have at optimal growing proliferation rate and thus often well-suited for the chondrogenic differentiation of cells. Unsuitable for the chondrogenic differentiation according to the three-phase model with individual preparations against it have egg-shaped or triangular cells proved.

**4.3 The Chondrogenic differentiation of hMSCs**

**4.3.1 The importance of growth factors**

As *in vivo,* the success of chondrogenesis in the simulation *in vitro* on the involvement of certain growth factors is dependent (Goldring et al, 2006;.. Hendriks et al, 2006). *In vitro,* have it especially different isoforms of TGF-ß and IGF-I and dexamethasone proved (In et al, 2006;. Sekiya et al, 2002b).. IGF-I was the first factor, the chondrogenic effect has been described (Salmon and Daughaday, 1957). For the participation of this anabolic factor in cartilage formation and its maintenance also speaks its presence in the synovial fluid (Schalkwijk et al.

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, 1989). The supportive effect of IGF-I in the differentiation of hMSCs (McQuillan et al, 1986,.. Neidel et al, 1994) and in the redifferentiation of dedifferentiated *in vitro* chondrocytes (. Van Osh et al, 2001) has already been described several times. These and similar findings justify the frequent use of IGF-I for experimental approaches to the chondrogenic differentiation of hMSCs.

For TGF-ß3 are very different functions described in the context of chondrogenesis. The seemingly contradictory findings be explained by the fact that the effect of this growth factor on the differentiation status of the target cell is dependent (Stalinska and Ferenc, 2005) (Jacob et al.). Thus, both mitogenic and chondrogenic effects of TGF-ß1 described (Giannouli and Kletsas, 2006; Yaeger et al, 1997).. In this study, in combination with bFGF and PDGF showed TGF-ß1 used a strong mitogenic effect. Similar effect has been shown in several studies (Bosse et al, 2006;.. In et al, 2006). In contrast, TGF-ß is very often attributed to a strong effect on the differentiation (Wang et al., 2005). All three isoforms, TGF-ß3 are as TGF-ß1 and TGF-ß2 formed among others by chondrocytes. The regulatory role of TGF-beta in cartilage formation during skeletal development was also shown (Kawaguchi et al, 2005). At the same time, TGF-ß apparently involved in the maintenance of cartilage function (Ballock et al, 1993,.. Bohme et al, 1995 ). Then that GAG content from the ECM of the cartilage in the absence of this growth factor (Cawston et al, 1999;. Lee et al, 2004a.). A synergistic effect of TGF-ß1 with IGF-I has been shown in serum-free medium upon stimulation of matrix formation in adult human chondrocytes (. Longobardi et al, 2006; Yaeger et al, 1997).. Also, the concentration dependence of the effect of IGF-I and TGF-ß3 was studied in detail in several publications (In et al., 2006)). The formation and secretion of TGF-ß are other factors such as bFGF or IGF-I and of the TGF-ß-dependent concentration itself (Ruscetti et al, 2005;. Wang et al, 2002)..

The role of glucocorticoids in the development stages of frühren of cartilage has been investigated for several decades (Jones and Addison, 1975; Kato and Gospodarowicz, 1985). Since then, dexamethasone is considered one of the most important differentiating factors for chondrogenesis and osteogenesis and used experimentally (Kim et al, 2005;.. Locker et al, 2004;. Tanaka et al, 2004). It was observed that the SOX9 is up-regulated expression with the addition of z as dexamethasone, which is considered a prerequisite for chondrogenesis (Sekiya et al., 2000) that the concentration of growth factors *in vitro* may play a crucial role, has also been shown (Richardson et al, 2003;. Behrens et al, 1975).. This is the effect of dexamethasone is a fine example: high concentration of

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Discussion

Dexamethasone served the osteogenic differentiation, whereas at lower concentrations it has a chondrogenic effect.

**4.3.2 The pellet culture as a differentiation model**

A number of recent publications on the topic "Chondrogenic differentiation of mesenchymal stem cells' differentiation based on the model of the so-called pellet culture (Johnstone et al., 1998). In this technique, cells are initially sedimented to a pellet. Subsequently, during several weeks of culture in the presence of an appropriate differentiation medium, the formation of an extracellular matrix is induced. The composition and concentration of the additives could be varied. This basically tries to simulate the process of mesenchymal Zellkondensierung during embryogenesis, are responsible for the specific cell-cell contacts as a trigger for the chondrogenic differentiation (Thorogood and Hinchliffe, 1975; Poole, 1991).

A typical example of the use of pellet culture as a model for chondrogenic differentiation of hMSCs is described by the 1998 Mackay Court Proceeding (Mackay et al., 1998). In this case, TGF-ß3 and dexamethasone are used to the sedimented hMSCs to support them in matrix formation. If the positive effect of these factors is well proven, the results remain qualitatively and quantitatively, to meet expectations (Yang et al., 2004).

This impression is also supported in this work confirms to compare data collected through the use of pellet culture as a differentiation model (direct pellet culture). In accordance with the findings of other authors is made ​​in the direct pellet culture no hyaline cartilage, but a biomechanically inferior matrix, which is characterized by the predominant synthesis of collagen type I and type II instead of a weak GAG production. In addition, observed in the direct pellet culture by various authors, the formation of significant amounts of collagen type X, which seems to point to a hypertrophic differentiation of chondrocytes (Ichinose et al., 2005). On the other hand, at this point noted critically that the early expression of collagen type X earlier than that of the collagen type II, not to order the activation of genes fits as of chondrogenesis known in vivo ago (Winter et al., 2003). Therefore, the conclusion can be drawn that the pellet culture for chondrogenic differentiation principle, not of hMSCs *in vitro* is, when the production plans of hyaline cartilage.

The cause for the purposes of *tissue engineering,* unsatisfactory results may lie in the heterogeneity of the isolated bone marrow cell population. It is conceivable that the different stages of differentiation in these cells

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respond to cell-cell contacts and the added growth factors vary widely. While immature cells enter the differentiation, mature chondrocytes begin with the hypertrophy. Of TGF-ß was already mentioned that its effect depends on the differentiation status of the target cell. Must be accepted as a result of an asynchronous culture. Remains unclear how adipogenic or osteogenic progenitor cells respond to this treatment.

Although the direct pellet culture, less than one method for cultivation of hMSCs in cartilage *tissue engineering* of the question, should not be dispensed with entirely on this method. For, as is known from experiments on the aggregation of primary chondrocytes, cell-cell contacts in the mature chondrocytes solve the formation of a hyaline extracellular matrix. Thus, the pellet culture is very well suited to assess the differentiation status of chondrocytes and their progenitor cells. Therefore, at the beginning of this work was started from the hypothesis that the pellet culture should be no more than differentiation model, but only used as a test system for potentially differentiated cells.

**4.3.3 The alginate gel as a differentiation model**

As a replacement for the pellet culture was chosen based on the accumulated experience in the working group to embed the cells in an alginate gel as a differentiation model. This method allows the differentiation of stem cells without the same matrix synthesis start to what was seen as the cause of the problems outlined. This is mainly achieved by preventing cell-cell contacts. In principle, therefore the two processes of differentiation and matrix synthesis are temporally separated from each other. The alginate gel in the differentiated cells are then only by intense cell-cell contacts in pellet culture synchronized to stimulate matrix formation. Because this process has been formally extended for this process, the term "three-phase model" is selected. In contrast, as described in the literature direct pellet culture, differentiation and matrix synthesis, while allowing in a reaction vessel, as a "two-phase model". In both cases separately performed proliferation is regarded as the first phase. The differentiation of hMSCs in alginate culture effectively prevents the unwanted, premature matrix synthesis, and still leaves open all possibilities of stimulation by growth factors. Moreover, in the three-phase model, where the additional advantage that stimulate two separate processes by selecting the most appropriate growth factors optimally.

The confirmation of the benefits of using the three-phase model in comparison to the two-phase model was central to this work. This model has been used successfully. Thus, in the pellet culture after differentiation in alginate

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Gel not only more GAG than in the direct pellet culture produces, but also improves the quality of the resulting matrix. As further evidence, a much higher rate of synthesis of collagen type II can be given. This was shown both at the protein level by Western blot and immuno-histologically and at the mRNA level using qPCR.

The use of the embedding of cells in an alginate gel as a culture for cartilage production *in vitro* is not new (Kavalkovich et al, 2002,.. Ma et al, 2003;. Majumdar et al, 2000; Mehlhorn et al, 2006;. Shakibaei and De Souza, 1997). Majumdar was able to show, for example, that which in the alginate gel matrix formed collagen II (Majumdar et al., 2000). More widely, several authors have tried these results by optimizing the conditions of alginate technology improve. Here, different parameters for the alginate culture studied such as the density of the gel, the shape of the alginate gel, the cell concentration and the duration of the culture (Steinert et al, 2003;.. Williams et al, 2005). In addition, was also tried both methods - the direct pellet culture and alginate culture - to compare directly. Thus, the advantages of chondrogenesis in alginate gel compared with the direct reports in pellet culture (Yang et al., 2004). In this example, but at the same time the main difference to one's own use of alginate technology significantly. It lies less in the way as in the type of use. For while the embedding of cells in an alginate gel found in the examples mentioned in the literature for the promotion of chondrogenesis, including differentiation and matrix formation is recommended, it is used as part of the three-phase model exclusively as a differentiation model. In contrast to the above examples, the cells within the three-phase model for the differentiation of the alginate gel can be isolated in order to subsequently stimulate through induction of cell-cell contacts in the pellet matrix formation.

To establish this system in the laboratory, the influence of several parameters, the differentiation of cells in alginate gel was examined, most notably various growth factors, the partial pressure of oxygen in the gas phase, the cell density in the gel as well as the duration of the alginate culture.

The positive effect of decreased oxygen partial pressure for the differentiation or redifferentiation of chondrocytes has been described (Domm et al., 2002). This also corresponds to the knowledge of the natural environment of the chondrocytes. As reported by Brighton, the oxygen content in the native cartilage to drop to about 1% (Brighton and Heppenstall, 1971). Thus, the findings presented in this work appear on a supportive role of decreased to 10% oxygen partial pressure in the chondrogenic differentiation in alginate culture is quite plausible. It is known that metabolic active cells, it was during the

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Proliferation and matrix synthesis, have a higher oxygen consumption. Accordingly, these processes can be promoted through a maximum with oxygen supply. It is different with less energy-consuming processes such as differentiation. Here, a lower oxygen partial pressure of advantage (Wang et al., 2000). Quite analogous considerations also led to the decision in the alginate culture medium to select one with a lower glucose concentration in the pellet and culture to such a use with higher glucose concentration. That this decision brought benefits could be experimentally confirmed by an increased GAG production (data in appendix).

The optimal cell density for the differentiation phase in the alginate culture was purely determined experimentally. Served as criteria for the formation rate of GAG and DNA content as a measure of the vitality of the cells in the following pellet culture. Recommended by other authors in higher cell density, which supposedly is conducive to the formation of matrix in the alginate gel (Majumdar et al., 2000), works in the alginate gel as a differentiation stage rather harmful. In a sub-optimal cell density, a disproportionate decrease in GAG synthesis rate is observed, indicating the support of the differentiation by paracrine factors first Be advantageous, the cell density was found from 2 million cells per ml of alginate culture. This value corresponds approximately to the cell density in the native cartilage (about 1,6 million per ml of matrix).

Another criterion for the optimization of differentiation in alginate culture was the duration of the alginate culture. The experimental data show that a period of two weeks is to be regarded as minimal for the cells to give optimal opportunity to achieve the differentiation state of mature chondrocytes. Measurements were made ​​in the form of matrix formation in a subsequent standard pellet culture. A longer-alginate culture has disadvantages due to cell loss. After three weeks of culture in alginate gel decreases the cell number, which was identified by DNA analysis in pellet culture.

Since the optimization of the method under the perspective of a therapeutic intervention was carried out later, was also tested using human serum (as a substitute for autologous serum) in the differentiation phase in alginate gel. The results provided in comparison to no visible differences calf serum (data in appendix).

One of the most important criteria for the success of the alginate culture as a differentiation model, the characterization of the isolated cells from the alginate gel with the

1 As used in the pellet cultures are always the same number of Alginatbeads was and the standard conditions in the pellet - culture were the same (see M & M), one can say with certainty that the only reason for the decrease in the number of cells, the cell density in alginate or the duration of the alginate - culture was.

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Discussion

Its characteristics of mature chondrocytes. For this purpose, the ability to form chondrones a used unique structure typical for chondrocytes (Poole et al., 1992). Evidence of the formation of chondrones place one hand by the typical morphology and the other by the immunological identification of a highly specific marker for the chondrones, the collagen type VI. In addition to the quantitative evaluation revealed that virtually all of the alginate gel-isolated cells (98% + - 2%) were stained with fluorescently labeled antibodies directed against collagen type VI. This means that we have succeeded with the three-phase model to gain a virtually homogenous cell population of mature chondrocytes from a heterogeneous population with regard to the differentiation status of hMSCs. The formation of collagen type VI was accompanied by the formation of collagen type I and II. The synthesis of collagen type II is characteristic of hyaline cartilage and is necessary for a meaningful tissue replacement (Poole et al., 1992). The simultaneous formation of collagen type I can be interpreted as an indication of the scarring response. You will be observed at suboptimal *in vitro* culture conditions (Shakibaei et al., 1993).

In the context of finding the optimal conditions for the differentiation in alginate gel were also tested different combinations of growth factors. As a start condition the same combination was used at the same concentrations as described for direct pellet culture as essential, namely dexamethasone, TGF-ß3 and IGFI. To limit the contribution of individual growth factors, singly and in combinations were omitted from the culture medium.

The omission of dexamethasone led to decrease in the proportion of cells that

after differentiation in alginate culture using antibodies against collagen type I, II and VI were stained. In addition, it was observed at the mRNA level, particularly a decrease in collagen type II expression and the expression of collagen type I remained virtually unchanged. The benefits derived from these results, positive influence of dexamethasone on the differentiation of stem cells is therefore in accordance with data that has been already shown by other authors (Derfoul et al, 2006;. Grigoriadis et al, 1989).. Also as a result of the omission of dexamethasone was a slight decrease in the expression of SOX9 and type X collagen found. This is in par with the observations of Chen and John Stone, who have examined the influence of dexamethasone and TGF-ß3 in pellet culture model of differentiation (Johnstone et al, 1998;. CW Chen et al, 2005)..

The most important criterion for assessing the effect of dexamethasone on the differentiation in alginate gel matrix, however, the educational potential of the cells in a subsequent pellet culture was viewed. It was found that the cells

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less GAG produced when they were differentiated in the absence of dexamethasone. Dexamethasone was obviously needed by the cells in the alginate culture to the differentiation status of chondrocytes to achieve. These data are consistent with the findings of other authors who report an essential role of dexamethasone in the dedifferentiated chondrocytes redifferentiation and chondrogenic differentiation of stem cells (Ochi et al., 2006).

A positive effect of TGF-ß3 on the differentiation of hMSCs into chondrocytes is already known (Lee et al, 2004b,.. Zhang et al, 2006). This role of TGF-ß3 could be confirmed by studies to this work. Thus, the presence of TGF-ß3 to a strong induction of collagen type II mRNA in addition to a weaker effect on collagen type I mRNA. In addition, a modest induction of expression of Sox9 and collagen X was observed. These effects are consistent with published data (Im et al., 2006).

At the protein level (Chondronbildung), caused the omission of TGF-ß3 heavy losses in collagen type I synthesis, a small increase in type II collagen synthesis and no visible changes in the collagen type VI. On the GAG synthesis in the pellet culture, the omission of TGF-ß3 during the differentiation phase has a positive effect. In the absence of TGF in the alginate culture hardly more GAG is formed. This confirms the essential role of TGF-ß3 for chondrogenic differentiation. This effect was already observed by other authors (Indrawattana et al., 2004).

The important role of IGF-I in the chondrogenesis has long been known (Blunk et al, 2002,.. Yaeger et al, 1997). This accounts for the frequent use of this factor in the differentiation phase. IGF-I is also in the alginate culture a positive effect, which manifests itself as increased GAG formation rate in the pellet culture. At the mRNA level does the presence of IGF-I during the Differenzierunsphase strong positive effect on the induction of collagen type II and to a somewhat lesser extent, on the induction of type I collagen and Sox9. The expression of type X collagen remained unchanged under these conditions. The formation of the alginate gel chondrones is promoted by IGF-I. The omission of IGF-I results in the alginate culture to a significant reduction in matrix synthesis.

In summary we can say that TGF-ß3, IGF-1 and dexamethasone may also be classified under the terms of the alginate culture as essential factors for the chondrogenic differentiation of hMSCs. The positive effects of these factors on the differentiation of cells in the alginate culture were both at the mRNA level using RT-PCR at the protein level by immunological analysis of cytospins, as well as functional in the form of GAG production in the

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subsequent pellet culture can be detected. Also observed the induction of collagen I synthesis, especially under the influence of TGF-ß3 and IGF-I in the alginate culture seems initially the target of chondrogenic differentiation disagree. This result should not be overstated. It has been shown that the differentiation status of the isolated cells from the alginate gel could not be reproducibly assessed by analysis of mRNA levels of collagen type I and II. This was not investigated further for experimental reasons. Contrast, has proven itself as the only reliable method to verify the putative functional chondrocytes in the subsequent pellet culture.

**4.3.4 The matrix formation in the pellet culture**

In addition to the evidence of successful differentiation of the cells in the alginate culture of the three-phase model, additional studies were undertaken with the goal of functional verification can be isolated from the alginate gel-differentiated chondrocytes. To the pellet culture was used as a model for the formation of hyaline cartilage matrix. The results of the pellet - cultures show the superiority of three-phase model, compared to the two-phase model. The GAG synthesis is significantly increased when additional differentiation stage was switched on. The result demonstrates the advantage of the quantitative differentiation of the cells used. Immuno-histological analysis confirmed the findings.

The difference is clear on when the effect is studied of the additional differentiation in the three-phase model on the gene expression of some markers chondrogenesis. Here are two examples over the two-phase model, a 30-70-fold found elevated levels of collagen II mRNA. Of equal importance is that the mRNA levels of collagen type I and X rise here at the same time by less than a factor 2.

To exploit the advantages offered by the three-phase model was also attempted to optimize the culture conditions for the pellet culture separate. As already known from the literature was that the formation of extracellular matrix in hyaline cartilage not only not supported by the presence of dexamethasone and TGF-ß3, but inhibited (Chadjichristos et al is 2002,. De Luca, 2006; Gerwin et al ., 2006), the effects of the omission of these factors were also examined. Such a inhibitory effect on the GAG formation by TGF-ß3 and DEX in the pellet culture of the three-phase model has been demonstrated. Here's an important contrast to the two-phase model is clear in which these two factors are considered essential (Mackay et al., 1998).

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However, it seems the effects of dexamethasone and TGF-ß3 terms of GAG synthesis is not to be additive and should not be dispensed with IGF-I. Similar findings arise when comparing the immuno-histological data: In the paraffin sections of pellet cultures after additional differentiation can be seen a much stronger staining of collagen II antibodies when dexamethasone and / or TGF-ß3 are omitted. In the presence of IGF-I and in the absence of dexamethasone and TGF increases not only the synthesis of collagen II, but also the formation of collagen type I. This suggests a complex effect of IGF-I. On the one hand, the addition of IGF-I is required for the GAG formation, on the other hand, is thus in the absence of TGF-ß3 and dexamethasone stimulates collagen type I synthesis.

Even the omission of all three factors is not acceptable, because in addition to a strong staining of collagen type II desired and collagen type I is favored, but not the GAG formation. Perhaps an optimization can only be more precise dosing of the concentrations of individual growth factors can be determined. Consistent with these data, the important role of IGF-I on proteoglycan synthesis shown by other authors (McQuillan et al., 1986, Neidel et al., 1994).

The observed negative effect of TGF-ß3 and dexamethasone on the GAG production and immunologically measured collagen synthesis is completed by qPCR measurements. There is a strong induction of collagen II expression observed when either dexamethasone or TGF-ß3 was omitted. Simultaneously, the mRNA levels of collagen I and SOX9 was hardly affected. Under these conditions, but partly also a relative increase in expression of collagen type X is found, but much more at a lower level. It is possible that the increased mRNA concentration of collagen type X biologically harmless to the formation of hyaline cartilage, because it is in the order of the value found in native cartilage. In contrast to these findings will a simultaneous omission of TGF-ß3 and dexamethasone from unfavorable. Although in this case, induction of collagen II mRNA is measured, but is simultaneously also stimulates the expression of collagen type I and X. This result is not satisfactory because it is close to the case of osteoarthritis (Gebhard et al, 2003;.. Ho et al, 2006). In summary we can say that in a three-phase model can be offered an opportunity for individual stimulation of differentiation and matrix formation can be used successfully. In particular, however, seen in a more precise dosage of the individual growth factors used in yet another way to target the synthesis of matrix components in the context of the formation of hyaline cartilage continues to improve.

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**Compared the findings with the three-phase model samples with native cartilage, human cartilage from the articular surface of the knee**

Since the present work should help clarify the question, how are far from the bone marrow isolable stem cells for *tissue engineering* of hyaline cartilage implants, is a comparison of the producible with the three-phase model of cartilage samples with the original of great interest. These two methods were used to analyze the gene expression of important gene markers using the qPCR and immuno-histological staining of paraffin sections. A direct comparison of gene expression of the gene markers used in this work shows that in the pellet culture after the three-phase model of the currently optimal conditions, ie in the absence of TGF-ß3 or dexamethasone, a strong induction of mRNA of Type II collagen was detected. What is also relevant with regard to the desired goal, remained the same levels for the mRNAs of collagen type I and type X in the range of values ​​found in native cartilage. Sox9 also no visible change was observed. The graph in Figure 45 also demonstrates the sensitivity of the system. If both factors - TGF-ß3 and dexamethasone - omitted the same time, this has bad consequences. Now, the genes of collagen type I and X is activated more strongly than that of type II collagen

The result of the immuno-histological comparison shows that among the previously determined best conditions as in the native sample a strong staining with antibodies directed against collagen type II, but has not been achieved with those against type I collagen. In addition to preferentially localized pericellular staining with collagen type VI antibodies is evident. Overall, the intercellular gaps appear in the native sample still larger than the product produced *in vitro.* But experience shows this is not a disability for use in *tissue engineering.*

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Summary

**Summary**

The aim of this study was to establish a procedure for chondrogenic differentiation of human mesenchymal stem cells from bone marrow.

These so-called three-phase model has been designed that the temporal separation and separate stimulation of the three main processes - enables proliferation, differentiation and matrix synthesis. To obtain a suitable cell population for this project from the bone marrow was a process for enriching and developing so rapidly proliferating only slightly differentiated progenitor cells. To monitor the quality of different methods of cell flow cytometry were used. The differentiation of the cells was reversible after embedding in a gel to prevent avoidance of cell-cell contacts early initiation of matrix formation. Supporting the process of differentiation by growth factors like IGF-I, TGF-ß3 and dexamethasone was verified by measurement of matrix formation and gene expression of collagen type I, II and X. The proof of the success of quantitative differentiation was achieved by immunohistochemical staining of collagen type VI, a highly specific marker for chondrones. For the functional verification of the differentiation of hMSCs into mature chondrocytes was analyzed by the cells in a three-week pellet-formed culture with regard to the matrix formed glycosaminoglycans and the expression of some genes characteristic of hyaline cartilage. It was found that after optimization of conditions, including the omission of TGF-ß3 or dexamethasone from the culture medium, could be achieved for the hyaline cartilage typical expression pattern. While the formation of strong mRNA of collagen type II could be stimulated, remained low on collagen type I and X level. The results were confirmed by immunohistochemical analysis of paraffin sections. Here, too, conditions are determined under which collagen type II and VI were increasingly formed, whereas collagen type I remained below the detection limit. Based on these parameters, the quality of the manufactured with the three-phase model of cartilage is far superior to that which could be achieved previously by other authors with which differentiation model of pellet culture. The comparative analysis of gene expression of important gene markers of cartilage and immuno-histological comparisons manufactured in vitro cartilage sample with native cartilage lead to the conclusion that the developed three-phase model is a very good opportunity for the use of hMSCs from bone marrow to produce cartilage implants for the treatment of articular surface damage is.

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Appendix

**Die *Screenshots* von der Auswertung der Cytospins A:B:**

**Die Auswertung der Cytospins**

Die Bilder zeigen exemplarisch die Auswertung der Cytospins (Screenshots)

Aufnahme der Bilder von den Cytospins mithilfe des Fluoreszenzmikroskops wurden

mithilfe des Programms Lucia G (Nikon GmbH, Düsseldorf) ausgewertet

A: DAPI gefärbte Zelle wurden von dem Programm gezählt.

B: Die Kollagen II Markierung konnte mithilfe des Programms ausgewertet.

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Appendix

**010203001020 LowHighFKSHSG/D**

**Ratio [**

**ng/ng]G/D Verhältnis [ng/ng]**

**Abbildung zeigt die Abhängigkeit der Differenzierung im Alginat von der Verwendung humanes bzw. fötales Serums (A) und der Anwendung High Glucose bzw. Low Glucose:**

Die GAG- und DNA-Analyse der Pellet-Kultur nach Alginat. Die Zellen wurde im Alginat mit High Glukose (High 4500 mg/L) Medium bzw. Low Glucose (Low 1000 mg/L) kultiviert. (A) (n=4). Die Anwendung humanes Serum (HS) statt fötales Kälberserum (FKS) hatte wieder Vorteile noch Nachteile hinsichtlich GAG-Produktion (B). (angegeben sind die Mittelwerte unter Verwendung der Standardbedingungen in Alginat- und Pellet-Kultur, (n= 3.)

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Appendix

**Immunohistologie von Pellet-Kulturen mit bzw. ohne Alginat-Kultur**

**(Maßstabbalken 100** μ **m Standardbedingungen)**

**Kollagen IKollagen IIdirekte Pellet-KulturPellet-Kulturnach AlginatKollagen Alginat**

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Appendix

**Immunohistologie von Pellet-Kulturen nach Alginat-Kultur**

**(Maßstabbalken 100** μ **m Standardbedingungen im Alginat)**

**Kollagen IKollagen IIDEXTGFIGF –+++–+––+ Pellet-Kulturnach AlginatKollagen Alginat**

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Appendix

**Kolonie-Bildene Zellen**

**(Maßstabbalken 100** μ **m, Standardbedingungen representative Bilder) 2 Tage4 Tage6 Tagenach der Isolierung…**

**Abhängigkeit der Morphologie der Zellen von der Aussaatdichte**

**(Maßstabbalken 100** μ **m, Standardbedingungen representative Bilder) 5001000Aussaatdichte [ Zellen/cm2 ]5000Passage 1Passage 35001000Aussaatdichte 3**

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