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DISSERTATION

In vitro recruitment and differentiation of human mesenchymal cells for the development of a scaffold-based meniscus repair approach

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List of abbreviations

3-D	three-dimensional
COMP	cartilage oligomeric matrix protein
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IKDC	International Knee Documentation Committee
IP10	chemokine CXCL10
LINK	cartilage link protein
Lymphotactin	chemokine XCL1
OA	osteoarthritis
real-time PCR	real-time polymerase chain reaction
PGA	poly(glycolic acid)
PI/FDA	propidium iodide/ fluorescein diacetate
RA	rheumatoid arthritis
SF	synovial fluid
SF-36	Short Form (36) Health Survey
TECK	chemokine CCL25
TGF-β3	transforming growth factor-β3
VAS	Visual Analog Scale

1. Abstract/ Abstrakt

Introduction: Meniscus lesions are a complex orthopedic challenge. Cell-free scaffold-based repair approaches for the non-vascular meniscus zone are rarely developed. Regenerative techniques based on PGA scaffolds with factors that stimulate cell recruitment and differentiation may be an alternative. This work aimed at investigating factors that support the *in vitro* recruitment and differentiation of human mesenchymal cells for the development of a cell-free scaffold-based meniscus repair approach.

Methodology: Isolation and expansion of human meniscus cells as well as evaluation of cell morphology and growth kinetics were performed. Therein, chemokines were identified in synovial fluids from normal, osteoarthritis and rheumatoid arthritis donors by chemokine antibody membrane array. Selected chemokines were tested for their recruiting potential on joint-associated mesenchymal progenitor cells. Human meniscus cells were evaluated for their migratory activity toward increasing doses of human serum. Further studies investigated factors that support re-differentiation of human meniscus cells. Herein, the potential of hyaluronic acid, human serum and TGF-ß3 to induce meniscal extracellular matrix was respectively evaluated on protein and gene expression level in high-density cultures. Subsequent studies assessed cell viability and the induction of typical meniscal genes and proteins after 3-D cell cultivation in hyaluronic acid loaded PGA scaffolds that have been cultured in the presence of hyaluronic acid and human serum.

Results: Human meniscus cells were successfully isolated from native meniscus tissue. Cells showed high proliferation and expansion capacities in monolayer cultures and a stable fibroblastoid phenotype. Cell migration assays confirmed that human serum and the chemokines TECK, IP10 and Lymphotactin have the potential to recruit human joint-associated mesenchymal progenitor and meniscus cells. High-density cultures of human meniscus cells showed the induction of meniscal extracellular matrix after stimulation with hyaluronic acid and human serum. Meniscus cells seeded in 3-D scaffold-based cultures showed a high cell viability and the induction of meniscus-like extracellular matrix.

Conclusion: Although it is highly speculative and results of these *in vitro* studies do not reflect in vivo situations, we conclude that a cell-free approach based on PGA scaffolds combined with hyaluronic acid and human serum may have the potential to

support *in vivo* meniscus tissue healing by recruiting resident mesenchymal cells of the knee joint and differentiating them into meniscal cells. Results of this work encourage preclinical studies and the further development of a cell-free approach based on PGA scaffolds in combination with hyaluronic acid and human serum for the repair of human non-vascular meniscus lesions.

Einleitung: Verletzungen des Meniskusgewebes stellen ein komplexes orthopädisches Behandlungsproblem dar. Derzeitige Reparaturtechniken sind effektiv für den vaskulären Außenbereich, der Behandlungserfolg lässt sich aber nicht auf den avaskulären Innenbereich übertragen. Eine Alternative zu den wenigen bisher klinisch etablierten zellfreien Trägermatrix-basierten Verfahren könnte hierbei die Entwicklung eines Ansatzes zur Meniskusregeneration auf Basis von resorbierbaren PGA-Trägerstrukturen in Kombination mit Induktionsfaktoren für die Rekrutierung und Differenzierung humaner mesenchymaler Zellen sein.
Methodik: Zunächst wurden robuste Methoden zur Isolierung und Vermehrung von humanen Meniskuszellen etabliert sowie deren Wachstumskinetik und Zellmorphologie untersucht. Weiterhin wurden Chemoattraktanten definiert und in einem *in vitro* Chemotaxis-Testsystem auf ihr Potential zur Rekrutierung von Meniskuszellen und Gelenk-assoziierten mesenchymalen Vorläuferzellen untersucht. Hierzu wurde die zellanlockende Wirkung von Humanserum und ausgewählten Chemokinen untersucht, die zuvor in Synovialflüssigkeiten von gesunden sowie an Osteoarthrose und Gelenkrheumatismus erkrankten Spendern identifiziert wurden. Fortführend wurden Faktoren bestimmt, die eine Re-Differenzierung von humanen Meniskuszellen und die Ausbildung von meniskusartiger extrazellulärer Matrix anregen können. Hierbei wurde der jeweilige Einfluss von Humanserum, Hyaluronsäure und TGF-ß3 auf die Matrixbildung in standardisierten Hochdichte-Pelletkulturen bestimmt und mittels Protein- und Genexpressionsanalysen typischer Meniskusmarker ausgewertet. Des Weiteren wurden humanen Meniskuszellen zusammen mit Hyaluronsäure in resorbierbare PGA-Vliese eingebettet und in Hyaluronsäure- und Humanserum-haltigem Medium kultiviert. Die 3D-Kulturen wurden mittels Zellvitalitätsfärbung und anhand von Protein- sowie Genexpressionsanalysen typischer Meniskusmarker ausgewertet.
Ergebnisse: Humane Meniskuszellen konnten erfolgreich aus dem Nativgewebe isoliert und in Monolayerkulturen vermehrt werden. Die Zellen zeigten ein hohes

Proliferationsvermögen, ein stabiles Wachstumsverhalten und einen fibroblastoiden Phänotyp. Die Auswertung der Zellmigrationsversuche ergab, dass Humanserum und die Chemokine TECK, IP10 und Lymphotactin das Potential zur Zellanlockung besitzen. Die Stimulation von Hochdichte-Pelletkulturen mit Hyaluronsäure und Humanserum wirkte jeweils begünstigend auf die Ausbildung von meniskusartiger extrazellulärer Matrix. Nach der Kultivierung von humanen Meniskuszellen in 3-D Trägerstruktur-basierten Kulturen konnte eine hohe Zellvitalität und die Induktion einer meniskusartiger extrazellulären Matrix nachgewiesen werden.

Schlussfolgerung: In den durchgeführten *in vitro* Studien konnten Faktoren herausgestellt werden, die eine *in vitro* Rekrutierung von humanen Meniskuszellen und Gelenk-assoziierten mesenchymalen Vorläuferzellen unterstützen und eine Induktion von meniskusartiger Matrix begünstigen. Diese Ergebnisse lassen vermuten, dass ein Trägermatrix-basierter Ansatz aus PGA und in Kombination mit Hyaluronsäure und Humanserum auch die *in vivo* Reparatur von Meniskusgewebe durch die Rekrutierung von ortsständigen Zellen und deren Differenzierung zu meniskusartigen Zellen unterstützen könnte. Die Ergebnisse dieser Arbeit bilden die Grundlage für präklinische Studien und die weitere Entwicklung eines zellfreien Ansatzes basierend auf PGA-Trägerstrukturen, Hyaluronsäure und Humanserum für die Behandlung von humanen avaskulären Meniskusläsionen.

2. Introduction

The wedge-shaped menisci in the knee joint are known to play an important role in load transmission, shock absorption, knee joint stabilization and joint congruity [1]. Meniscus function is reflected by its morphology showing distinct tissue zones (outer vascular zone and inner non-vascular zone) and biomechanical resistant fibro-cartilaginous tissue, which contains mainly of water (72%), collagens (22%) and extracellular matrix components like aggrecan and glycosaminoglycans [2]. In the meniscus, radial and circumferential bundled type I collagen maintains the structural integrity during load bearing and represents over 90% of the total collagen content besides a number of minor collagens (e.g. type II, III, V collagen). Aggrecan supports the viscoelastic properties, compressive stiffness and tissue hydration of the meniscus [1, 2].

Meniscus injuries are among the most frequent injuries and pose a complex problem in orthopedic surgery. Only injuries of the outer vascular meniscus zone may heal spontaneously or upon suturing, while the inner non-vascular zone shows a low self-regeneration capacity [3]. Traumatic or degenerative injuries of this inner meniscus zone often fail to heal and lead to a partial or complete tissue resection (meniscectomy). Disruption of the fibrous meniscus architecture impairs load transmission and initiates degeneration of the adjacent articular cartilage surface [3]. Consequently, meniscectomy can lead to the development of osteoarthritis at 5-10 years post-injury [4]. Current surgical repair techniques are effective in the outer vascular meniscus zone but show an insufficient therapeutic outcome for the inner non-vascular zone. Regenerative strategies based on Tissue Engineering are a promising opportunity for meniscus repair of the inner non-vascular zone to avert the onset of knee joint osteoarthritis. Thereby scaffold materials supporting cells to restore functional tissue offer new treatment strategies for the repair of the non self-healing inner meniscus zone [1]. Until now, cell-free scaffold-based techniques for partial meniscus repair are rarely developed. Currently, only two cell-free implants based on animal type I collagen (CMI®) or polyurethane (ACTIFIT®) are tested clinically. Patients receiving the medial CMI® meniscus implant showed a significant lower VAS pain score, higher objective IKDC score, Tegner activity level and SF-36 score and a significantly less medial joint space narrowing compared to patients treated with partial meniscectomy at a minimum 10-year follow-up [5]. ACTIFIT® has

been clinically proven in first pilot case series [3]. Therein, second-look evaluation showed the integration of the scaffold with the native meniscus tissue at 12 months follow-up [3]. Histological analysis of taken biopsy samples showed viable cells within the polyurethane scaffold [3, 5].

Clinical development of a cell-free scaffold-guided meniscus repair approach requires the attraction of cells into the scaffold and their stimulation to form meniscal repair tissue. Factors for cell recruitment and cell differentiation are necessary to support these needs. The combination of bioactive factors with scaffolds may help to attract cells from the adjacent tissue into the defect area and to induce meniscal tissue matrix. In recent studies, human serum [6, 7] and synovial fluid [7] have been shown to recruit human mesenchymal cells *in vitro*. Therefore, these factors or components thereof are promising candidates for the recruitment of cells into the scaffold and may also show a migratory effect on human meniscus cells and joint-associated progenitor cells. Regarding factors for meniscus matrix induction, transforming growth factor-ß3 (TGF-ß3) has been described to stimulate proteoglycan synthesis in meniscus cell cultures [8, 9] and may support the formation of meniscal extracellular matrix. Another bioactive factor may be hyaluronic acid as it has been shown to promote the *in vitro* proliferation of meniscus cells without altering of cell morphology or glycosaminoglycan synthesis [1]. Hyaluronic acid also has been found to support meniscus healing in the rabbit model [10]. Development of a cell-free scaffold-guided meniscus repair approach requires a suitable scaffold material for cell immigration and tissue ingrowth. Synthetic bioresorbable polymers are advanced scaffold materials since their material properties like porosity, degradation rate, three-dimensional (3-D) structure and mechanical stability are controllable and can be adapted to biological and biomechanical requirements for implants. Among synthetic biodegradable polymers, poly(glycolic acid) (PGA) polymers are suitable scaffolds for cell ingrowth due to their good biomechanical stability, cytocompatibility and 3-D architecture. Based on the good long-term experience with PGA and its derivates in the field of articular cartilage repair [11, 12], PGA seems to be also a suitable material for the development of a cell-free meniscus repair approach. Thus, studies of this work will aim at the identification of bioactive factors that support cell attraction and cell re-differentiation and will evaluate these factors in combination with PGA scaffolds with regard to the development of a cell-free scaffold-guided repair approach for non-vascular meniscus lesions.

3. Objectives

The focus of this doctoral thesis is to identify factors for the *in vitro* recruitment and meniscal differentiation of human mesenchymal cells with regard to the development of a cell-free scaffold-based approach for the treatment of non-vascular meniscus lesions.

Studies will pursue following sub-objectives:

1) To establish robust methods for isolation of human meniscus cells from native meniscus tissue and for *in vitro* cell expansion. To investigate their cell morphology and growth kinetics under sub-cultivation.

2) To identify potential factors for mesenchymal cell recruitment *in vitro*. Thereby, synovial fluid from normal, rheumatoid arthritis and osteoarthritis donors will be analyzed for their chemokine profiles. Human meniscus cells and joint-associated mesenchymal progenitor cells will be tested for their migratory activity toward chemotactic factors, such as human serum and selected chemokines derived from chemokine profile analysis of synovial fluids.

3) To identify stimulating factors for human meniscus cell re-differentiation and meniscus-like matrix induction. Herein, hyaluronic acid, human serum and TGF-ß3 will be investigated for their potential to induce meniscal extracellular matrix formation using standard high-density differentiation assays. The effect of selected factors on cell re-differentiation and meniscus-like matrix induction will be evaluated on protein and gene expression level using typical meniscal marker proteins and genes.

4) To investigate meniscus-like matrix induction in 3-D scaffolds seeded with human meniscus cells. *In vitro* expanded human meniscus cells will be cultivated in resorbable PGA scaffolds supplemented with factors stimulating cell migration and cell re-differentiation. Therein, cell viability and cytocompatibility with the scaffold will be investigated. The induction of typical meniscal markers will be verified on protein and gene expression level and compared to typical markers found in native meniscus tissue samples.

4. Methodology

4.1. Cell isolation, expansion, growth kinetics and morphology of human meniscus cells

Human meniscus cells were harvested by enzymatic digestion of post mortem non-degenerative donor menisci and were expanded in monolayer cultures under defined conditions as described in the publications 1 and 2 in details. For investigation of growth kinetics, isolated cells from 3 human adult donor menisci were expanded in monolayer cultures up to passage 8. Total cell numbers of each cell passage were obtained and plotted against the cultivation time in days. After reaching a confluence of about 80%, primary cultures were trypsinized and re-seeded with a cell density of 10,000 cells/cm². Cell morphology of human meniscus cells was evaluated directly after cell attachment at passage 0 and after cell sub-cultivation at passage 3.

4.2. Identification of potential cell recruiting factors

Chemokine levels of collected synovial fluids (SF) from normal, rheumatoid arthritis (RA) and osteoarthritis (OA) donors were determined by human chemokine antibody membrane arrays as described in publication 1. The migratory activity of human joint-associated mesenchymal progenitor cells (cells derived from subchondral cortico-spongious bone) toward selected chemokines was determined using 96-well chemotaxis assays as described in detail in publication 1.
Furthermore, human serum derived from the German Red Cross was analysed for its dose-dependent migratory effect on human meniscus cells as described in detail in publication 2.

4.3. Identification of potential cell re-differentiation stimulating factors in high-density cultures

A detailed description of this work is given in publication 2.

Induction of meniscus-like extracellular matrix formation was analyzed in high-density cultures of human meniscus cells after stimulation with TGF-ß3, hyaluronan or human serum and in a serum-free control group. Therein, 1,260 cell pellets were

examined in total including cell preparations of 5 individual donors. The effect of TGF-ß3, human serum or hyaluronic aicd on cell re-differentiation was examined by gene expression analysis (real-time PCR) of marker genes like *type I collagen, type II* and *type IX collagen, aggrecan* and *cartilage oligomeric matrix protein (COMP)* at defined time points. The formation of meniscus-like extracellular matrix proteins was also evaluated on protein level. For that purpose cell pellet sections were prepared and analyzed for the presence of proteoglycans and type I and II collagen by histological and immuno-histochemical stainings.

4.4. Three-dimensional cultivation of *in vitro* expanded human meniscus cells in factor-loaded scaffolds

A detailed description of this work is given in publication 3.

Three-dimensional cultures of human meniscus cells were prepared using PGA scaffolds. Human meniscus monolayer cells were expanded up to passage 3 prior to cultivation in 3-D scaffolds. Three scaffolds were seeded each with human meniscus cells derived from 5 individual donors using 8.8 million cells per scaffold. Cells were mixed with hyaluronic acid, homogenously dispensed in PGA scaffolds and fixed with fibrin glue. 3-D scaffold-based cell cultures were cultivated in the presence of human serum and hyaluronic acid for 3 weeks.

Samples were taken at defined time points to verify cell viability and to perform histological and gene expression analyses. Cell viability and cytocompatibility with the scaffold material were assessed by propidium iodide/ fluorescein diacetate (PI/FDA) staining. The de- and re-differentiation potential of *in vitro* expanded human meniscus cells was investigated by comparative gene expression analyses of corresponding native meniscus tissue samples, expanded monolayer cells at passage 0 and 3 and of 3-D scaffold-based cell cultures at week 1 to 3. For evaluation of the cell re-differentiation capability, expression levels of respective marker genes in expanded monolayer cells were evaluated statistically against levels found in 3-D scaffold-based cell cultures and those in native meniscus tissue samples.

Sections of the 3-D cell cultures were analyzed by histological and immuno-histochemical stainings for the presence of proteoglycans and type I collagen.

Staining intensities were additionally quantified by a subjective scoring system.
Native meniscus tissue samples served as positive controls for histological and immuno-histochemical stainings.

5. Results

5.1. Cell isolation, expansion, growth kinetics and morphology of human meniscus cells

Detailed results have been reported in publication 3 (Freymann et al. 2012).

Summary

Robust methods for isolation of human meniscus cells from native meniscus tissue and for the *in vitro* cell expansion could be established. Human meniscus cells showed a high proliferation capacity under monolayer expansion. Evaluation of growth kinetics showed a stable cell proliferation and increasing cell numbers over the observed cultivation time of 50 days and 8 cell passages. Isolated cells adhered fast on the plastic surface and showed a stable fibroblastoid morphology after sub-cultivation.

5.2. Evaluation of potential cell recruiting factors

Detailed results have been reported in publication 1 (Endres et al. 2010) and in publication 2 (Freymann et al. 2013).

Summary

As described in publication 1, analyses of chemokine antibody arrays showed that SFs from normal, RA and OA donors contain a broad range of chemokines. Chemotactic analysis was performed for 10 chemokines that showed significantly reduced levels in RA or OA compared to normal SF or robustly high levels in all SF. Results revealed a migratory activity of joint-associated subchondral progenitor cells toward 3 (TECK, IP10, Lymphotactin) out of 10 selected chemokines.
Human serum showed a dose-dependent chemotactic effect on human meniscus cells (publication 2). Migratory activity of these cells increased significantly upon stimulation with human serum in doses of 1-20% when compared to negative controls (without human serum). Stimulation with human serum dose higher than 20% led to a decreased chemotactic effect on human meniscus cells. Cells showed a minimal migratory activity upon stimulation with human serum in a dose of 100%.

Results of publication 1 and 2 revealed that human serum and the chemokines TECK, IP10 and Lymphotactin, which are present in the SF, have the potential to induce cell migration of joint-associated mesenchymal cells.

5.3. Evaluation of potential cell re-differentiation stimulating factors in high-density cultures

Detailed results have been shown in publication 2 (Freymann et al. 2013).

Summary

Hyaluronic acid, human serum and TGF-ß3 were each investigated for their potential to induce meniscal extracellular matrix formation in standard high-density differentiation assays. The effect of selected factors was verified by gene expression and protein analysis of typical meniscal markers.

High-density cultures of human meniscus cells stimulated with 10% human serum showed significantly increased expression levels for *COMP* and *aggrecan* when compared to non-stimulated controls at day 7. Gene expression analysis of both genes (*COMP* and *aggrecan*) in high-density cultures stimulated with 25% hyaluronic acid revealed slightly increased expression levels for *COMP* and *aggrecan* compared to controls at day 7. High-density cultures stimulated with 10ng/ml TGF-ß3 showed increased expression levels for *type II* and *type IX collagen* in comparison to controls. Histological findings confirmed these results on protein level. Human serum and hyaluronic acid stimulated high-density cultures showed each the presence of proteoglycans and type I collagen and the absence of type II collagen as assessed by histological and immuno-histochemical stainings. Stimulation of high-density cultures with TGF-ß3 led to the additional formation of type II.

5.4. Investigation of meniscal matrix induction after 3-D cell cultivation in factor-loaded scaffolds

Detailed results have been reported in publication 3 (Freymann et al. 2012).

Summary

The induction of meniscal matrix formation was investigated in 3-D cultures with factor-loaded scaffolds with regard to the development of a potential cell-free scaffold-based meniscus repair approach. Cultivation of *in vitro* expanded human meniscus cells was performed in resorbable PGA scaffolds with cell re-differentiation stimulating factors.

Human meniscus cells showed a high cell viability during 3-D cultivation in hyaluronic acid loaded PGA scaffolds and when culture medium contained human serum and hyaluronic acid.

Overall histological evaluation of 3-D cultures revealed the formation of meniscus-like extracellular matrix over the cultivation period of 3 weeks. Stained samples of native meniscus tissue showed the presence of proteoglycans and type I collagen in the extracellular tissue matrix. Stained sections of meniscus cell-seeded 3-D cultures showed the formation of proteoglycans after 1 week, with increasing staining intensities after 2 and 3 weeks of cultivation. Immuno-histochemical analysis showed the presence of type I collagen, which has been synthesized around the embedded meniscus cells, and the absence of type II collagen.

The de- and re-differentiation potential of human meniscus cells was investigated by comparing of gene expression levels found in native meniscus tissue samples with levels found in monolayer expanded meniscus cultures at passage 1 and 3 and those in scaffold-based 3-D cultures at day 7 and 14. Expression analysis of typical meniscus marker genes, like *type I collagen* and *type III collagen*, showed continuously decreased levels during monolayer cell expansion when compared to levels found in native meniscus tissue. Re-differentiation of meniscus cells after 14 days of cultivation in factor-loaded PGA scaffolds was accompanied by an increase in the expression level of marker genes when compared to levels found in expanded meniscus cells at passage 3. Gene expression analysis of native tissue samples showed significantly higher expression levels for all examined genes when compared

to levels found in expanded monolayer cells at passage 3. The expression of *type III collagen* in scaffold-based 3-D cultures at day 14 was significantly increased in comparison to expanded monolayer cells at passage 3. The expression of *COMP* showed a decreased level in expanded cells at passage 3 and an increased level in scaffold-based 3-D cultures at day 7. Gene expression levels of other matrix molecules, like *aggrecan* and *cartilage-link protein (LINK)*, decreased after cell expansion in monolayer and after cell cultivation in hyaluronic acid loaded PGA scaffolds up to day 14.

Results demonstrated that *in vitro* expanded human meniscus cells have the potential to induce meniscal extracellular matrix when cells have been cultivated in hyaluronic acid loaded PGA scaffolds and in the presence of human serum and hyaluronic acid.

6. Discussion

In vitro studies of this work identified factors that stimulate the recruitment of human mesenchymal joint-associated progenitor and meniscus cells and that support the re-differentiation of meniscus cells with regard to the further development of a cell-free scaffold-based approach for the repair of non-vascular meniscus lesions. Herein, human serum in doses of 1-20% and the chemokines TECK, IP10 and Lymphotactin were shown to recruit human mesenchymal cells. Further studies indicated that the stimulation with hyaluronic acid and human serum led to the induction of typical meniscal proteins and genes in high-density cell cultures. The use of hyaluronic acid loaded PGA scaffolds for 3-D meniscus cell-seeded constructs and of culture medium containing hyaluronic acid and human serum was shown to induce meniscal extracellular matrix formation.

The mechanisms that underlie the recruitment of human mesenchymal cells into the defective meniscus tissue site as well as their subsequent differentiation towards mensical cells are rarely understood. Tissue-derived joint-associated progenitor cells or resident meniscus cells may enter the tissue defect upon stimulation with growth factors or local chemokines that are released from the SF in the knee joint and may form meniscal repair tissue. Studies of this work identified particular chemokines that are present in SFs of normal, OA and RAs donors and showed that three (TECK, IP10 and Lymphotactin) out of 10 selected chemokines have the potential to recruit joint-associated human mesenchymal progenitor cells. Results are in line with previous studies that reported the recruiting effect of SF from normal and OA donors on human mesenchymal progenitor cells [7] and that showed the migration of human mesenchymal stem cells upon stimulation with the chemokines CXCL-10 (IP10) and CXCL-11, identified from the chemokine profile of human blood serum [6]. Results of present and previous studies indicate that SF and human serum contain chemokines that may contribute to the recruitment of human mesenchymal cells. Since human meniscus cells may take part in cell populating of the defective meniscus tissue site, the recruiting potential of human serum was also tested on human meniscus cells. *In vitro* results revealed a significant increase in the migratory activity of human meniscus cells upon stimulation with media containing 1-20% human serum doses, whether media with 50% and 100% human serum doses did not significantly stimulate cell migration. In brief, cell migration results of this work indicate that human

serum or components of the serum or synovial fluid like growth factors or chemokines may contribute to the recruitment of human meniscus cells and joint-associated mesenchymal progenitor cells and that human meniscus cells show a reduced migratory activity when media with high human serum doses is used for stimulation. Results of this work are comparable to other studies, showing the recruitment of other human mesenchymal cells like articular chondrocytes and bone marrow mesenchymal cells by serum [13]. Therein, fetal bovine serum was reported to dose-dependently stimulate the migration of articular chondrocytes and human bone marrow mesenchymal cells were shown to have a significant increased migratory activity upon stimulation with 5% fetal bovine serum and upon several growth factors and cytokines [13]. With regard to the development of a cell-free scaffold-guided meniscus repair approach, the examined chemotactic properties of human serum and/or selected chemokines may help to attract cells from the adjacent tissue and/or the residual meniscus tissue into a cell-free scaffold.

Tissue repair requires among cell attraction and immigration also the formation of meniscus-like repair tissue in the defective site. Cell differentiation stimulating factors and growth factors may support differentiation into meniscus cells and subsequent repair tissue formation. In this work we evaluated the potential of human serum, hyaluronic acid and TGF-ß3 to induce mensical genes and extracellular matrix proteins in 3-D cultures with regard to the use of these factors in a cell-free scaffold-based meniscus repair approach. Therein, stimulation of high-density cell cultures with differentiation media containing 10% human serum dose resulted in a significant induction of *aggrecan* and *COMP,* genes commonly found in fibrocartilage meniscus tissue, and a significant reduction of *type II collagen*, a prior marker gene of hyaline cartilage, when compared to untreated controls. Histological findings confirmed gene expression results on protein level and showed the formation of proteoglycans and type I collagen without type II collagen formation. Gene expression results of our work are in line with previous studies, showing increased gene expression levels for *aggrecan* and *COMP* after stimulation of bovine meniscus pellet cultures with media containing 10% fetal bovine serum [14]. Stimulation of high-density cell cultures with differentiation media containing 25% hyaluronic acid resulted in a significant induction of *COMP* and no induction of *type II collagen* and the formation of typical meniscal extracellular matrix proteins (proteoglycans and type I collagen) when compared to untreated controls. Results are comparable to previous studies, showing

a positive effect of hyaluronic acid on tissue healing after meniscus injury in the rabbit model [10]. In brief, *in vitro* results of our studies suggest that human serum as well as hyaluronic acid may support the induction of meniscus-like extracellular matrix formation when used in a potential meniscus repair approach. In contrast to that, high-density cell cultures stimulated with media containing 10ng/ml TGF-ß3 resulted in an induction of genes coding for *type II* and *type IX collagen,* markers that are preferentially found in hyaline cartilage, and led to the formation of type II collagen on protein level. Results are in line with previous studies [1, 9] showing the formation of proteoglycans and type II collagen after stimulation with TGF-ß3. Type II collagen is the major protein in hyaline-like cartilage in contrast to native meniscus tissue, where type I collagen is predominantly found [2]. Due to these results, TGF-ß3 is suggested to be not useful in a potential meniscus repair approach.

Further *in vitro* results of this work showed that 3-D cultivation of monolayer expanded human meniscus cells in hyaluronic acid loaded PGA scaffolds led to the induction of meniscal genes and extracellular matrix proteins when culture medium contained 25% hyaluronic acid and 10% human serum. Cells embedded in the scaffold showed the formation of proteoglycans and type I collagen in the extracellular matrix over an observational period of 21 days, comparable to histological findings in native meniscus tissue preparations [2]. Gene expression analysis indicated a de-differentiation of the cells after expansion in monolayer cultures at passage 3, as shown by significant decreases in *type III* and *IX collagen, LINK, COMP* and *aggrecan* expression levels compared to levels found in native meniscus tissue preparations. In contrast to that, meniscus cells showed a re-expression of meniscal genes after 3-D scaffold-based cultivation as indicated by significant increases in *type III, type I collagen* and *COMP* expression levels when compared to levels found in monolayer-expanded cells at passage 3. Since meniscus cells are supposed to de-differentiate in monolayer cultures and to maintain their differentiated phenotype under 3-D cultivation conditions [15], results of this work suggest that the examined 3-D culture conditions, using hyaluronic acid loaded PGA scaffolds and culture medium with 25% hyaluronic acid and 10% human serum, may support cell re-differentiation into a meniscal phenotype and subsequent meniscus-like tissue formation.

Further characterization of human meniscus cells confirmed a fibroblastic spindle-shaped morphology and a high *in vitro* cell proliferation capacity under monolayer

expansion, as already described by other groups [2]. Cells embedded in the hyaluronic acid soaked PGA scaffold, showed a high cell viability and a good cytocompatibility with the scaffold material next to the induction of mensical genes and proteins. Results of our studies are comparable to preclinical studies in the nude mouse and sheep model that showed meniscus-like tissue formation after 3-D cultivation of meniscus cells in synthetic PGA–polymer scaffolds [15]. Results of previous studies indicate that it is high likely that the use of PGA scaffolds may also induce *in vivo* meniscal matrix formation, as shown in the present work under *in vitro* conditions. Furthermore, the combined use of hyaluronic acid and PGA scaffolds may support meniscus repair, since hyaluronan was shown to improve meniscus tissue healing in the rabbit model [10] and plays an important role in the frictionless movement of the knee joint *in vivo* [1]. Based on the good clinical experience with this scaffold-based technique in articular cartilage repair [11, 12], we suggest that it is highly likely that the use of PGA scaffolds combined with hyaluronic acid and human serum may also induce *in vivo* meniscal matrix formation.

Conclusion

Although it is highly speculative and results of our *in vitro* studies are limited and do not reflect in vivo situations, this cell-free scaffold-based approach may support *in vivo* cell recruitment to the defective meniscus site, meniscal cell differentiation as well as subsequent induction of meniscal repair tissue formation. We conclude that PGA scaffolds combined with hyaluronic acid and human serum may have the potential to support tissue healing of non-vascular meniscus lesions by recruiting and differentiating resident mesenchymal cells. Results of this work encourage further preclinical studies and the further development of a cell-free meniscus repair approach based on PGA scaffolds in combination with hyaluronic acid and human serum for the treatment of human non-vascular meniscus lesions.

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Affidavit

I, Undine Freymann certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "*In vitro* recruitment and differentiation of human mesenchymal cells for the development of a scaffold-based meniscus repair approach". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Declaration of any eventual publications

Undine Freymann had the following share in the following publications:

Publication 1:

Endres M, Andreas K, Kalwitz G, Freymann U, Neumann K, Ringe J, Sittinger M, Häupl T, Kaps C. *Chemokine profile of synovial fluid from normal, osteoarthritis and rheumatoid arthritis patients: CCL25, CXCL10 and XCL1 recruit human subchondral mesenchymal progenitor cells.* Osteoarthritis Cartilage. 2010 Nov;18(11):1458-66.

Impact Factor: 4.262 (2013). The journal "Osteoarthritis and Cartilage" is on rank 2 in the Impact Factor-sorted list of all journals in the category "Orthopedics".

Contribution in detail: 25%

Undine Freymann performed the chemotaxis assays, the statistical analysis of data and analyzed the gene expression data. She also revised the manuscript critically.

Publication 2:

Freymann U, Endres M, Neumann K, Scholman HJ, Morawietz L, Kaps C.*Expanded human meniscus-derived cells in 3-D polymer-hyaluronan scaffolds for meniscus repair*. Acta Biomater. 2012 Feb;8(2):677-85.

Impact Factor: 5.093 (2013). The journal "Acta Biomaterialia" is on rank 2 in the Impact Factor-sorted list of all journals in the category "Materials Science and Biomaterials".

Contribution in detail: 75%

Undine Freymann took part in developing of the study concept and design. She performed the experimental work, the statistical analysis of data, analyzed the gene

expression data and drafted the manuscript.

Publication 3:

Freymann U, Endres M, Goldmann U, Sittinger M, Kaps C. *Toward scaffold-based meniscus repair: effect of human serum, hyaluronic acid and TGF-ß3 on cell recruitment and re-differentiation.* Osteoarthritis Cartilage. 2013 May;21(5):773-81.

Impact Factor: 4.262 (2013). The journal "Osteoarthritis and Cartilage" is on rank 2 in the Impact Factor-sorted list of all journals in the category "Orthopedics".

Contribution in detail: 75%

Undine Freymann performed the experimental work, analysis and interpretation of the data and drafted the manuscript. She also approved the final manuscript.

Selected publications

Publication 1:

Endres M, Andreas K, Kalwitz G, Freymann U, Neumann K, Ringe J, Sittinger M, Häupl T, Kaps C. *Chemokine profile of synovial fluid from normal, osteoarthritis and rheumatoid arthritis patients: CCL25, CXCL10 and XCL1 recruit human subchondral mesenchymal progenitor cells.* Osteoarthritis Cartilage. 2010 Nov;18(11):1458-66.

URL: http://dx.doi.org/10.1016/j.joca.2010.08.003.

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URL: : http://dx.doi.org/10.1016/j.actbio.2011.10.007.

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URL: http://dx.doi.org/10.1016/j.joca.2013.02.655.

Curriculum vitae

My curriculum vitae will not be published in the electronic version of my work due to data protection provisions.

Complete list of publications

1. Krüger JP, Freymann U, Vetterlein S, Neumann K, Endres M, Kaps C. *Bioactive Factors in Platelet-Rich Plasma Obtained by Apheresis.* Transfusion Medicine and Hemotherapy. in press.

2. Freymann U, Petersen W, Kaps, C. *Cartilage regeneration revisited: entering of new one-step procedures for chondral cartilage repair.* OA Orthopaedics. 2013 Jun 05;1(1):6.

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