THE INFLUENCE OF CELLULAR INTERACTIONS IN TISSUE ENGINEERING FOR CARTILAGE REPAIR

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Het schilderij op de omslag is een abstracte interpretatie van de kraakbeen tissue engineering waarin de verschillende factoren die met elkaar interacteren tijdens kraakbeenregeneratie in vitro en in vivo gerepresenteerd zijn.

THE INFLUENCE OF CELLULAR INTERACTIONS IN TISSUE ENGINEERING FOR CARTILAGE REPAIR

PROEFSCHRIFT

ter verkrijging van
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Voor Erwin
&
Voor Irin Lynn,
Max en Jip

een zachte hand heeft ons gemaakt
uit grijs schemerlicht
en zachte zomerregen
(Thé Lau)

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List of abbreviations

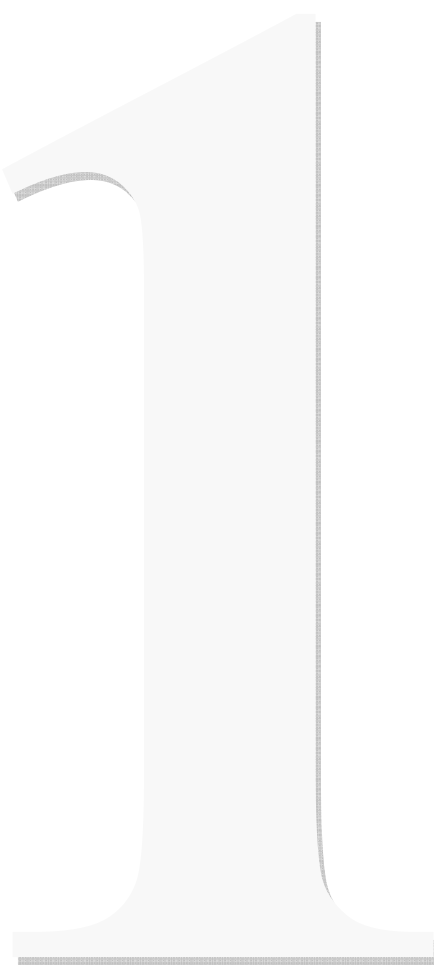
2D	Two-dimensional
3D	Three-dimensional
3T3 cell	3T3 fibroblastic feeder cell
ACI	Autologous Chondrocyte Implantation
AsAP	Ascorbic Acid 6-phophate
BM-MSC	Bone marrow mesenchymal stem cell
BPC	Bovine primary chondrocytes
CGF	Chondrogenic growth factors
CM	Culture medium in relation to cells or compression moulding in relation to porous scaffold manufacturing
DMMB	1,9 dimethyl methylene blue
EC	Expanded chondrocyte
ECM	Extracellular matrix
E(SEM)	Environmental scanning electron microscopy
FGF	Fibroblast growth factor
Fn	Fibronectin
GAG	Glycosaminoglycans
HDF	Human dermal fibroblast
HEC	Human expanded chondrocytes
M	Matching (refers to dynamic stiffness properties of a scaffold matching to dynamic stiffness properties of cartilage
MESC	Mouse embryonic stem cell
MPa	Mega Pascal
MSC	Mesenchymal stem cell
NEAA	Non essential amino acids
NM	Non-matching (refers to dynamical stiffness of a scaffold not matching to dynamic stiffness of articular cartilage)
PC	Primary chondrocyte
PM	Proliferation medium
TGF	Transforming growth factor
PDGF	Platelet derived growth factor
PEG	Poly(ethylene glycol)
PEGT	Poly(ethylene glycol) terephthalate
PEOT	Polyethyleneoxide-terephtalate
PBT	Poly (butylene terephthalate)
SEM	Scanning electron microscopy

List of definitions

Allogenic	Cells or a graft derived from a donor of the same species as the recipient
Arthroplasty	Surgical repair or replacement of a joint
Articular	Of or relating to the joint
Autologous	Cells or a graft derived from the recipient of the graft
Chondrocyte	Cartilage forming cell
Condyle	Rounded projection at the end of a bone
Dynamic stiffness	Frequency dependent, apparent stiffness of a linear elastic member under cyclic or shock loading
Embryonic stem cell	A totipotent stem cell from embryonic origin, capable of all possible differentiation pathways with unmet self-renewal capacity.
Femur	The long bone between the hip and knee joint
Hyaline	Clear, transparent, granule free
Hydrophilic	The ability of a surface or volume to attract liquid
Hydrophobic	The ability of a surface or volume to repel a liquid
Interconnectivity	The degree to which a single phase within a medium is joined to form continuous paths
Lacunuae	A space or cavity around cells
Mesenchymal stem cell	A multipotent cell usually from adult origin, capable of multiple differentiation pathways
Osteoarthritis	A disease of the joint cartilage associated with secondary changes in the underlying bone, which may ultimately cause pain and impair the function of the joint
Primary chondrocyte cultured	Chondrocytes isolated from cartilage tissue without being
Scaffold	Porous biomaterial with an specific architecture and composition
Subchondral	Located beneath cartilage
Synovial joint	Diarthrosis or freely moving joint. The ends of the adjoining bones are covered with a thin layer of cartilage and the bones are linked by a ligament lined with synovial membrane, secreting the joints lubricant, synovium.
Tibia	The larger, medial bone of the lower leg
Vascular	Pertaining to blood vessels or indicative of a copious blood supply

Chapter 1

General introduction and aims



Chapter 1

General introduction and aims

Already in 1743 it was recognized by Hunter that cartilage defects are troublesome and when inflicted not repaired (1). Worldwide millions of people each year suffer from the results of traumatized or degenerated cartilage (2). Only 5% of the potential patient population receives treatment each year, other patient either receives temporary pain relief or they can't receive treatment thus their activity level is compromised or in the worst-case they are immobilized (3). The major impact of loss of function of articular cartilage in for example the knee or hip is obstruction or loss of mobility. With the increase in life expectation and higher expected activity level at older age, this problem is expected to become even more important in the near future.

In the last 2 decades, tissue engineering evolved from the combination of research in biology, material engineering and the need for replacement surgery as a new scientific field of research.

Tissues are complex 3-dimensional structures with a highly organized architecture made up of cells and matrix. The cells and matrix in a tissue are continuously interacting with each other and (cells from) their surrounding tissues to maintain their form and function. Interactions of cells with their surrounding cells and matrix are equally important for a successful repair reaction. While in tissue engineering significant progress has been made, still a number of challenges need to be overcome for the development of clinically applicable treatments. One of the challenges in cartilage tissue engineering is to direct cellular interactions involved in either engineering of a repair tissue outside of the body or an appropriate repair reaction in situ. Cellular (inter)actions can be influenced indirectly via scaffolds or directly by supplying the cells with growth factors, extracellular matrix proteins or other cells. This thesis reviews and discusses research data and model systems related to the influence of cellular interactions between chondrocytes and extracellular matrix proteins, scaffolds and other cell types on cartilage tissue formation.

In this chapter, the nature of articular cartilage, the implications of cartilage damage, current clinically available repair strategies and recent advances in cartilage repair are introduced. In chapter 2, mechanisms involved in cellular interactions during co-culture of different cell types in general and more specific the influence they have on cartilage tissue engineering are reviewed.

Articular cartilage and implication of cartilage defects

Three types of cartilage are distinguishable in the body: fibro-, elastic and hyaline cartilage. Studies described in this thesis focused on cellular interactions in tissue engineering of hyaline cartilage. Hyaline cartilage is found mainly at the distal parts of bones of articulating joints, for example in the knee and hip but also in ankle or finger joints (Figure 1). Besides articulating joints, hyaline cartilage is found between bones, when resilience together with strength is required, like between vertebrae or between ribs and sternum. Furthermore, hyaline cartilage is found when function is related to form, like in the nose. Hyaline cartilage in articulating joints is also referred to as articular cartilage. Articular cartilage is a resilient load bearing tissue that provides a smooth almost frictionless surface to the distal ends of bones in synovial joints. The extracellular matrix is provided and maintained by the only cell type residing in cartilage, the chondrocytes which are scattered through this matrix (5% of wet weight) (Figure 2). The extracellular matrix contains a fibrilar collagen mainly made up with type II collagen (10-20% of wet weight) (Figure 2), which provides the tissue with tensile strength. It has a second major class of matrix molecules, proteoglycans (10-15% of wet weight) the most common of which is aggrecan. Proteoglycans form a porous structure in the matrix, providing the tissue with compressive strength and a network to regulate matrix hydration (water content 60-80% of wet weight) (5),(6).

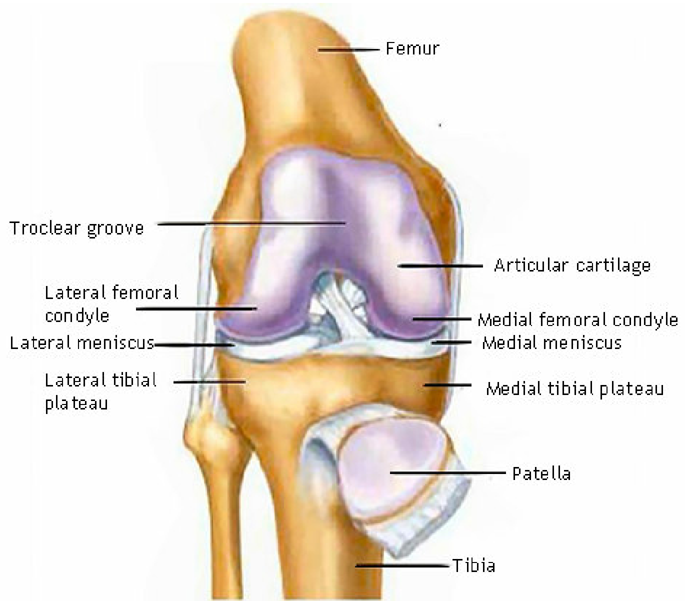


Figure 1 Human knee anatomy.

The weight bearing capacity of cartilage tissue is made possible by regulating regional water content, while the chondrocytes themselves have adapted to withstand this, by developing a unique lacuna surrounded by a type VI collagen rich region around them (7). Cartilage is a unique tissue in that it consists of only one cell type; chondrocytes. It holds no vasculature or nerve system and as a result its capacity for self-repair is limited (8).
A normal repair reaction is initiated by innervations of the nerve system, disruption of the blood circulation system upon which activation of factors and cells involved in an inflammatory response. This is followed by homing in of cells and factors involved a tissue repair reaction. When cartilage lesions are extending into the subchondral bone (osteochondral defects) access to blood flow and bone marrow is established and cells and factors enter the defect, possibly contributing to a repair reaction.

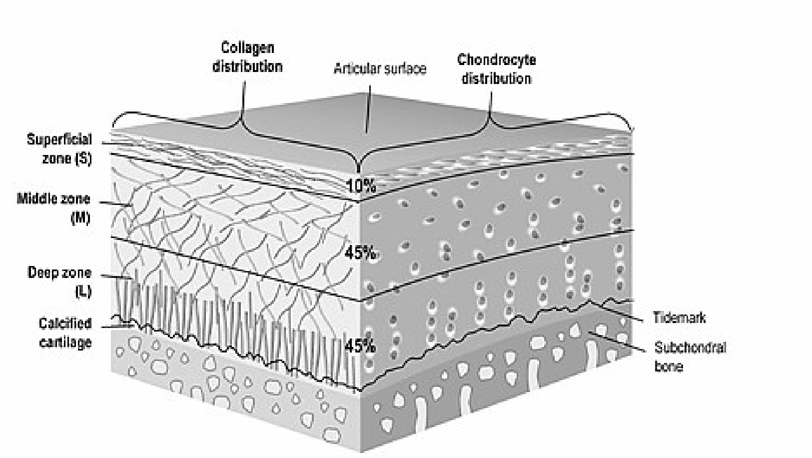


Figure 2 Distinguished zones, collagen distribution and chondrocyte distribution in articular cartilage. Illustration adapted from Woodfield (4) et al.

This however, seems not to lead to mechanically functional tissue but rather fibrocartilage is formed (9),(10). When critical size defects are limited to the chondral part of the knee (Figure 3) such defects fail to heal spontaneously. Damaged cartilage possibly results in loosening of cartilage pieces, which obstruct smooth function of the joint. Although cartilage itself contains no nerves, wear and tear aflicted cartilage damage causes release of pain-mediating molecules affecting the surrounding tissues. Moreover, inadequate loading of the joint can cause pain in the surrounding tissues which are nerve innervated. Current approaches in cartilage repair available in the clinic are mainly focusing on pain relief and/or overcoming mechanical obstruction. A description of clinically available cartilage tissue repair techniques are briefly discussed in the following paragraph (Figure 3).

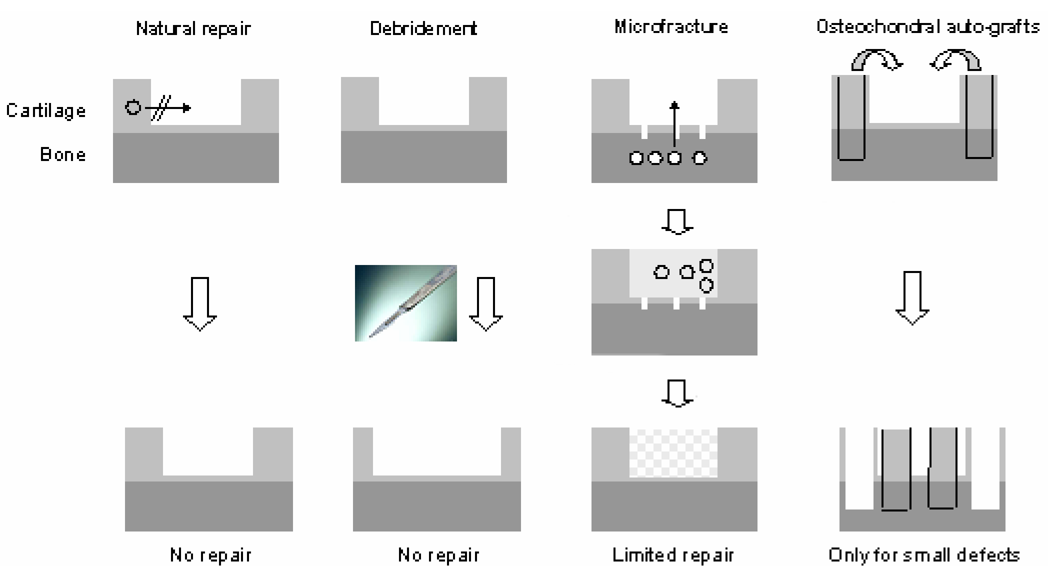


Figure 3 Schematic representation of natural repair and various treatments clinically available for osteo(chondral defects).

Current approaches in cartilage tissue repair available in the clinic

Arthroscopic techniques: lavage, shaving and debridement, abrasion chondroplasty, drilling, microfracture. These treatments are performed mainly to remove pain-mediating molecules, cartilage debris inflicting mechanical impediment or to penetrate the subchondral bone to stimulate a repair response via the vascular/bone marrow spaces. The average results are short-term pain relief, limited long-term benefit and mechanically inferior fibrocartilage repair tissue (10-12). The success rate of treatments differs between 10-50% (13),(14),(15),(16),(17).

Open surgery procedures: osteotomies, total joint arthroplasty. For osteotomy, the limb is realigned to transfer joint load away form the damaged cartilage surfaces. Procedures result in long-term pain relief but overloading of the joint can aggravate osteoarthritis. For severe joint degeneration the joint is replaced by a total prosthetic joint typically made up of metallic alloy combined with polyethylene liner and cup. Although this is a major surgery, the procedure results in functional relief for 10-15 years. However it is generally limited to elderly patients since possibilities for revision surgery are suboptimal (18),(19,(20),(21),(22).

Autogenic and allogenic tissue transplantation (open surgery): mosaicplasty, peri-chondral and periosteal grafts as well as osteochondral allografts. Transplantation of autologous or allogenic osteochondral plugs with an intact articular cartilage layer from low-weight bearing areas to fill a defect. It results in short-term pain relief and variable long-term benefit. The donor site for osteochondral plugs shows morbidity.The success rate of these treatments differ between 70-90%. However only limited defects can be treated depending on their size (23),(24-26),(27),(28).

Although these treatments are widely accepted and do initiate a healing response, the tissue formed is mostly fibrocartilagenous with poor mechanical properties. In addition, while temporary outcomes in pain reduction and increased motility are established, few of these procedures yield long-term functional tissue repair. Other concerns are: high invasiveness of procedures, donor site morbidity and possible pathogen transfer with allogenic transplantation techniques.

Autologous chondrocyte implantation ACI, open surgery

With the cell therapy available in the clinic already for more than 15 years, good to excellent clinical results are seen in 70-80% of the cases in isolated post-traumatic lesions of the knee joint in younger patients, with the formation of hyaline or hyaline-like repair tissue (10),(29),(30).

The major complications are periosteal hypertrophy, delamination of the transplant, arthrofibrosis and transplant failure (10). Recent advances in tissue engineering wherein cells are combined with resorbable biomaterials have contributed to the next generation of cell therapies. The biomaterials applied secure the cells in the defect area and can be designed to enhance their proliferation and differentiation (31). First clinical trials with the combination of scaffold and expanded chondrocytes are encouraging, with 97% of hyaline cartilage-like repair tissue (32). However, these procedures still require two surgical interventions, complicated logistics to ship the biopsy and cells or construct from and to the hospital and a controlled culture environment for the expansion of chondrocytes (cleanroom) (Figure 4).

Consequently increasing interest is raised on either development of single surgery therapies or of bioreactors in which the procedures after biopsy retrieval up to a tissue engineered construct is performed in a small-scale controlled environment, which is patient dedicated and can be operated at the hospital facilities. Novel therapies for cartilage defects with growth factors, cells or scaffolds alone or in combination are entering the clinical trail phase(s) with varying results

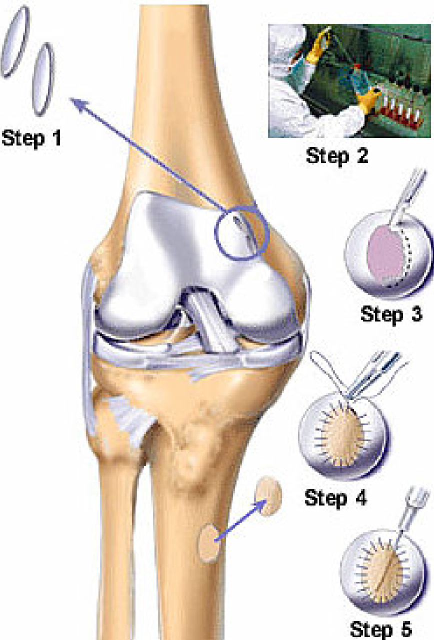


Figure 4 ***Schematic presentation of Autologous Chondrocyte Implantation.*** *Step 1: harvest cartilage biopsy, step 2: isolation and expansion of chondrocytes in a cleanroom, step 3: defect preparation, step 4: harvest of periosteum and suture over the cartilage defect and step 5: injection of chondrocytes under periosteal flap and closing with fibrin glue.*

Advances in cartilage repair therapies

Microfracture combined with scaffold

Only a few studies describe intra-operative therapies as a possible future tissue engineering treatment for articular cartilage defects. In a study from Dorotka R (33) et al. microfracture was combined with implantation of a collagen scaffold. The microfracture procedure allowed an influx of bone marrow mesenchymal stem cells and the scaffold was expected to mediate chondrogenic differentiation. However, influx of bone marrow mesenchymal stem cells from the underlying bone did not enhance tissue formation compared to scaffold implantation alone, whereas compared to untreated defects, combining scaffold with microfracture showed a slight increase in quality and quantity of the repair tissue (33). Several other studies similarly show that access to the bone marrow from underlying bone combined with a scaffold seems not to result in hyaline cartilage repair (33).

Scaffold combined with growth factors

In another study hydroxyapatite/pol-L,D-lactate/poly-ethylene-glycol composite scaffolds were impregnated with BMP2 and implanted in osteochondral defects of a rabbit model.

Results showed that repair tissue was not significantly different between defects implanted with composite scaffold only or hydroxyapatite scaffold only compared to an empty defect. However, when a combination of the composite scaffold with BMP2 was implanted, a 3-fold increase in histological score was found (34),(35). Clinically, a combination of scaffolds and chondrogenic growth factors possibly also would yield cartilagenous tissue repair and it would allow for intra-operative treatment. Even though promising the following has to be considered for clinical application: growth factor side effects on the surrounding tissues, particularly for cartilage repair growth factor induced vascularization is undesirable and an adequately controlled delivery method is yet to be identified (36),(37),(38).

Scaffold combined with cartilage fragments

A method for cartilage defect treatment with both cartilage fragments and scaffolds was recently described by Lu Y et al 2006 (39). Cartilage defects in a goat model were either left empty, treated with polyglycolide/polylactate scaffold alone or treated with scaffold seeded with autologous cartilage fragments. Although no histological scoring was performed, histological pictures of the repair tissue showed more cartilagenous tissue in the group treated with scaffold and autologous cartilage compared to the other 2 groups. However, current efforts to combine crushed cartilage tissue with growth factors in clinical trials suggest limited effectiveness.

Cartilage tissue engineering

Recent advances in cartilage tissue engineering aim at repairing cartilage defects with constructs that both functionally and biologically resemble the surrounding tissue. For this, a combination of growth factors, extracellular matrix proteins, scaffolds or cells are applied to repair a tissue in situ or engineer (part of) a tissue in vitro. Major progress has been made in improving seeding efficiency onto a scaffold, identifying culture parameters significant for tissue formation, identifying scaffold requirements that support tissue formation homogenously throughout a scaffold and identifying and addressing crucial safety and quality assurance issues. However, one should be aware of (a) the complex structure of connective tissue, particularly at the interphase, (b) the interplay between cellular environment, matrix and cell phenotype, (c) the plasticity of connective tissue lineages, (d) how repair is dependent upon structural anatomy of the tissue and the nature of the injure and (e) the natural phenomena that influence the application of tissue engineered constructs in each clinical setting (40). The different parameters and their influence on cartilage tissue formation are described in the next few paragraphs.

Cell sources for cartilage tissue engineering and cell therapy

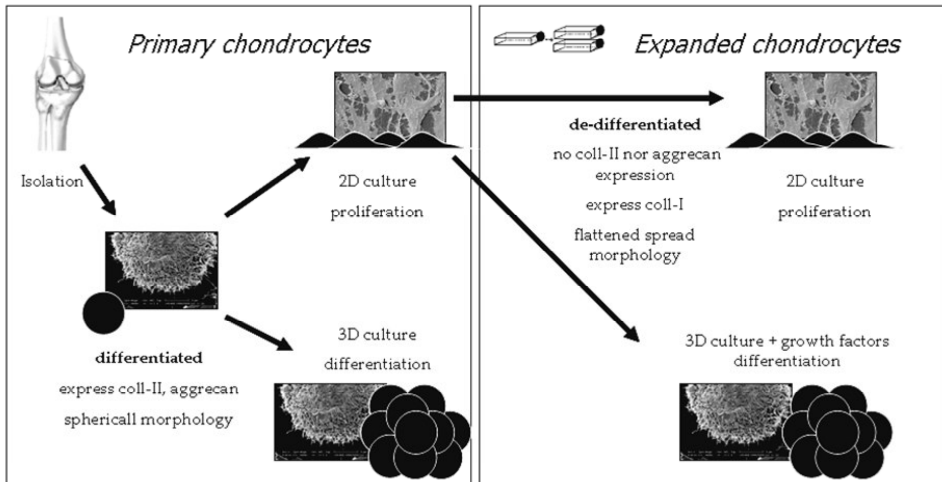


Figure 5
In general cells are referred to as primary cells when they and their progeny orginate directly from a tissue. In this thesis isolated chondrocytes are referred to as primary chondrocytes to distinguish them from their cultured progeny (expanded chondrocytes). (In this schematic drawing the differences between primary chondrocytes and expanded chondrocytes are depicted.)

A possible source of cells for cartilage regeneration are the chondrocytes residing in the cartilage itself. However, they are limited in number and expansion easily leads to de-differentiation and/or loss of phenotype (41),(42). In general, primary cells are described as cells taken from a tissue source and their progeny grown in culture before subdivision and transfer to a subculture. For this thesis, it is essential to recognize that immediately after isolation chondrocytes maintain a cartilaginous phenotype and posses high differentiation capacity, unmet by cultured chondrocytes (Figure 5) (43). The limited availability of primary chondrocytes and their dedifferentiation during culture illustrates the need for other suitable cell types in cartilage tissue engineering. The different cell types described hereafter were previously applied in studies with a focus on chondrogenesis or cartilage tissue formation.

Bone marrow is a highly complex and organized tissue where several types of differentiated cells coexist. Immature cells called hematopoietic stem cells (HSC) which can self-renew and differentiate into all the mature peripheral blood types coexist with mesenchymal stem cells or stromal cells (BM-MSC) in the bone marrow. The later serve as long-lasting precursors for many tissue and posses self renewal capacity (44),(45),(46). Mesenchymal stem cells residing in the bone marrow are a source of replacement cells for injury or disease inflicted defects in the body and have been identified as suitable

candidates for cartilage tissue repair and engineering (47),(48),(49). However, injection of mesenchymal stem cells alone into cartilage defects resulted in a discontinuity between host and neocartilage and a progressive thinning of the repair tissue with subnormal mechanical properties (50). Numerous experimental data suggest that mesenchymal stem cells require some chondrogenic stimuli from scaffold or growth factors to regenerate cartilage (49),(51),(52). Mesenchymal stem cells also were found to be present in adipose tissue and synovium (53),(54),(55-57).

The multipotency of adipose tissue derived mesenchymal stem cells (AT-MSC) has been shown by their differentiation into adipogenic, myogenic, osteogenic and chondrogenic lineage (58),(59),(60). Comparison studies with BM-MSCs and AT-MSCs show varying results. In several papers, it is claimed that chondrogenic capacity of AT-MSC is comparable or equal to that of BM-MSCs (53),(61),(62), while in other studies chondrogenic capacity of AT-MSC was shown to be less (60),(63),(64).

Dermal fibroblasts from skin can be isolated and induced to undergo chondrogenic differentiation (65),(66). When pretreated with IGF-1 and subsequently cultured on aggrecan, a dermal fibroblast cell line RAB-9 stained positive for safranin O and type II collagen. mRNA levels for type II collagen increased 3-fold compared to untreated dermal fibroblasts (65). Dermal fibroblast showed increased levels of aggrecan and type II collagen expression upon stimulation with lactic acid, while type I collagen expression decreased (67),(68). Chondrogenic differentiation of dermal fibroblasts was shown when cultured in the presence of demineralized bone powder and correlated to this several signal transduction pathways among which the TGFß, insulin and Hedgehog signal transduction pathway, where found to be modulated (68). Until recently, dermal fibroblasts were considered a cell type not capable of differentiation into the chondrogenic lineage. These findings indicate that with the right cues, dermal fibroblast can be persuaded to produce cartilage specific extracellular matrix proteins and change their signal transduction pathways possibly more similar to a chondrogenic cell.

Embryonic stem cells, although concerned with ethical issues, offer great potential in tissue engineering and cell therapy applications for cartilage repair. Chondrogenic differentiation of embryonic stem cells is observed in embryoid bodies without any further cues or when stimulated with BMP-2 and-4 or TGFß (2),(69),(70),(71). Controlling the differentiation of embryonic stem cells differentiation into a specific lineage might be a challenge because of their pluripotency. Not surprisingly, recently it was shown with micro-array analysis that a significantly higher number of genes are expressed in embryonic stem cells compared to adult differentiated cells (72). Very likely, many of these genes expressed, determine the variance in embryonic stem cell response to external cues. However, Levenberg S. et al (71) showed that embryonic stem cells can be directed to differentiate into tissue with cartilage characteristics and that this was maintained upon subcutaneous implantation in a mouse model. They, however also showed that vasculature which developed before implantation anastamosed to host

vasculature upon implantation, which would be detrimental for cartilage tissue formation if occurring in an articular cartilage defect (71).

Pellet/micromass culture

One of the prerequisites for chondrogenic differentiation seems to be that cells are able to regain or adapt a spherical morphology. With micromass culture or pellet culture cellular interactions between chondrocyte are stimulated and they adapt a spherical morphology. This model is often applied to study chondrogenic differentiation (73),(67),(74).

Scaffolds in cartilage tissue engineering

Scaffolds ideally provide a cartilage defect with immediate resilience and strength and stimulate cells to (re)generate biological and mechanically functional cartilage tissue. Scaffold preferred requirements have been extensively described in the literature and are discussed briefly in table 1, followed by an overview of recent developments and visions.
Several techniques are available to manufacture porous biomaterials among which is compression molding and particle leaching or foaming. Porogens like gas or salt particles, generate the pores in such scaffolds. While the size, form and distribution of pores with these engineering techniques can be controlled to a certain degree, their position and orientation to one another is inherently random. Non-woven fiber meshes generated by fiber bonding techniques have the advantage of highly interconnected porosity and surface to volume ratio. A major disadvantage of such manufactured scaffolds is that they hold very limited mechanical properties. Recent advances in computational topology design (CTD) and solid free form deposition (SFFD) made it possible to define architecture, shape and porosity of fiber deposited scaffolds with highly interconnected pores (4),(75).
Being able to design scaffold with fiber deposition techniques opens the possibility to control architecture and interconnectivity of scaffolds and at the same time provide them with tissue specific mechanical properties. Resulting scaffolds have been examined for their possible influence on cartilage tissue formation (76),(77),(78),(79),(75, 80),(81).
Besides scaffolds architecture also the composition of scaffold materials have shown to influences cell physiology and differentiation. Materials used in cartilage tissue engineering are numerous and natural materials can be distinguished from synthetic materials.

Scaffold requirements	description
Biocompatible	Scaffold material does not raise an immune response or foreign body reaction upon implantation
Porous 3D environment	Scaffolds provide the cells with a 3D environment which allows natural proliferation and differentiation, nutrient supply and metabolic waste deposition
Cell seeding	Pores throughout the scaffold are accessible for cells to allow efficient homogenous cell seeding
Interconnected/permeable	Interconnection of scaffolds allow for efficient nutrient, gas and metabolic waste flow
Biodegradable	Polymeric materials break down due to macromolecule degradation with dispersion in vivo
Mechanical integrity and integration	Scaffolds withstand stresses and loading applied on them before and after implantation. This is an important factor in determining the successful integration of a construct with its surrounding tissue
Structural anisotropy	Structure of a scaffold applies to the requirements of the surrounding tissue at the level of implantation. For example, cartilage distinguishes 4 zones; superficial gliding zone, transitional zone, radial zone and tide mark all with different cellular and extracellular matrix compositions which might be reflected in a scaffold
Sized and shaped	Construct or scaffold can be shaped to match the structure of the site of implantation or even match a whole joint of part of a joint
Surgical application	Scaffold or construct suitable for minimal invasive techniques; for example injectable, solidify in situ or are preformed that can be temporarily reshaped for delivery arthroscopically
Bioactivity/gene delivery	Scaffold can be used to deliver bioactive signals supporting cartilagenous tissue formation. Scaffold can control release of bioactive signals or act as a gene delivery vehicle.

Table 1.Tissue engineering scaffold requirements as identified by several experts in the field. References (4),(75),(76),(77),(82),(83).

Natural materials

Hydrogels materials are cross-linked polymeric systems that can absorb large amounts of aqueous solution. By regulating the crosslinks in hydrogels their porosity can be changed, which is important for cell viability and tissue formation. While upon implantation in a cartilage defect their mechanical properties would not allow early loading of a joint, their matrix is expected to accelerate tissue formation to provide mechanical integrity. Hydrogels examined for their suitability in cartilage tissue engineering include Pluronic, chitosan, alginate, agarose and fibrin (2),(84),(85). Polymerization or gelation is the formation of crosslinks and ones initiated; this process cannot be stopped or accelerated. Control over the shape and size of the hydrogels during polymerization of gelation in the clinical setting would be a further requirement (84).

Other natural biomaterials for cartilage repair are collagen and hyaluronate, which are insoluble and have mechanical properties matching the surrounding tissue more. Because of their limited porosity the cells that are seeded onto these scaffolds remain on the surface. Moreover, the source of these extracellular protein based scaffold is xenogenic or allogenic. These sources are accompanied with risk in transfer of prion or virus diseases.

Synthetic materials

The synthetic materials applied by far most in manufacturing of copolymer scaffolds for cartilage tissue engineering studies are Poly(α)-hydroxyesters such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly (lactide-co-glycolide acid) (PLGA). In the studies described here we focused on the application of poly(ethylene glycol)-terephtalate (PEGT) and poly(butylene terephthalate) (PBT) blocks. A major advantage of these copolymers is that by varying the amount and the length of the two building blocks a whole range of polymers can be obtained with differences in surface properties, swelling capacity, degradability and mechanical strength. The next paragraph describes and discusses material properties of (PEGT/PBT) scaffolds and their application in cartilage tissue engineering.

Poly(ethylene glycol)-terephtalate/poly(butylene)-terephtalate (PEGT/PBT) scaffold

Mechanically functional PEGT/PBT scaffolds are highly suitable candidates for a cartilage repair treatment strategy. This co-polymer, has an extensive safety file and has received FDA and CE approvals (SynPlug cement restrictor). PEGT/PBT co-polymer offers flexibility through variations in molecular weight and ratios of the polymer segments. PEGT/PBT polymers are a segmented block co-polymer consisting of soft, hydrophilic PEGT segments and hard, hydrophobic PBT segments, denoted as *a*/*b*/*c*, whereby, *a* represents the poly(ethylene glycol (PEG) molecular weight (MW, g/mol), and *b* and *c* represent the weight percentage of PEGT and PBT blocks respectively. By varying the PEGT MW and the weight percentage ratio between PEGT and PBT, it is possible to modulate the wettability, protein adsorption, swelling and mechanical properties of the substrate (86),(81).

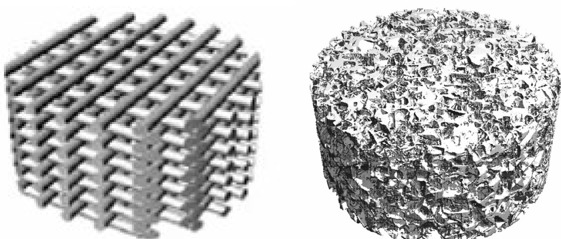


***Figure 6** 3D examples of 3D fiber deposited scaffold (left) and compression moulded scaffold. Pictures adapted from Woodfield.*

Furthermore, by changing the fabrication technique (i.e. compression molding or 3D fibre deposition Figure 6) for a given PEGT/PBT composition, it is possible to fabricate 3D scaffolds with the same bulk composition and overall porosity but different interconnecting pore architectures (87). Novel technologies have been developed that enable the fabrication of scaffolds with appropriate mechanical properties through a combination of computer aided design and free-form fabrication technologies that allow complex three-dimensional porous architectures to be designed and accurately reproduced. The use of this 3D deposition system allowed the modulation of cartilage formation by the polymers 3D scaffold structure. It has further been demonstrated that scaffolds can be fabricated which match the static and dynamic properties of healthy cartilage (81). Using this scaffold technology in the inductive scaffold system will allow weight to be applied to the treated joints much earlier, reducing rehabilitation time by half compared to other treatments like microfracture alone.

Clinical safety of PEGT/PBT scaffolds implanted into mosaicplasty donor sites was established in ten patients by the absence of synovitis, inflammatory responses or any other adverse events at 9 months follow up (88) The capacity of these co-polymers to maintain or induce a chondrocytic phenotype has been extensively studied using isolated (differentiated) and expanded (de-differentiated) chondrocytes from various origin.

2D films of PEGT/PBT produced from 1000/70/30 compositions best retained chondrocytic phenotype of primary bovine (89) or human (86) articular chondrocytes as compared to 300/55/45 compositions. In addition, by applying controlled and selective modifications of chemico-physical scaffold parameters, it was demonstrated that both scaffold composition and architecture are instructive for expanded dedifferentiated human chondrocytes in the generation of 3D cartilaginous tissues (90). Specifically it was demonstrated that the 1000/70/30 polymer composition enhanced chondrogenesis of dedifferentiated cartilage cells 2 orders of magnitude above cell culture plastic control substrates (91).

Cell seeding and scaffold retention in cartilage research

Initial chondrocyte density proved to be important for efficacious cartilagenous tissue formation, thus seeding efficiency is essential (7),(92),(93),(94). Cell seeding in cartilage tissue engineering is done through dynamic seeding, for example in rotating or perfusion bioreactors or through static seeding potential aided by hydrogels (95),(96),(97). The application of hydrogels was discussed in the scaffold section (page 22) and in this section attachment mediated seeding and aggregation mediated seeding are described. Although major improvements have been made in enhancing seeding efficiency, many studies applied immature chondrocytes or chondrocytes from animal models with a high metabolic rate like rabbit. Unlike cells from adult human or bovine source, chondrocytes from an immature animal or rabbit model have shown to generate hyaline like tissues throughout clinical relevant size scaffolds. However, for clinical applications in adult human patients, seeding efficiency needs further improvements.

Attachment mediated seeding

To improve seeding efficiency the surface properties of a scaffold can be influenced. Several surface properties such as wettability, surface chemistry and roughness have shown to influence cell attachment and several research groups have explored grafting bioactive proteins or peptides onto scaffold surfaces to enhance cell attachment and therefore increase seeding efficiency (2),(98). In previous work of our group, on hydrophobic surfaces like PEGT/PBT 300/55/45 enhanced cell attachment correlated with a spread chondrocyte morphology, elevated expression of fibronectin (FN) integrin receptor and concomitant reduced differentiation capacity (91).

Aggregation mediated seeding

Several studies with chondrocytes showed a correlation between aggregation and enhanced cartilagenous tissue formation (99),(100). Additionally a relationship is apparent between cell seeding on hydrophilic surfaces like PEGT/PBT 1000/70/30 and chondrocyte aggregation, acquiring a spherical morphology and enhanced cartilagenous metabolism. When aggregation of cells is stimulated with fibronectin, initial cell-cell interactions are facilitated by fibronectin and intercellular cohesion in the aggregates are enhanced (101),(102). Furthermore fibronectin and fibronectin fragments play a crucial role in cartilage metabolism, and a specific fibronectin splice variant is uniquely expressed in cartilage *in vivo* (103),(104),(105),(106). Nevertheless, fibronectin was also shown to mediate cell attachment on materials and extracellular matrices via $\alpha5\beta1$ integrin and concomitantly correlated with dedifferentiation of chondrocytes (91).

Bioreactors in tissue engineering

For development of clinical relevant sized, 3-dimensional engineered tissues for implantation, circulation of gas, nutrients and waste products is essential. Bioreactors facilitate these functions through circulation of culture medium. The flow of the medium itself already supplies mechanical stimulation and systems build in a bioreactor can provide other or more intense mechanical stimulation during culture. In a bioreactor medium is refreshed in a closed system through continuous controlled flow. During conventional tissue culture, medium is refreshed in specialized flow cabinets in a cleanroom by manually dispersing the medium off and replacing this with fresh medium. Thus, bioreactors additionally decrease the threat of microbiological contamination which is an important issue during culture of every tissue engineered construct (95),(107).

Growth factors

Numerous growth factors have been identified to stimulate chondrogenic differentiation of chondrocytes, but also mesenchymal stem cell from bone marrow or fat tissue, dermal

fibroblasts and embryonic stem cells. In table 2 (growth) factors shown to stimulate chondrogenic differentiation of several cell types are identified.

Cell type	Growth factors	references
chondrocytes	TGFβ1, IGF-1, BMP-2, BMP-7, dex, PDGF-2, PDGF2α	(108),(109), Nischida Y 2000,(110), (111), (112)
mesenchymal stem cells derived from bone marrow or fat	dexamethasone, TGFβ1, TGFβ2,TGFβ3, insulin, BMP-2, BMP-6, BMP-7	(113), (114), (115), (53),(116),(117),(118)
dermal fibroblasts	CDMP-1, TGFβ1, IL-1α, IGF-1	(65), (119), (120)
embryonic stem cells	BMP-2, BMP-4, TGFβ1,	(69), (71), Legner CJ 2004

Table 2 Growth factors stimulating chondrogenic differentiation of different cell types in vitro.
TGF= transforming growth factor, IGF=Insuline-like Growth Factor, BMP=bone morphognetic protein, IL=InterLeukin CDMP=cartilage derived morphogenetic protein, PDGF=platelet derived growth factor, dex= dexamethasone.

In this thesis, we focused on the influence of cellular interaction on cartilage tissue formation with less conventional approaches. Always starting with the question

“ in what ways can cellular interactions support chondrocyte proliferation or cartilage tissue formation ?”

Aims and Outline of this Thesis

The aims of this thesis were to examine the influence of cellular interactions among chondrocytes and between chondrocytes and extracellular matrix proteins, scaffolds or other cell types in tissue engineering and cell therapy for cartilage tissue repair. The following paragraph defines the aims and outline of studies described in this thesis.

I. Influence of cell-cell interaction on proliferation of chondrocytes

Aim: Study the potential of chondrocyte-chondrocyte interactions to enhance 3-D proliferation and at the same time decrease dedifferentiation of chondrocytes in a fully autologous culture system intended for Autologous Cell Therapy

Chapter 3 focuses on development of a culture system for efficient expansion of human chondrocytes in the presence of autologous serum for application in cell therapy or tissue engineering for cartilage repair.

II. Influence of cell-cell interactions on tissue formation during co-culture or co-implantation of primary chondrocytes with other cell types

Aim: Investigate the influence of cellular interactions on cartilage tissue formation of primary chondrocytes co-cultured or co-implanted with different cell types.

In **Chapter 4** the influence of cellular interaction on cartilage tissue formation of primary chondrocytes co-cultured with expanded chondrocytes, dermal fibroblasts, 3T3 fibroblasts and embryonic stem cells are examined. The contribution of each cell type in co-culture is determined with collagen type II specific in situ hybridization and a specie specific antibody. **Chapter 5** describes the influence of cellular interactions on cartilage tissue formation of primary chondrocytes co-cultured or co-implanted with either expanded chondrocytes or bone marrow mesenchymal stem cells. Primary chondrocytes and expanded chondrocytes or mesenchymal stem cells are combined at different ratios for co-culture or co-implantation. Cartilagenous tissue formation is evaluated quantitively and qualitively.

III. Influence of cell-fibronectin interaction and cell-scaffold interaction on seeding efficiency and cartilage tissue formation in constructs

Aim: Investigate the influence of cell-extracellular matrix protein interaction and cell-scaffold interaction on seeding efficiency into a porous scaffold and cartilage tissue formation in the resulting construct.

In **chapter 6** the influence of chondrocyte-fibronectin interaction through aggregation compared to attachment mediated seeding on cartilage tissue formation is examined. Chondrocytes were either, aggregated with fibronectin and subsequently seeded onto a porous PEGT/PBT scaffold or a scaffold is coated with fibronectin prior to seeding. The influence of both treatments on seeding efficiency and cartilage tissue is examined. Additionally in **chapter 7** the influence of chondrocyte-scaffold interaction upon aggregation mediated seeding on 3D deposited PEGT/PBT scaffolds with different surface properties and porosity on cartilage tissue formation was evaluated. The thesis ends with a section regarding the conclusions of the studies described and some interesting future prospectives.

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Chapter 2

Co-culture in cartilage tissue engineering

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Co-culture in cartilage tissue engineering

For biotechnological research in vitro in general and tissue engineering specifically, it is essential to mimic the natural conditions of the cellular environment as much as possible. In choosing a model system for in vitro experiments, the investigator always has to balance between being able to observe, measure or manipulate cell behavior and copying the in situ environment of a cell. Most tissues in the body consist of more than one cell type. Such organization is essential for normal development, homeostasis and repair reactions. In a co-culture system two or more cell types are brought together in the same culture environment enabling them to interact and communicate. Co-culture proved to be a powerful tool in unraveling the importance of cellular interactions during physiology, communication and homeostasis. In this review the introduction and applications of co-culture systems in research and specifically cartilage tissue engineering are discussed. The first studies wherein co-culture was applied, focused on oocyte maturation to a pre-implantation blastocyst. Therefore, a brief overview of these studies is given in the first paragraph. Later on in the history of co-culture it was applied to study cell-cell communication. After which, almost immediately as the field of tissue engineering was recognized, it was introduced in tissue engineering to study cellular interaction and their influence on tissue formation. The major part of this review describes findings of co-culture studies in tissue engineering.

The developmental and differentiation status of cell type donors are identified in this review as: 1) embryonic stem cell, 2) differentiated embryonic cell, 3) immature differentiated, 4) immature undifferentiated (stem) cells, 5) mature differentiated and 6) mature undifferentiated (stem) cells.

History in co-culture studies

Over the last 4 decades, co-culture has extensively been used in biological research to investigate cellular interactions and function (1-3). The very first co-culture study reported heterologous communication by means of gap junctions between rat ovarian granulosa cells and mouse myocardial cells when combined (1). Thereafter, many co-culture studies focused on reproducing the natural embryonic development of pre-implantation embryos in vitro (4),(5),(6),(9).

These experiments were performed not only to study pre-implantation development, but also to support fertilization programs for clinical application (6),(7). Research on the influence of coculture of somatic cells with pre-implantation embryos on normal development resulted in defined co-culture protocols still used today for clinical applications (8),(9). Until recently, co-culturing with tubul ampular, endometrial epithelial, oviduct endothelial, trophoblastic cells or specific cell lines like the Vero cell line was considered to be the most viable way to mature fertilized oocytes through normal development to an implantable blastocysts (7),(6),(10). Studies on development of pre-implantation embryogenesis lead to successful reproducible support of the signals exchanged during co-culture, with several carefully composed culture media which are sequentially applied (11).

Totipotent embryonic stem cells in the embryo maintained their integrity during co-culture in vitro, while factors released from a supporting somatic cell type were necessary for their normal development in vitro. Evidently, in an embryo the organization level is high; this might prevent these totipotent cells to differentiate into the lineage the embryo is co-cultured with. If and how somatic cell types in co-culture influence each others physiology or differentiation in similar ways as described for embryonic cells and somatic cells was investigated subsequently.

In the next paragraphs the nature of cellular interaction and their influence on cell physiology and/or differentiation is reviewed. Being able to influence cellular interactions is considered of great interest in tissue engineering, because through these interactions tissue formation of one or all cell types is regulated. The goal for tissue engineering is to simulate and stimulate natural physiology and differentiation of cells in order to engineer a tissue in vitro or induce the formation of a repair tissue in situ. The cellular interaction involved in tissue engineering possibly involves cell-cell communication. Other cellular interactions include cell-extra cellular matrix interaction which play an important regulating role in normal cell physiology and differentiation. Cellular interactions examined in co-culture studies mainly focus on cell-cell communication.

Types of cell communication during co-culture

Cellular communication in animals can be established via a variety of signalling pathways. Signalling pathways identified include endocrine signalling (via the blood stream), synaptic signalling (via nerve innervations), paracrine or autocrine signalling (signals released by one cell bind to membrane receptors of other cells), juxtacrine signalling (signals exposed on the membrane of one cell are bound by a membrane receptor of another cell) or gap junctional communication (intracellular signal exchange). When different cells types are co-cultured, they can interact and communicate via several different pathways depending on their proximity and mutual ability to interact or communicate. In the next paragraph

cell-cell signalling pathways reported to occur during co-culture are described in more detail.

While for gap junctional or juxtacrine communication direct, cell-cell contact is required, for paracrine signalling close proximity alone is sufficient for transfer of signalling molecules. Besides this, gap junctional communication enables exchange of intracellular signals since a direct connection is made between the cytoplasm of two cells. Paracrine or juxtacrine signalling involves extracellular secretion of signalling factors (12).

In co-culture experiments, the types of cell communication between different cell types has been examined. Gap junctional communication during co-culture has been shown to occur between cells from different cell types (heterotypic) but also between cells from different species (heterologous) (1),(13) (Figure 1). From the studies described in Table 1 it is imperative that during co-culture cells from different tissues and even different species can communicate via paracrine, juxtacrine or gap junctional signalling pathways. No correlation has been found between the communication pathway of cell types in co-culture and the developmental stage of the donor of these cells (embryonic vs. immature vs mature) or the influence they have on each others physiology or differentiation status. At the same time it is clear from these experiments that cellular interactions between different cell types in co-culture contributes to their tissue formation in vitro. Understanding which cellular communication pathways are important for tissue formation and how these cellular interactions are regulated could contribute significantly to the engineering of specific tissues in vitro, or development of therapies to stimulate tissue repair in situ.

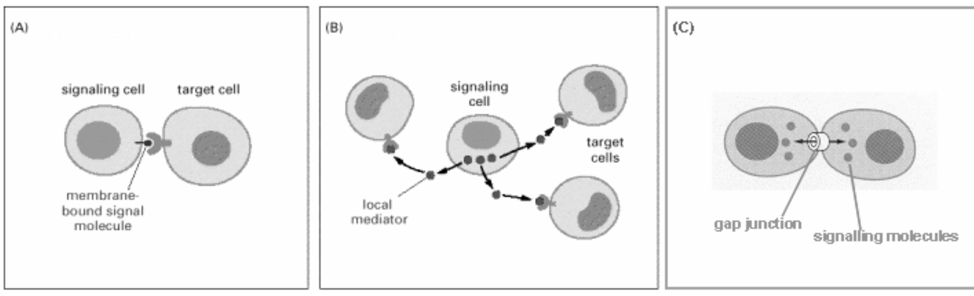

Figure 1 Forms of intercellular signalling during co-cultureof differentiated cell types

(A) Contact-dependent signalling requires cells to be in direct membrane-membrane contact (juxtacrine) (B) Paracrine signalling depends on signals that are released into the extra cellular space and act locally on neighbouring cells (C) cells connected intracellular by gap junctions share small molecules and can therefore respond to extracellular signals in a coordinated way (adapted from Alberts et al 4th edition).

Co-culture in tissue engineering

In tissue engineering co-culture was introduced to study the role of cellular communication and interactions between cell populations involved in organ or tissue development. Cell-cell interactions are important in tissue and organ development and influence cell proliferation, differentiation and physiology (14),(15). Co-culture in tissue engineering can be applied for two reasons. Firstly, two cell types are co-cultured to enhance tissue formation into one specific lineage and secondly, two cell types are co-cultured to form a multicellular tissue or organ replacement and are expected to maintain and support each others specific lineages respectively. To examine under which co-culture conditions cell type 1 (trans) differentiate into the lineage of cell type 2 and when they influence each others normal physiology or lineage specific differentiation is therefore of particular importance for tissue engineering (Figure 2).

In co-culture experiments it is a challenge to identify the different cell types involved, when cell-cell contact is established and cell mixing is allowed. Cell specific markers or labeling could contribute to discriminate the cell types in co-culture. Furthermore, it is difficult to compare results between co-culture studies performed (Table 1), because a wide variety of different cell types, cells from different species and cells in different developmental stages are applied. Proliferation, differentiation or communication of all of these cells might be influenced differently in similar culture circumstances.

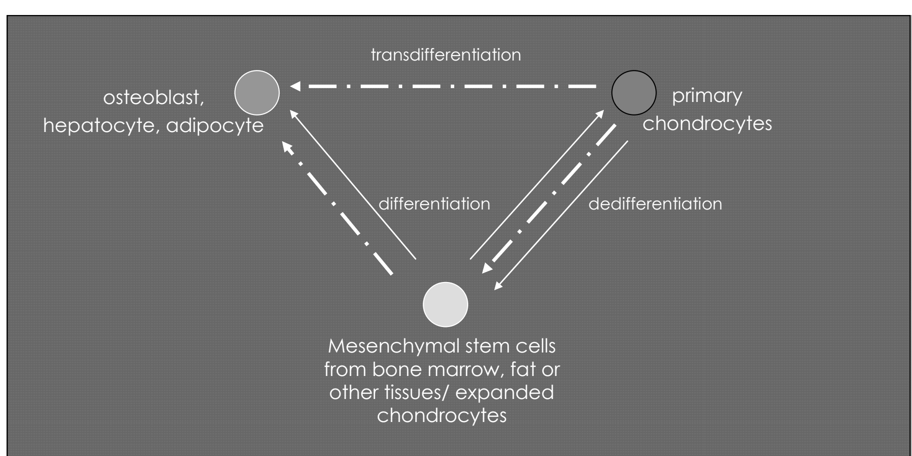

Figure 2 Differentiation, dedifferentiation and transdifferentiation of cells occurring in situ or in vitro.
Differentiation = the process by which cells undergo a change towards a more specialized form or function, Dedifferentiation = regression of a specialized cell to a simpler more embryonic unspecialized form. Transdifferentiation= an already differentiated (stem)cell forms cells outside its already established differentiation (extremely rare). transdifferentiation is also referred to when a non-stem cell transforms into a different type of cell, which might actually takes place through dedifferentiation and subsequent differentiation.

Additionally, the culture environment itself may also influence cell behavior of one or more cell types that are present during co-culture. Finally, many of the co-culture studies focus on either the effect or on only one cell type but rarely on all. Scientist designing co-culture studies should be aware of the influence of all of these factors on both cell types. Cellular interactions occurring during co-culture and the influence this has on cell differentiation and/or physiology was reviewed in the next paragraph and in Table 1.

(Trans)differentiation (Figure 2) is reported to occur when either embryonic stem cells or mature undifferentiated (stem) cells like mesenchymal stem cells from bone marrow are co-cultured with differentiated cells (16),(17),(18),(19),(20).

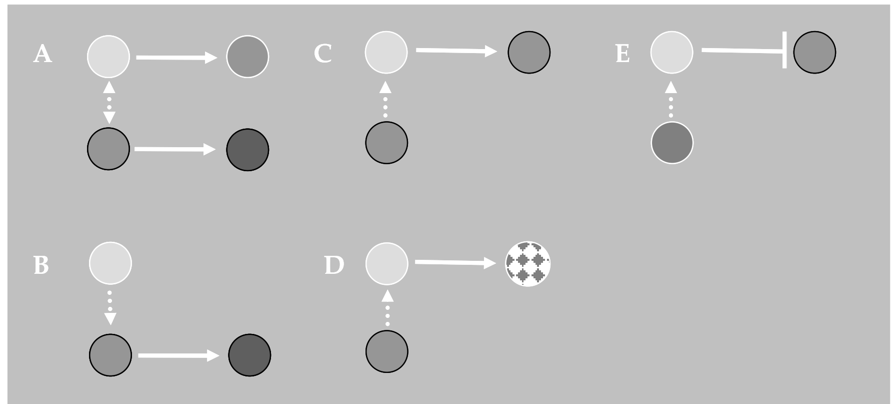

Figure 3 Cell behavior in co-culture studies(as described in Table 1)
(A) cell types support physiology or differentiation of one another, (B) cell type 1 supports differentiation or physiology of cell type 2, (C) one cell type (trans) differentiates towards the lineage of the cell type it is co-cultured with or (D) one cell type differentiates into a tissue specific lineage which is different from the cell that initiates or enhances the differentiation, (E) cell type 1 inhibits terminal differentiation of cell type 2.

In contrast, mature mesenchymal stem cells do not differentiate when co-cultured in conditioned medium or co-cultured with immature differentiated cells without cell-cell contact (16). However, in other studies it has been reported that differentiation was initiated during co-culture when cell types were separated by means of a membrane insert (15),(20),(16). Whether cells in co-culture (trans)differentiate or not is apparently not necessarily depending on cell-cell contact and might be depending on the developmental stage of the cell donor.

Co-culture of differentiated cells with a second differentiated cell type in most cases influenced lineage specific physiology of both cell types (Table 1). For example, when primary immature chondrocytes are co-cultured with primary immature osteoblasts, they showed to either support or suppress each others physiology depending on the co-culture environment (21),(22). From the studies described in Table 1 no general correlation can

be found between the influence of co-culture on physiology or (trans)differentiation of cells and the developmental stages (e.g. mature vs. immature) of the donor nor on difference in differentiation status of the cell types (e.g. mesenchymal vs. differentiated hepatocyte). Even specie difference between cell types did not seem to influence cellular interaction in a specific way. However, interspecies difference should be considered, when extrapolating results obtained with cells from an animal model to other species or the human model (23),(24),(25). It has also been suggested that in some cases (trans)differentiation might be in fact cell fusion. There is no evidence that cell fusion is contributing to the stimulatory effect of co-culture on chondrogenic differentiation (26),(27),(28). Finally, while cell-cell contact in some co-culture experiments seems to be crucial for cellular interactions in other experiments co-culture in the same medium without cell-cell contact is sufficient. To be able to draw more conclusions about the influence of cellular interaction on tissue formation in vitro, more systematic studies on cell behavior of all cell types which are brought into co-culture is required. Although chondrocytes are the only cells residing in adult cartilage tissue, during cartilage (re)generation cellular interactions between prechondroblasts and other cell types is expected to determine the fate of chondrogenic cells.

Co-culture in cartilage research

In the last two decades co-culture was introduced in cartilage research (29). Cartilage is a unique tissue in that it consists of only one cell type; chondrocytes. Cell-cell interactions between chondrocytes and other cell populations mainly take place at the border of cartilage. Therefore, in cartilage research co-culture has been used to study the development of osteoarthritis by looking at cellular interactions between articular chondrocytes and synovial cells (29-31) and chondrocytes and osteogenic cells (22),(32).

Co-culture of chondrocytes with synovial fibroblasts

Studies on interactions between chondrocytes and synovial fibroblasts focus on the influence of rheumatoid arthritic synovial, cartilage homeostasis and neocartilage formation. Results from different studies showed that while synovial cells from healthy tissue support chondrogenesis of chondrocytes but also mesenchymal stem cells, synovial cells from a donor with rheumatoid arthritis invaded neocartilage (31),(33). More surprisingly, it was shown that upon co-culture, heterologous rabbit chondrocytes and human synovial cells communicate both through intercellular calcium signalling through gap junctions and ATP-mediated paracrine stimulation (34). Together these studies suggest that synovial cell-chondrocyte interactions seem to more important for homeostasis than cartilage tissue formation and show that the co-culture system is a suitable model to study these interactions. Another cell type interacting with chondrocytes in vivo is osteoblasts from the underlying bone.

Co-culture of chondrocytes with osteoblasts

When a cartilage defect proceeds to the underlying bone or when loss of blood supply to subchondral bone results in osteochondritis dissecans (OCD), the subchondral bone is also affected, resulting in poor mechanical properties (35). Consequently, treatment of these defects preferentially would be with an osteochondral construct. Osteochondral constructs are engineered by co-culturing two cell types (chondrocytes and osteoblasts) or two tissue types (cartilagenous and bone-like tissue) in close proximity mostly in bioreactors. However, the studies reviewed here have quite different outcomes and sometimes the conclusions drawn from them seem to be contra dictionary.

In the study of Jiang (22) glycosaminoglycan deposition of primary immature chondrocytes was significantly lower when co-cultured with a layer of primary immature osteoblasts compared to chondrocytes alone. Also cell-mediated mineralization of osteoblasts in co-culture was considerably lower compared to osteoblasts controls. Interactions between osteoblasts and chondrocytes apparently modulate their cell physiology. In contrast with the results of Jiang, a study by Spalazzi (21) showed that chondrocytes maintain their phenotypic morphology for a longer time in the presence of osteoblast compared to chondrocytes only control. Moreover, more extensive matrix production and cell growth was observed in the co-culture group compared to the chondrocyte and osteoblast control group. The difference in outcome possibly come the difference in culture system used.

When cells from a mesenchymal stem cell line were co-cultured with endochondral chondrocytes, osteoblasts or fibroblasts in a transwell system, osteogenesis of mesenchymal stem cells was strongly induced when co-cultured with endochondral chondrocytes but not with osteoblasts or fibroblasts. (15). Finally, mature osteoarthritic articular chondrocytes were co-cultured with subchondral osteoblasts isolated from sclerotic and non-sclerotic areas of the underlying bone. The findings suggest that sclerotic osteoarthritic osteoblasts could initiate chondrocyte phenotype shift towards hyperthrophic differentiation although chondrocytes did not express type I or X collagen nor Alkaline Phosphatase (32). The latter finding confirms on a cellular level, what is already known from clinical practice. It emphasizes that for treatment of osteochondritis dissecans certainly but maybe for many other chondral defects, cartilage as well as underlying bone should be treated or replaced.

Co-culture of chondrocytes in cartilage tissue engineering

The few co-culture experiments performed in hyaline cartilage tissue engineering were mainly focusing on generating nucleus pulposus tissue. Mesenchymal stem cells originating from bone marrow or fat tissue were co-cultured with nucleus pulposus cells. Upon co-culture, nucleus specific markers Sox-9, aggrecan and Collagen type II expression increased significantly in both mesenchymal stem cells as nucleus pulposus cells. Results suggested that paracrine signalling between the two cell types initiated chondrogenic

stem cells differentiation (36). Also in co-culture with chondrocytes it is evident that multipotent cells such as mesenchymal stem cells can support chondrogenesis either through transdifferentiation or mediated through the secretion of trophoblastic factors supporting physiology of chondrocytes.

In a study of Jikko (37) immature articular chondrocytes were combined with immature growth plate chondrocytes to study mechanisms involved in inhibited terminal differentiation of articular chondrocytes. When co-cultured in a transwell insert system, hence without direct cell-cell contact, articular chondrocytes inhibited terminal differentiation of growth plate chondrocytes. In contrast medium conditioned by articular chondrocytes could not prevent terminal differentiation of growth plate chondrocytes (37). Apparently signalling between articular chondrocytes and growth plate chondrocyte in co-culture was not mediated via paracrine signalling.

Finally, Tsuchiya K et al (2004) (38) was the first to co-culture (expanded) chondrocytes with bone marrow mesenchymal stem cells in pellets. Results showed elevated safranin O staining with increasing initial percentages of chondrocytes (passage 2). Nevertheless, to our understanding, in the Tsuchiya study, pellets were cultured in medium containing dexamethasone and TGFβ3, which in itself might be responsible for differentiation of the expanded chondrocytes present in the pellets. Thus far no studies have been performed combining isolated primary chondrocytes with other cell types with the purpose to initiate and support chondrogenic differentiation and physiology for cartilage tissue engineering.

In conclusion co-culture proves to be a powerful tool in tissue engineering not only to generate tissues and organs consisting of multiple cell types, but also to guide and support tissue formation of cartilage through cellular interactions with other cell types. In this thesis, co-culture was examined not only as a tool to support cartilage tissue engineering but also to contribute to the knowledge of the influence of cellular interactions during co-culture of primary chondrocytes with a whole range of cell types.

The findings in co-cultured studies summarized in this review and studies described in this thesis emphasize the importance of examining cellular interaction for engineering of tissue with different cell type but maybe surprisingly also for tissues containing only one celltype like cartilage.

Tissue	Cell type 1	Cell type 2	Culture system	Co-culture effect	Reference
Bladder	Embryonic bladder smooth muscle (rat)	Embryonic bladder epithelium (rat)	cell-cell contact	B	Liu W 2000(39)
Bladder	Primary mature Smooth muscle cells (bovine)	Primary mature urothelial cells (bovine)	unclear	A	Zhang Y 2000(40)
Blood vessels	Immature expanded Endothelial cells (bovine)	Immature expanded smooth muscle cells (bovine)	cell-cell contact	B	Williams C 2004(41)
Blood vessels	Umbilical vein endothelial cells (human)	mature primary fibroblasts (human)	cell-cell contact	B	Wenger A 2005(42)
Bone	Embryonic stem cells (murine)	embryonic osteoblasts (murine)	no cell-cell contact	C	Buttery LD 2001(20)
Bone	Mature mesenchymal stem cell line (C3H10T½) (murine)	Immature endochondral chondrocytes (avian)	no cell-cell contact	C	Gersten-feld LC 2003(15)
Bone	Umbilical Vein Endothelial cells (HUVEC)(human)	Mature Osteoprogenitor cells (human)	cell-cell contact	B	Guillotin B 2004(43)
Cartilage	Immature primary articular chondrocytes (rabbit)	Immature primary growth plate chondrocytes (rabbit)	no cell-cell contact	E	Jikko A 1999(37)
Cartilage	Embryonic Notochordal cells (canine)	Mature and immature nucleus pulposus cells (bovine)	no cell-cell contact	B	Aguiar DJ 1999(44)
Cartilage	Immature Keratinocytes (human)	immature elastic chondrocytes (human)	unclear	B	Neovius EB 2003(45)
Cartilage	Mature bone marrow mesenchymal stem cells (sheep)	Mature synovial cells (sheep)	no cell-cell contact	D	Chen J 2005(33)
Cartilage	Mature fat mesenchymal stem cells (rabbit)	Mature intervertebral disc tissue from nucleus pulposus		C	Li X 2005(36)

Hart	Fibroblastic immature ventricular fraction (rat)	Immature cardiomyocyte (rat)	cell-cell contact	B	Van Luyn MJ 2002(46)
Hart	Mature mesenchymal stem cells (murine)	Mature cardiomyocytes (rat)		C	Fukuhara S2003(18)
Hart	Embryonic stem cells (human)	Mytomycin treated visceral endoderm like cells (embryonic cells)(murine)	cell-cell contact	D	Mummery C 2003(17)
Hart	Immature cardiomyocytes (murine)	Mature micro vascular endothelial cells (murine)	cell-cell contact	A	Narmoneva DA 2004(47)
Hart	Mature mesenchymal stem cells (rat)	Immature cardiomyocytes (rat)	no cell-cell contact	C	Yoon J. 2005(16)
Hart	Mature bone marrow cells (murine)	Embryonic cardiac explants (avian)	cell-cell contact	C	Eisenberg CA 2006(48)
Liver	NIH 3T3-J2 fibroblasts (murine)	Mature hepatocytes (Rat)	cell-cell contact	B	Bhatia 1998(49)
Liver	Mature sinusoidal liver cells (rat), liver epithelial cells (rat), 3T3 fibro-blasts (murine), dermal fibroblasts (human) and oarta endothelial cells (bovine)	Mature hepatocytes (rat)	cell-cell contact	B	Goulet F 1988(50)
Liver	Mature bone marrow mesenchymal stem cells (female rat)	Mature hepatocytes (male rat)	cell-cell contact	B	Mizuguchi T 2001(51)
Liver	Mature aortic expanded endothelial cells (human)	Immature hepatocytes (rat)	cell-cell contact	B	Harimoto M 2002(52)
Liver	NIH 3T3 fibroblasts (mouse)	Primary mature hepatocytes (rat)	cell-cell contact	A	Kang IK 2004(53)
Mucosa	Mature fibroblasts (human)	Mature Keratinocytes (human)	unclear	B	Imaizumi F 2004(54)

Mucosa	Mature mesenchymal stem cells (human)	Mature respiratory epithelial cells (human)	no cell-cell contact	B	Le Visage C 2004(55)
Muscle	Myoblasts cell line (C2C12) (murine)	Embryonic endothelial cells and HUVEC endothelial cells (human)	cell-cell contact	A	Levenberg S 2005(56)
Neuron	Mature fibroblasts and Keratinocytes (human)	Embryonic dorsal root ganglia neurons (murine)	cell-cell contact	B	Gingras M 2003(57)
Neuron	Mature bone marrow mesenchymal stem cells (rat)	Mature Schwann cells (rat)	cell-cell contact	C	Zurita M 2005(58)
OC construct	Primary immature chondrocytes (bovine)	expanded immature osteoblasts (bovine)	cell-cell contact	A	Spalazzi JP 2003(21)
OC construct	Primary Immature chondrocytes (bovine)	Primary Immature osteoblasts (bovine)	cell-cell contact	A	Jiang J 2005 (22)
OC construct	Immature epiphyseal chondrocytes (human)	Embryonic osteoblasts (human)	cell-cell contact	A	Mahmou-difar N 2005(59)
Retina	Mature endothelial cells (bovine)	Mature retinal cells (human)		A	Dutt K 2003(60)
Neuro sensory retina	Mature bone marrow stem cells (human)	Irradiated mature retinal epithelium cells (human)	cell-cell contact	C	Chiou SH 2005(61)
Skin	Mature fibroblasts (human)	Mature keratinocytes (human)	cell-cell contact	A	Zacch i V 1998(62), Wang TW 2003(63), El-Ghalb-zouri A. 2004(64)
Urethra	Mature fibroblasts and smooth muscle cells (human)	Mature urothelial cells (human)	cell-cell contact	A	Fossum M 2004(65)

Table 1. Influence of co-culture on cell behavior of different cell types when either in direct contact or not.

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Chapter 3

The effect of stratified culture compared to confluent culture in monolayer on proliferation and differentiation of human articular chondrocytes.

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Chapter 3

Abstract

With conventional tissue culture of cells, it is generally assumed that when the available 2D substrate is fully occupied, growth ceases or is greatly reduced. However, in nature wound repair mostly involves proliferation of cells that are attracted to the defect site in a 3D environment. Hence proliferation continues in 3D until the defect site is filled with cells contributing to repair tissue. With this in mind, we examined the growth behavior of human articular chondrocytes during stratified culture as opposed to routine culture to confluency. Additionally we studied the influence of growth factors on proliferation during stratified culture and differentiation thereafter. Chondrocytes were cultured in monolayer on tissue culture plastic to confluency or stratified for an additional 7 days. Culture medium was based on Dulbecco's minimal essential medium with 10% serum and either supplemented with high concentrations of non essential amino acids and ascorbic acid, or in stead with basic fibroblastic growth factor, platelet derived growth factor and/or transforming growth factor. After expansion cells were harvested, counted and their differentiation capacity was examined in pellet culture assays. It was shown that chondrocytes, cultured stratified proliferate exponentially for up to an additional 4 days and that cell yield increased 5 fold. Furthermore, during stratified culture the amount of cells increased even further in the presence of either fibroblastic growth factor, platelet-derived growth factor and transforming growth factor or high concentrations of non essential aminoacids and ascorbic acid. Depending on donor variation and factors supplemented the cell yield ranged from 0.06 up to 1.1 million cells/cm^2 at the 2nd passage. During stratified culture in the presence of either bFGF and PDGF or high concentrations of NEAA and AsAP exponential growth continued for up to 7 days. Finally cells maintained their differentiation capacity when cultured stratified with or without growth factors (bFGF, TGFβ and PDGF), but not when cultured with high levels of AsAP and NEAA. In contrast to other 3D culture techniques like micro-carrier or suspension culture, nutrient consumption remained the same as with conventional expansion. Since this allowed to culture clinical relevant amounts of chondrocytes without increasing the amount of serum, chondrocytes can be fully cultured in the presence autologous serum avoiding the risk of viral and/or prion disease transmission associated with the use of animal derived serum or serum substitute with animal derived constituents.

Introduction

In healthy functional articular cartilage, theoretically chondrocytes stay in the G1/G0 phase indefinitely and thus are not prone to go into cell cycle and start proliferation. However, when cultured in monolayer they have shown to attach, loose their rounded morphology, become fibroblastic and are able to proliferate exponentially. Thus these cells are able to regain their ability to proliferate exponentially. And they are able to regain their ability to proliferate exponentially under the right conditions. With conventional tissue culture of mammalian cells in 2D it is generally accepted that when the cell concentration exceeds the capacity of the medium or when the available substrate is fully occupied, growth ceases or is greatly reduced. However, in vivo, when a defect is created in articular cartilage, cells released from the surrounding tissue start to proliferate to some extend in an attempt to fill the defect (1). This observation indicates that during an initial attempt to fill a defect chondrocytes are capable of proliferation in a 3D environment at least to some extend. Studies on micro carriers, have shown that proliferation of human chondrocytes are as efficient in this 3D model as during monolayer culture, even though they regain their rounded morphology readily on the surface of micro carriers (2),(3). Additionally, during in-gel culture, chondrocytes have been shown to maintain their rounded morphology, while surrounded by a substrate continued proliferation (4),(5). Together these findings illustrate that chondrocytes are still able to expand in a 3D-environment and that they do this regardless of their morphology.

Previous studies have shown that proliferation of chondrocytes is further supported by the presence of specific growth factors and supplements in culture medium. More specifically basic fibroblast growth factor (bFGF), transforming growth factor β (TGFβ), platelet derived growth factor (PDGF) and high concentrations of non essential amino acids (NEAA) and ascorbic acid 2-phosphate (AsAP) have been shown to enhance the proliferation rate of chondrocytes (6),(7),(8), (9),(10),(11),(12),(13),(14),(15).

Our hypothesis is that chondrocytes can continue exponential growth in a 3D environment, for example, when cultured stratified. And that they can overcome cell contact related growth inhibition depending on supplements or growth factors present in the medium. It has been shown that serum can overcome contact inhibition. Thus, we further hypothesize that growth factors like bFGF, TGFβ and PDGF identified to be present in serum and shown to influence proliferation of chondrocytes, might be involved in this process. In this study we examined to what extend exponential growth of chondrocytes is continued during stratified culture. Additionally the influence of bFGF, TGFβ, PDGF, NEAA and AsAP on exponential growth of chondrocytes during stratified culture is studied. Finally, we examined if chondrocytes cultured stratified either in the presence or absence of growth factors or high concentration of supplements maintain their ability to differentiate and form cartilage specific extra-cellular matrix.

Materials & Methods

Cell isolation

After informed consent was obtained and in accordance with the local ethical committee and blood bank procedures, human articular chondrocytes were isolated form full thickness cartilage knee biopsies and approximately 100ml of blood was retrieved from patients undergoing Anterior Cruciate Ligament replacement. None of the biopsies showed macroscopic signs of cartilage pathology. Patient's age on average was 29 ranging from 19 to 36 and biopsies were taken from 2 females and 7 males. Cartilage was separated form underlying bone and connective tissue and digested for 20-22 hrs in collagenase type II (0.15% Worthington) in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100U/ml) and streptomycin (100μg/ml). Digested suspension was filtered through a 100μm mesh nylon filter (cell strainer Nucleon) and washed 2 times with phosphate-buffered saline (PBS) supplemented with penicillin (100U/ml) and streptomycin (100μg/ml)(Sigma-Aldrich).

Cell culture

Chondrocytes were plated at a density of 3.5 * 10^4 cells/cm^2 at each passage and unless noted otherwise cultured in proliferation medium 1 (PM1) containing DMEM (Gibco) supplemented with 10 % serum (Human autologous serum), 0.1 mM Non-essential amino acids (Sigma-Aldrich), 100mM HEPES buffer (Biowhitakker), 0.2 mM ascorbic acid 2-phosphate (InVitrogen), 4 mM proline (Sigma-Aldrich) , 100U/ml penicillin (InVitrogen) and 100μg/ml streptomycin (Sigma-aldrich). Additionally in PM2, PM3 and PM4 extra supplements or growth factors were added according to the scheme described in table 1. Plated cells were cultured in a humidified 37ºC/5% CO_2 incubator until the surface area was fully occupied, followed by 4 days culturing at stratification. Cells were either passaged or harvested with trypsin/EDTA (0.25% /0.1 mM InVitrogen,) stained with Trypan blue for viability and counted with a Burker-Turk counting chamber. Population doublings, cell yield, number of passages and culture time of cells are described in table 2.

In vitro redifferentiation.

Subcultured chondrocytes were tested for their re-differentiation capacity in a pellet culture assay. 5 * 10^5 cells were centrifuged at 500 g for 2 min. Micromasses were cultured in a polypropylene Falcon centrifuge tube containing 3 ml of medium. Pellets were cultured in DMEM supplemented with ITS^{+1} (10 μg/ml insulin, 5.5 μg/ml transferrin,
5 ng/ml selenium, 0.5 mg/ml human serum albumin and 4.7 μg/ml linoleic acid; Sigma) 1.25-mg/ml human serum albumin, 10^{-7}M dexamethasone and 10 ng/ml TGFβ1 in a humidified 37ºC/5% CO_2 incubator. From each experimental group 6 pellets were harvested after 21 days and processed for histology or GAG- and DNA-assay

medium	extra neAA	extra ASAP	FGF2	PDGF	TGFβ
	(2 x)	(1 mM)	(5 ng/ml)	(10 ng/ml)	(1 ng/ml)
PM1					
PM2	+	+			
PM3			+	+	
PM4			+	+	+

Table 1 Composition of proliferation media.

Histology

Pellets for histology were fixed in 1.5 % glutaraldehyde in cacodylate buffer (0.14M, pH 7.2-7.4), dehydrated, embedded in glycolmethacrylate (Merck) and cut to yield 5μm sections. Sections were stained for sulphated glycosaminoglycans (GAG) with safranin O and counterstained with haematoxyline (Gill's 3) and fast green respectively for nuclei and cytoplasm.

Quantitative GAG and DNA assay

Pellets (n=3) for quantitative analysis of GAGs and cell number were washed with PBS and frozen at -80°C. Subsequently they were digested with 1 mg/ml proteinase K (SIGMA) in Tris/EDTA buffer (pH7.6) containing 185 μg/ml iodoacetamide and 1 μg/ml pepstatin A (SIGMA-Aldrich) for >16 hrs at 56°C. GAG content was spectrophotometrically determined with 9-dimethylmethylene blue chloride (DMMB) (Sigma-Aldrich) staining in PBE buffer (14.2 g/l Na_2HPO_4 and 3.72 g/l Na_2EDTA, pH 6.5) with a micro plate reader (Bio-TEK instruments) at an absorbance of 520 nm. Cell number was determined via quantification of total DNA with CyQuant DNA kit according to the manufacturer description (Molecular probes) and fluorescent plate reader (Perkin-Elmer).

Statistical analysis

Average and standard deviations are shown in all Figures. Data were analyzed with 2-tailed student t-test with two sample equal variances using $p < 0.05$ as the criteria for statistical significance.

Results

Cell yield after and average growth rate during confluent- or stratified culture

In this study we investigated the ability of chondrocytes to overcome contact inhibition and continue exponential growth after confluency. Results showed that when cultured stratified, the number of cells per square cm that could be harvested was 4-fold higher after 26 days of culture compared to the confluent culture (Figure 1). This indicates that

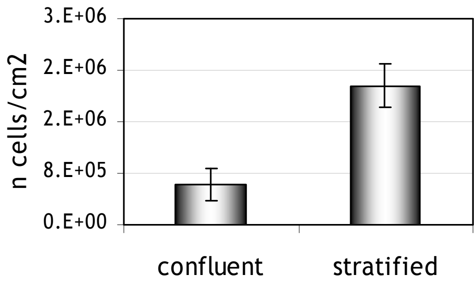
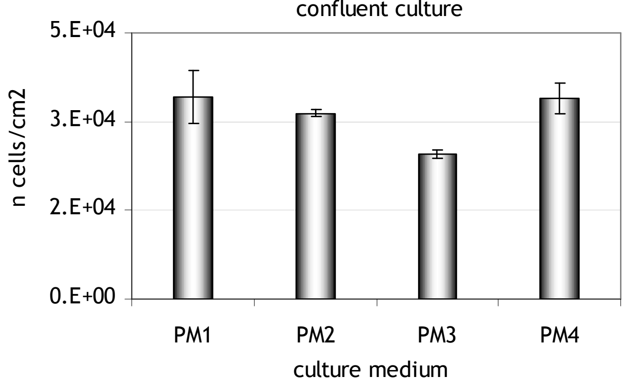


Figure 1 Culture efficiency (cells/ cm^2) of chondrocytes cultured to confluency or stratification.
Chondrocytes from human articular cartilage were expanded in PM1 (DMEM$^+$ with 10% HAS) and harvested either just before reaching confluency or 4 days beyond confluency (stratified). The amount of cells/cm^2 harvested was monitored and shown. ($P<0,05$)($n=6$).

3

cells continue proliferation when cultured stratified. To determine the influence of growth factors on cell yield and average growth rate we compared their influence on stratified culture to confluent culture. Figure 2A and B show the cell yield per square cm of chondrocytes from a patient cultured either confluent or stratified in PM1-PM4. This is representative for ratio differences between the experimental groups for all donors. When cells were cultured to confluency in PM1, cell yield at the 3rd passage on average was $3.4 \pm 0.4 \cdot 10^4$ cells/cm^2. In PM2, PM3 or PM4, although confluency was reached earlier, cell yield was significantly lower or not different from PM1 (respectively, $3.1 \pm 0.1 \cdot 10^4$, $2.4 \pm 0.1\ 10^4$ and $3.4 \pm 0.3 \cdot 10^4$ cells per square cm; figure 2A). In contrast, cell yield of stratified cultured chondrocytes in the presence of extra supplements (PM2) or growth factors (PM3 & PM4) increased significantly, 3.2 fold , 5.1 fold and 7.0 fold respectively, compared to stratified culture in PM1 (Figure 2B). Although patient to patient variation was high (Table 2), similar differences in cell yield between stratified cultures in PM1-PM4 compared to confluent culture in PM1 are observed for chondrocytes from all donors. Further detailed information on growth characteristics of chondrocytes cultured under described conditions are shown in Table 2. Average population doublings increased from 5.6 for confluent cultured chondrocytes in PM1 to 8.9, 10.4, 11.8 and 12.4 population doublings for chondrocytes cultured stratified in PM1, PM2, PM3 and PM4, respectively. As a result median cell yield showed an increase in cell yield/cm^2 of 3.5-fold, 7.7-fold, 12.3-fold and 17.1-fold in PM1, PM2, PM3 and PM4, respectively, compared to confluent cultured cells in PM1. Although median cell yield/ cm^2 shows clear differences between experimental groups, the minimum and maximum show that as a result of intra patient variation, the net amount of cells that can be harvested, overlap between the experimental groups.



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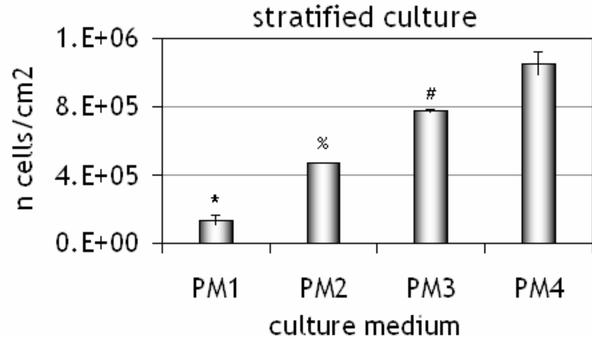

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Figure 2 Influence of supplements and growth factors on culture efficiency after culturing to confluency (A) or stratification (B).
Culture efficiency (cell number/cm^2) of chondrocytes cultured to confluency (A) or stratified; 4 days beyond confluency (B) in PM1, PM2 (PM1 + extra NEAA/AsAP), PM3 (PM1 + bFGF, PDGF-BB) or PM4 (PM1 + bFGF, PDGF-BB and TGF). Graphs are representing cell yield of 1 donor as an example of differences between experimental groups. P < 0.05; significant differences are indicated as follows:
** PM1 to PM2, $^{\%}$ PM2 to PM3, $^{\#}$ PM3 to PM4.*

We next determined to what extend chondrocytes stayed in log phase during stratified culture and at what time they entered the stationary phase. At the start of any culture in a culture vessel, cells adjust to their culture environment. Then their in the stationary phase and growth rate is very low. This is followed by a log phase during which cell uptake of nutrients and excretion of waste products is in equilibrium and the cells expand exponentially. Hence, growth rate increases at first and stabilizes when cell growth is in log phase.
Finally, it is assumed that when either total surface area is occupied or cell number is high and cells go into stationary phase during which growth rate decreases again. This is because more waste products are excreted and fewer nutrients are available.

	Culture condition	Total average population doublings	Median cell yield (min-max) $*10^5$ cell/cm^2	Number of passages	Average culture time (days)
1	Confluent PM1	5.6 ± 0.2	0.37 (0.12-0.57)	3	22
2	Stratified PM1	8.9 ± 0.6	1.3 (1.1-1.6)	2	26
3	Stratified PM2	10.4 ± 2.8	2.7 (0.6-4.8)	2	26
4	Stratified PM3	11.8 ± 2.3	4.3 (1.1-7.8)	2	26
5	Stratified PM4	12.4 ± 1.8	6.0 (1.7-11)	2	26

Table 2 Number of population doublings vs. number of passages of chondrocytes culture to confluency or stratified in different media. *Chondrocytes were cultured to subconfluency in PM1 (1) or stratified for an additional 4 days (2-5) in different media (PM1-PM4). Initially cells were plated at a density of 3.5 * 10^4 cells/cm^2 for all conditions. Cells from all patients were cultured in autologous serum (n=6 for 1 and 2, n=3 for 3-5).*

3

We compared the change in average growth rate (d^{-1}) of chondrocytes when confluency is reached (t=0 days) and in stratified culture at 2 time points (t=4 and t=7 days). Figure 3 shows that in proliferation mediums PM1, PM2 and PM4, average growth rate increased or stabilized from day 0-4 but stabilized or decreased from day 4-7 of stratified culture. This showed that up to 4 days of stratified culture, chondrocytes were still in log phase in these media but thereafter the growth curve started to enter the stationary phase for chondrocytes of most donors. Chondrocytes cultured in medium PM3 show continues decrease in growth rate for donor 2 and 3, which implies that cells are in stationary phase. In contrast, cells from donor 1 are still in log phase; the growth rate is increases through day 7 (Figure 3).

Redifferentiation capacity of chondrocytes cultured stratified

Chondrocytes which were expanded stratified, were harvested and subsequently tested for differentiation capacity in pellets applying chondrogenic medium. Safranin O-stained sections showed that the capacity to produce sulphated GAGs is maintained in chondrocytes cultured stratified in PM1, PM3 and PM4 (Figure 4A). There was no apparent difference in intensity of the safranin O staining between these 3 groups. Usually after pellet culture, the cells in the outer layer of the pellet maintain their fibroblastic morphology and do not contribute to proteoglycan formation. Interestingly after safranin O staining we saw that in the PM 4 group the fibroblastic blue stained outer rim was not present. In contrast, when chondrocytes were expanded stratified in PM2, no safranin O staining for GAGs whatsoever could be detected in pellet sections (Figure 4A). These data were supported by quantitative GAG assay results (Figure 4B). Only low amounts of GAGs (8.1 ± 3.9 μg GAG/pellet) could be detected in pellets from PM2 expanded chondrocytes. More interestingly, PM3 and PM4 expanded chondrocytes produced respectively 54,6 ± 3,9 and 56,4 ± 13,9 μg GAG/pellet.

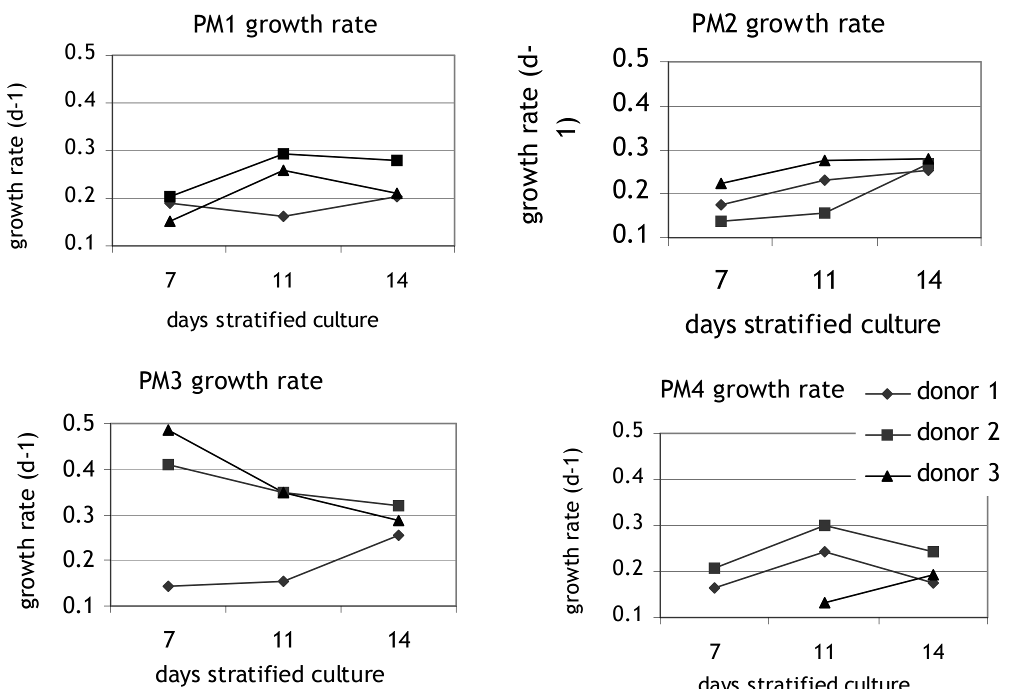


Figure 3 Trends in influence of supplements and growth factors on growth curve of chondrocytes culture to stratification.
Graph shows the trend in growth rate of human chondrocytes culture stratified for 0, 4 and 7 days in PM1, PM2 (PM1 + extra NEAA/AsAP), PM3 (PM1 + bFGF and PDGF-BB) or PM4 (PM1 + bFGF, PDGF-BB and TGFβ1) (n=3).

This was significantly more GAG/pellet compared to PM1 (37.3 ± 2.2 μg GAG/pellet). These results show that the presence of bFGF and PDGF-BB (PM3) during stratified culture stimulates redifferentiation of chondrocytes thereafter. The presence of TGFβ in addition to bFGF and PDGF (PM4) did not seem to have an additional effect on the redifferentiation capacity of chondrocytes (Figure 4). Finally, from DNA assay results we learned that in pellet cultures of chondrocytes that were expanded in PM2, PM3 or PM4, the cell number after 3 weeks is close to the initial cell density, ranging from 520.000 to 580.000 cells/pellet while pellet cultures of PM1 expanded chondrocytes contain 350.000 cells/pellet (Figure 4C).

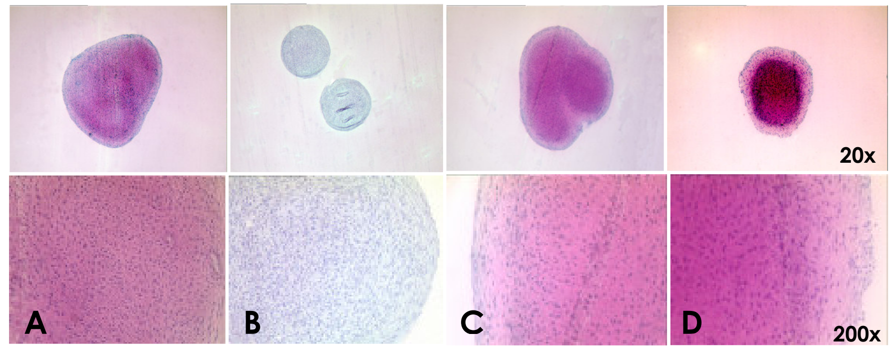
Discussion & Conclusions

It is commonly assumed that proliferation of mammalian cells in monolayer is inhibited by direct cell contact when the total surface area is occupied (16). However, our results show that chondrocytes continue to grow exponentially for at least 4 days when cultured stratified in PM1 (Figure 1). Continues culture through stratification resulted on average in

a 4-fold increase in cell yield/cm^2 compared to normal sub-confluent culture. Because chondrocytes during stratification form several cell-layers on top of each other, apparently their growth is not inhibited just by cell-cell contact. Already in 1968 it was suggested that with the presence of serum in the medium contact inhibition could be overcome (17).

Although high density cultures primarily are described to promote (re)differentiation of chondrocytes in vitro and in vivo (5),(18,(19), it also has been shown that initial high cell density during monolayer culture promotes cell survival (20), high density conditioned medium supported prolonged chondrocyte survival in low density cultures (21) and proliferation rate was maintained during the first 3 days of high density culture (De Mattei M. et al 2001). Because exponential growth of cells is the net result of the equilibrium between proliferation rate and apoptosis or cell death, these findings could explain our results. Based on these results and studies of others, we propose that autocrine or paracrine signaling occurring at high cell density plays a crucial role in tolerance of cells to nutrient limitation or accumulation of growth inhibitors in the medium. This is further supported by the findings of Trojani and Tun T. (22), that 3D culturing of cells supports survival and proliferation for hematopoietic stem cells or hematopoietic progenitor cells from bone marrow and osteogenic cells.

Growth factors like bFGF, PDGF and TGFβ are known for their mitogenic activity during monolayer culture of many cell types and in particular chondrocytes (8),(10),(23),(24). Additionally, these growth factors are present in serum and thus might be involved in prolonged exponential growth of cells cultured in 3D. Next to this, NEAA and AsAP enhance proliferation of chondrocytes in culture (9),(15),(25),(26). We investigated the influence of these growth factors and supplements on cell growth during stratified culture. In accordance with what has been shown during normal culture in monolayer, we demonstrate that bFGF, PDGF and TGFβ as well as high concentrations of ASAP and NEAA enhance growth of chondrocytes during stratified culture. Results described in this paper show that during stratified culture the presence of NEAA together with ASAP both at relatively high concentrations (PM2) or bFGF together with PDGF (PM3) keeps the growth curve of chondrocytes in the exponential phase for a longer time then without growth factors (PM1). The presence of TGFβ in addition to bFGF and PDGF (PM4) does not have an additional proliferative effect on stratified cultured chondrocytes after 4 days of stratified culture (Figure 3). However final cell density at passage 2 is higher for cells cultured in PM4 than in PM3 and for cells cultured in PM3 compared to PM2. During exponential growth of chondrocytes cells can, depending on cell density proliferate, go into apoptosis or differentiate. An explanation for our results is that the influence of specific growth factors like TGFβ, bFGF and PDGF on proliferation, differentiation or apoptosis changes during culture depending on cell density (6),(8),(24),(27-30) (Table 3).



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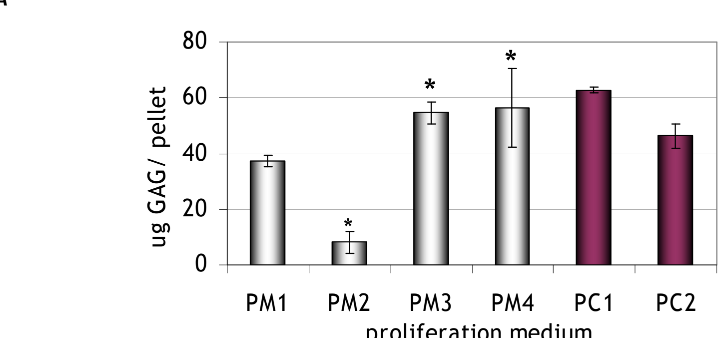

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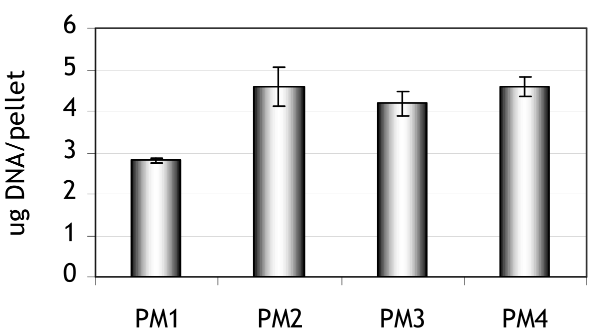

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Figure 4 Redifferentiation capacity of chondrocytes cultured to stratification in PM1, PM2, PM3 or PM4.
*Qualitative (safranin O staining) (A) and quantitative GAG (B) results, quantitative DNA assay results (C) on cell numbers of cell pellet cultures. Prior to pellet culture chondrocytes were expanded to stratification for 4 days in PM1 (A), PM2 (PM1 + extra NEAA/AsAP)) (B), PM3 (PM1 + bFGF/PDGF-BB) (C) or PM4 (PM1 + bFGF/PDGF-BB/TGFβ1) (D) for 2 passages. Pellets were cultured in redifferentiation medium for 3 weeks (n=3). PC1 and PC2; primary chondrocytes from two human donors immediately after isolation cultured in pellets (3 wks). * sign different from PM1.*

Whereas in chondrogenesis bFGF and PDGF are mostly involved in enhancement of proliferation and differentiation capacity and influence apoptosis differently, TGFβ is involved in all of these processes but as a chameleon can chance its action depending on cellular context. Apparently, TGFβ signaling is strongly depending on the signal transduction system made available by the cell that is experiencing specific circumstances. This might explain why addition of TGFβ when bFGF and PDGF are present doesn't enhance exponential growth further during stratification. Rather after 4 days of stratified culture, the cellular context might have changed to such an extend that addition of TGFβ does not result in further stimulation of growth but instead other cellular reactions (31),(32). If these cellular reactions include differentiation, apoptosis or others remain to be investigated.

Finally we examined the redifferentiation capacity of chondrocytes that are expanded stratified either with or without growth factors or a high concentration of supplements. Going through increasingly more population doublings and being passaged for more then 4 times during conventional monolayer culture, chondrocytes loose their differentiation capacity (33-35). During stratified culture within 2 passages chondrocytes undergo up to 14 population doublings whereas during conventional monolayer culture they undergo 5-7 population doublings (10). Results described here show that the differentiation capacity of chondrocytes in stratification is maintained when they are cultured in standard proliferation medium (PM1) or with growth factors (PM3 and PM4). In contrast, when the number of population doublings increased because of high concentrations of NEAA and AsAP (PM2), cells loose their redifferentiation capacity (Figure 4). If we then compare the number of population doublings between different culture media, we see that in PM1 population doublings are 8.9 and thus increased 1.6 fold compared to confluent culture (Table 2). When cultured stratified in PM2, PM3 and PM4 population doublings chondrocytes undergo are respectively 10.4, 11.8 and 12.4. Thus, whereas in PM2 the amount of population doublings is less then in PM3 or PM4, these cells loose their differentiation capacity earlier. This is in line with previous data showing that the presence of bFGF and PDGF-BB during expansion preserves the differentiation capacity of chondrocytes thereafter (10, 36-38). Even more striking is the observation that pellets of cells expanded in PM4 have no fibroblastic blue stained outer rim. This suggests that all cells in the pellet had a differentiated phenotype and produced cartilage specific matrix (Figure 4A).

In summary with these findings we show that chondrocytes can continue exponential growth for at least 4 days when cultured stratified. The presence of bFGF, TGFβ and PDGF or a high concentration of NEAA and AsAP is further supporting exponential growth during stratified culture for at least 4 days and can, depending on donor variation yield, from 0.6 up to 11 $*10^5$ cell/cm^2 at the second passage. When cultured stratified either or not in the presence of bFGF, PDGF and/or TGFβ chondrocytes maintain their redifferentiation capacity, whereas when cultured in the presence of high concentration of NEAA and AsAP,

although undergoing fewer population doublings, chondrocytes loose their capacity to redifferentiate. Culture efficiency was enhanced tremendously by stratified culture as shown here, nonetheless medium consumption remained at the same level as during conventional monolayer culture. For tissue engineering or cell therapy applications in cartilage repair, this results in a dramatic increase in cell yield per ml of medium. When bFGF, TGFβ and PDGF were added to the culture medium, during stratified culture $1.7\text{-}11 \cdot 10^6$ cells/cm^2 could be harvested. With confluent culture cell yield was ranging from $0.12 - 0.57 \cdot 10^5$ cells/cm^2. Other culture techniques like micro-carrier culture, suspension culture or bioreactor culture intended for clinical application focused on either high proliferation rate or maintaining differentiation capacity of chondrocytes (2)(26)(39). In comparison, this adjustment to an already established culture method has proven to yield high cell numbers which maintain their differentiation capacity and moreover consume low amounts of medium and therefore autologous serum and/or growth factors. This allows for application in cell therapy of this truly autologous cell culture method; being very efficient, yielding cells that are potent to contribute to a repair reaction and being resource and cost effective. Finally with this autologous culture method the risk of viral and/or prion disease transmission associated with the use of fetal bovine serum can be avoided.

Growth factor	Prolifer-ation	Apop-tosis	Re-differen-tiation capacity	Differen-tiation	References
NEAA	+				(Malda J. & Hendriks JAA. unpublished)
AsAP	+	+		+	(9),(40),(25),(41),(42),(43),(15)
bFGF	+/≈/-	≈ /+	+	-/≈	(44),(45),(10),(30),(46),(14),(47),(48),(49)
PDGF-BB	+	≈/-	+	-/≈	(50),(21),(10)
TGFβ	+/-/≈	+	+	+/≈	(50),(44),(31),(8),(51),(10),(9),(24),(52)

Table 3 Literature survey of influence of bFGF, TGFβ, PDGF-BB, NEAA and AsAP on proliferation, apoptosis and differentiation of chondrocytes.
+; stimulation, -; inhibition, ≈; no influence.

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Chapter 4

Primary chondrocyte enhance their cartilage tissue formation upon co-culture with expanded chondrocytes, dermal fibroblasts and 3T3 feeder cells and embryonic stem cells

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Chapter 4

Abstract

The objective of this study was to investigate the influence of cellular interactions on cartilage tissue formation when bovine primary chondrocytes are co-cultured with human expanded chondrocytes, human dermal fibroblasts, mouse embryonic stem cells, Mouse-3T3 feeder cells. Additionally, to identify cartilage tissue generating cell type(s) in co-cultures of primary chondrocytes with other cell types. Bovine primary chondrocytes were co-cultured (1:5 ratio) with human expanded chondrocytes, human dermal fibroblasts, mouse 3T3 feeder cells or mouse embryonic stem cells. After 4 weeks of micromass culture, formed tissue was examined for glycosaminoglycans (GAG) with safranin O staining and type II collagen with immunostaining. Type I collagen and II mRNA was detected using In Situ Hybridization. Upon co-culture of cells from bovine origin with cells from human origin, micro-mass cultures were analyzed for origin of cells with human cell specific antibody. Micromass co-cultures from all experimental groups were further quantitively analyzed for GAG and DNA. Safranin O and type II collagen data showed that in all experimental groups cartilaginous tissue formed. In Situ Hybridization results showed that in co-culture groups a range of 16-27% of the cells expressed type II collagen mRNA. When bovine primary chondrocytes were combined with human expanded chondrocytes, human cells showed to be homogenously distributed through the micromass. However, in micromass cultures combining bovine primary chondrocytes with human dermal fibroblasts, areas containing bovine chondrocytes only showed to coincide with safranin O stained areas, whereas areas with human dermal fibroblasts only showed no safranin O staining. Finally, the amount of GAG produced per primary chondrocytes in all experimental group increased ranging from 3- to 5-fold. Results shown suggest that primary chondrocytes enhance their cartilage tissue formation when co-cultured with expanded chondrocyte, dermal fibroblasts, embryonic stem cells and 3T3 feeder cells.

Introduction

Over the past 4 decades, co-culture has been extensively used in biological research to study cell-cell interactions (1-3). Cell-cell interactions play a major role in tissue and organ engineering as shown by co-culture experiments (4),(5). Interestingly, in some studies co-cultured cells influenced each others behavior without loosing their own phenotypic characteristics (6). In contrast, other co-culture studies showed that one cell population can adapt to the phenotype of the other cell population (7),(8).

In the last two decades co-culture was introduced in cartilage research mainly to cellular interaction of chondrocytes with synovial cells lining the joint cavity and osteoblasts residing in the subchondral bone (9). Cartilage is a unique tissue in that it consists of only one cell type, chondrocytes. It holds no vasculature or nerve system and therefore has limited capacity for self-repair. Cell-cell interactions between chondrocytes and other cell populations mainly take place at the interface of cartilage. Accordingly, co-culture has been used to study interactions between articular chondrocytes and synovial cells in relation to the development of osteoarthritis (9-11). In addition, co-culture has been used to study interactions between articular chondrocytes and osteogenic cells at the bone-cartilage interface (12),(13).

Recently, we have shown that co-culture or co-implantation of primary chondrocytes with expanded chondrocytes or mesenchymal stem cells enhanced cartilage tissue formation (14). In this study, we examined the capacity of various cell types (dermal fibroblasts, 3T3 feeder cells, embryonic stem cells and expanded chondrocytes) to enhance cartilage tissue when in co-culture with primary chondrocytes.

Interestingly, some of the growth factors and extracellular matrix components that stimulate chondrogenic metabolism or initiate chondrogenesis are produced and secreted by primary articular chondrocytes (15-18). However, it also has been shown that expanded chondrocytes as well as mesenchymal stem cells produce and excrete cytokines and growth factors (18),(19),(20). Furthermore, 3T3 cells are commonly used as a feeder cell line (21),(22) and are known to produce several cytokines, growth factors and extracellular matrix proteins supporting proliferation and maintenance of other cell types (23),(24). Finally, only few studies showed the chondrogenic differentiation capacity of dermal fibroblast (25),(26),(27). At the other side of the spectrum embryonic stem cells are able to form any tissue in the body and are considered totipotent (28),(29). We examined the potential to stimulate cartilage tissue formation of primary chondrocytes, dermal fibroblasts, 3T3 feeder cells, embryonic stem cells or expanded chondrocytes in micromass co-culture. Our hypotheses are that upon coculture of primary chondrocytes with other cell types cartilage tissue formation is enhanced either by 1. chondrogenic differentiation of other cell types or 2. enhanced cartilage tissue formation of primary chondrocytes upon interaction with other cell types.

Materials and Methods

Cell isolation & culture

The same batch of fetal bovine serum (FBS) was used for the culture of all cells and during micromass culture.

Chondrocytes

In co-culture experiments bovine primary chondrocytes (BPCs) were applied immediately after isolation. For isolation of these cells, full thickness articular cartilage was dissected from the patellar femoral groove of adult bovine. Human expanded chondrocytes (HECs) were obtained from full thickness cartilage dissected from knee biopsies of patient undergoing total knee replacement (# CARTD-K011) after obtaining consent from a local medical ethical committee. Dissected cartilage was incubated for 20-22 hrs in collagenase type II solution containing 0.15% collagenase (Worthington), Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The suspension was filtered through a 100 μm mesh nylon filter (cell strainer Nucleon) and cells were washed 2 times with phosphate buffered saline (PBS) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

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For expansion, HECs were plated at a density of $3.5 * 10^4$ cells per square cm and cultured in culture medium 1 (CM1) consisting of DMEM medium supplemented with 10% fetal bovine serum, 1x non-essential amino acids (Sigma-Aldrich), 10 mM HEPES buffer (Biowhitakker), 0.2 mM ascorbic acid 2-phosphate (Sigma-Aldrich), 0.4 mM proline (Sigma-Aldrich), 100 U/ml penicillin (InVitrogen) and 100 μg/ml streptomycin (InVitrogen). After cells were released with trypsin-EDTA and viable cells was counted with trypan blue staining and a Burker-Turk counting chamber. Cells were replated at a density of $3.5 * 10^4$ cells per square cm. After 2-3 passages expanded cells were mixed with primary chondrocytes for micromass culture.

Mouse 3T3 feeder cell line (3T3s)

After thawing, ATCC-3T3 feeder cells were plated at a density of $5*10^3$ cells per square cm and cultured in culture medium 2 (CM2) consisting of Alpha MEM (InVitrogen 22-571-038), 2 mM L-Glutamine (InVitrogen 25030-123), 1 mM Sodium pyruvate (Sigma Aldrich S8636) and 10% FBS. Medium was refreshed every 2-3 days and cells were subcultured for at least 1 additional passage after thawing as described in the "chondrocytes" section above. After a total of 3-4 passages, 3T3 feeder cells were mixed with primary chondrocytes for micromass culture

Human dermal fibroblasts (HDFs)

Human dermal fibroblasts were isolated from dermis of adult human breast tissue by enzymatic digestion. Isolation and culture protocols were adapted from Wang, HJ (30). Briefly, dermis was minced into small pieces and digested with 0.25% (w/v) collagenase

(Worthington) and 0.25% (w/v) dispase (Worthington) at 37°C for 2.5 hrs. After isolation cells were cultured in culture medium 3 (CM3) consisting of DMEM, 10 % Fetal Bovine Serum (FBS)(Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). Cultures were refreshed every 2-3 days and after 1 passage suspended in freezing medium containing 10% DMSO and 20% FBS and frozen in liquid nitrogen. After thawing, cells were seeded at a density of $5 * 10^3$ cells per square cm and cultured for two additional passages as described in the (chondrocytes" section above. After in total 3-4 passages, HDFs were mixed with primary chondrocytes for micromass culture.

Mouse ES cell culture

Murine ES cell line E14, sub clone IB10 was cultured as described previously (35). In brief, cells were plated at a density of 5000-10000 cells per square cm on gelatin-coated tissue culture flasks. Mouse ES cells were cultured in 50% mES proliferation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker) containing 4.5 mg/ml D-glucose, 10% fetal bovine serum (selected batch for mES cell culture, Greiner), 0.1 mM non-essential amino acids (NEAA, Sigma), 4 mM Lglutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 50% of Buffalo rat liver cell-conditioned mES proliferation medium (35). Prior to use 1000 U/ml Leukemia Inhibitory Factor (Esgro, Chemicon International) and 50 μM 2-mercapto-ethanol (Gibco) were added to the medium. Cells were grown at 37˚C in a humidified 5% CO2 incubator and passaged with 0.05% trypsin/EDTA before reaching confluence.

Micromass culture

100,000 primary chondrocytes were mixed with 400,000 human expanded chondrocytes, human dermal fibroblasts, mouse embryonic stem cells or mouse 3T3 feeder cells. $5 * 10^5$ cells were centrifuged at 500 g for 2 min in 3 ml of CM1 in a polypropylene Falcon centrifuge tubes to forma micromass. Micromass's were cultured in CM1 for 3 weeks and medium was refreshed every 3-5 days. Each experimental group (n=9) was further processed for histology, in situ hybridization, immunohistochemistry or quantitative biochemical analysis.

Histology

Micromass cultures were fixed with 1.5 % glutaraldehyde in cacodylate buffer (0.14 M / pH 7.2-7.4). Samples were washed in PBS, dehydrated and embedded in Paraffin. Sections (5 μm) were cut with a microtome, stained for sulphated Glycosaminoglycans (GAG) with safranin O, and counterstained with haematoxyline (Gill nr3) and fast green to visualize nuclei and cytoplasm respectively.

In Situ Hybridization

ISH was performed essentially as described previously (van der Eerden 1999). In short, micromass cultures were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS

and subsequently embedded in paraffin. Sections (6 μm) were cut with a microtome, deparaffinized and rehydrated. The sections were treated with proteinase K (Sigma-Aldrich) at 37°C (5 μg/ml, Boehringer) in Tris/HCL pH 8.0/ 50 mM EDTA and post fixed at room temperature (RT) with 4% paraformaldehyde for 5 minutes. After washing with PBS, endogenous alkaline phosphatase was removed applying 0.2 M HCl for 10 minutes at RT. The slides were acetylated with acetic anhydride in 0.1 M Triethanolamine (TEA) pH 8.0 and washed in 2 * sodium chloride/sodium citrate buffer (SSC). Sections were hybridized overnight at 60°C with 1μg/μl probe solution consisting of 1 μg/μl yeast tRNA, 50% formamid, 2 * SSC and 1 * Denhardt solution, 10% Dextrane sulphate. The next day, slides were washed twice in 2 * SSC, in 50% formamide/2 * SSC at 60°C and again in 2 * SSC. Sections were treated with RNase A solution (20 μg/ml) containing 10 mM TRIS pH 8.0, 0.5 mM NaCl and 1 mM EDTA for 30 minutes at 37°C to digest unbound probe. Slides were washed twice in 2 * SSC. To block aspecific antibody binding, sections were incubated for 30 minutes in blocking buffer containing 10 * Tris buffered Saline (TBS), 10% sheep serum and 0.3% Triton. Anti-DIG antibody (1:1250) was incubated overnight at 4°C in blocking buffer. The next day slides were washed 3 times in TBS-I and once in TBS-II (0.1 M TRIS-HCl pH 9.5, 0.1 M NaCl and 50 mM MgCl2). Slides were stained for 5 ½ hours with Nitrobluetetrasodium/ 5-bromo,4-chloro3-indolylphosphate (NBT/BCIP) solution (0.375 mg/ml / 0.188 mg/ml) containing 59 mg/ml polyvinyl alcohol (PVA), 75 mM NaCl, 75 mM TRIS-HCL pH 0.5, 0.19 M MgCl2 and 0.2 * TBS-II. Subsequently slides were washed in TRIS/EDTA buffer pH 8.0, TBS-I, counter stained with methyl green (2%) for 25 seconds and embedded in EUPARAL (BioQuip Products, Rancho Dominguez, CA, USA). TRIS, SSC and TBS buffers are made according to protocols described (31). Sequence of type I collagen and II probes were previously described by van de Eerden, B (32). In previous in situ hybridization experiments it was validated that probes detect type II collagen mRNA in human, bovine and mouse cells (data not shown). The amount of type II collagen expressing cells and the total amount of cells were determined in 5 ad random fields in ISH sections by visual quantification.

Immunohistochemistry

Micromass were embedded in OCT compound (Tissue-Tek) and immediately frozen at -80°C for immunostaining. Sections (5 μm) were cut with a cryotome and fixed with acetone for 10 min. Cryo-sections were stained overnight at 4°C for Type II collagen (1:100, DSHB # II-II6B3). Blocking was done with 10% human serum and, as a secondary antibody, goat anti-mouse (1:100, DAKO) was used. Staining was visualized with 3 diaminobenzidine (DAB)-solution (DAKO) for 10-20 minutes.

Specificity of Human specific MHC Class I antibody (1/100) was verified with human chondrocytes. this antibody did not cross react with bovine chondrocytes. The antibody was diluted in washing buffer (PBS containing 10% blocking buffer DAKO Cytomation X0909). Slides were preblocked in 100% blocking buffer for 1 hour and incubated with the

1st antibody overnight. The next day, slides were washed 3 times in washing buffer and incubated with the 2nd antibody goat anti-mouse (1:100, DAKO) for 1 hour. Slides were washed 3 times with PBS and staining was visualized with Fluorescent microscope.

Quantitive GAG- and DNA assay

Micromass for quantitative analysis of GAG's and cell number were washed with PBS and frozen o/n at -80°C. Subsequently they were digested with 1 mg/ml proteinase K (SIGMA-Aldrich) in Tris/EDTA buffer (pH 7.6) containing 18.5 μg/ml iodoacetamide and 1 μg/ml pepstatin A (SIGMA-Aldrich) for >16 hrs at 56°C. GAG content was spectrophotometrically determined with 9-dimethylmethylene blue chloride (DMMB, SIGMA-Aldrich) staining in PBE buffer (14.2 g/l Na2HPO4 and 3.72 g/l Na2EDTA, pH 6.5) with a micro plate reader (Bio-TEK instruments) at an absorbance of 520 nm. The standard curve for the GAG analysis was generated using chondroïtin sulfate A. Cell number was determined via quantification of total DNA with CyQuant DNA kit according to the manufacturer description (Molecular probes) and fluorescent plate reader (Perkin-Elmer). The standard curve for DNA analysis was generated with λ DNA provided with the CyQuant DNA kit.

Statistical analysis

Normality of the data was analyzed by determining skewness, which was -3 and 3 and showed a normal distribution all data groups. Data were analyzed for differences of the means with the ANOVA test and $p < 0.05$ as the criterion for statistical difference. When statistical differences were characterized by $p \leq 0.01$ this was indicated in the legend. Additionally in the legend of Figure 1, the lack of significant differences compared to the 100% primary chondrocyte group was indicated with addition of the accompanying p-value. Data are present as the mean with standard deviations.

Results

Medium conditioning during co-culture of primary chondrocytes with expanded chondrocytes?

Expanded chondrocytes (Figure 1A) and primary chondrocytes (Figure 1B) were cultured in a micromass assay without addition of any growth factors. Either a primary chondrocyte micromass were co- cultured in the same medium as a expanded chondrocyte micromass, without direct cell contact (Figure 1C) or primary chondrocytes and expanded chondrocytes are mixed and co-cultured in a micromass allowing direct cell-cell contact (Figure 1D). Results show that no GAG or type II collagen was found in expanded chondrocytes micromass in co-culture without cell contact, while type I collagen was detected (Figure 1C). When primary chondrocytes were mixed with expanded chondrocytes and cell contact allowed, intense GAG staining as well as type II collagen was shown to be present in the micromass (Figure 1D). Type I collagen was only detected

in the outer rim of the micromass, where cells also took a fibroblastic morphology (Figure 1B).

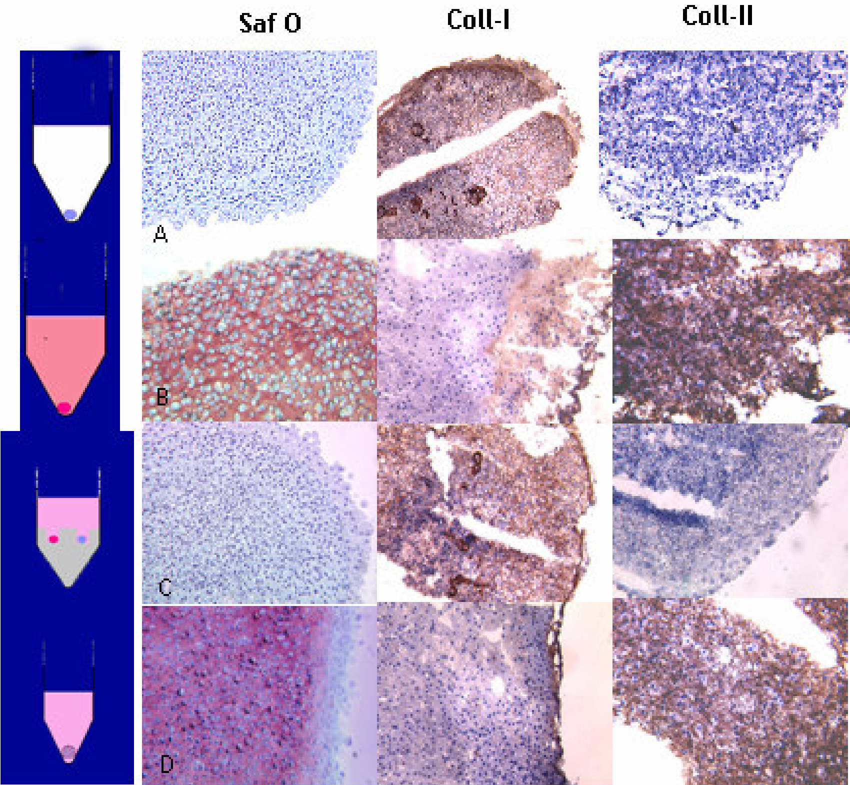


Figure 1 **Is medium conditioned when primary chondrocytes are co-culture with expanded chondrocytes?**
Primary and expanded chondrocytes were co-cultured either by mixing and formation of micromass cultures (D) or micromass cultures of each were cultured in the same medium without direct cell-cell contact (C). Controls are micromass cultures of expanded (A) and primary chondrocytes(B) (n= 3). All micromass cultures were analyzed for sulphated proteoglycans with safranin O (Saf O) and type I (Coll-I) and II collagen (Coll-II) with immunostaining.

Cartilaginous nature of tissue of co-cultured bovine primary chondrocytes (BPC) with human dermal fibroblasts (HDF), human expanded chondrocytes (HEC), mouse 3T3 feeders (M3T3) and mouse embryonic stem cells (MESC)

Micromass co-cultures of primary chondrocytes combined with different cell types were analyzed for cartilagenous tissue formation. Abundant safranin O staining was shown in 1:5 co-culture groups of primary chondrocytes combined with all cell types (Figure 2A). GAGs were absent in any of the control groups containing no primary chondrocytes (0:5) (Figure 2B) as shown by the lack of safranin O staining except for the MESC 0:5 control group (Figure 2-IIIB). That tissue generated in the 1:5 co-culture groups of all cell types was cartilaginous, is further exemplified by the presence of type II collagen in all co-

culture groups (Figure 2C). However, distriburion of type II collagen in sections of co-culture (1:5) groups was rather different between these experimental groups.

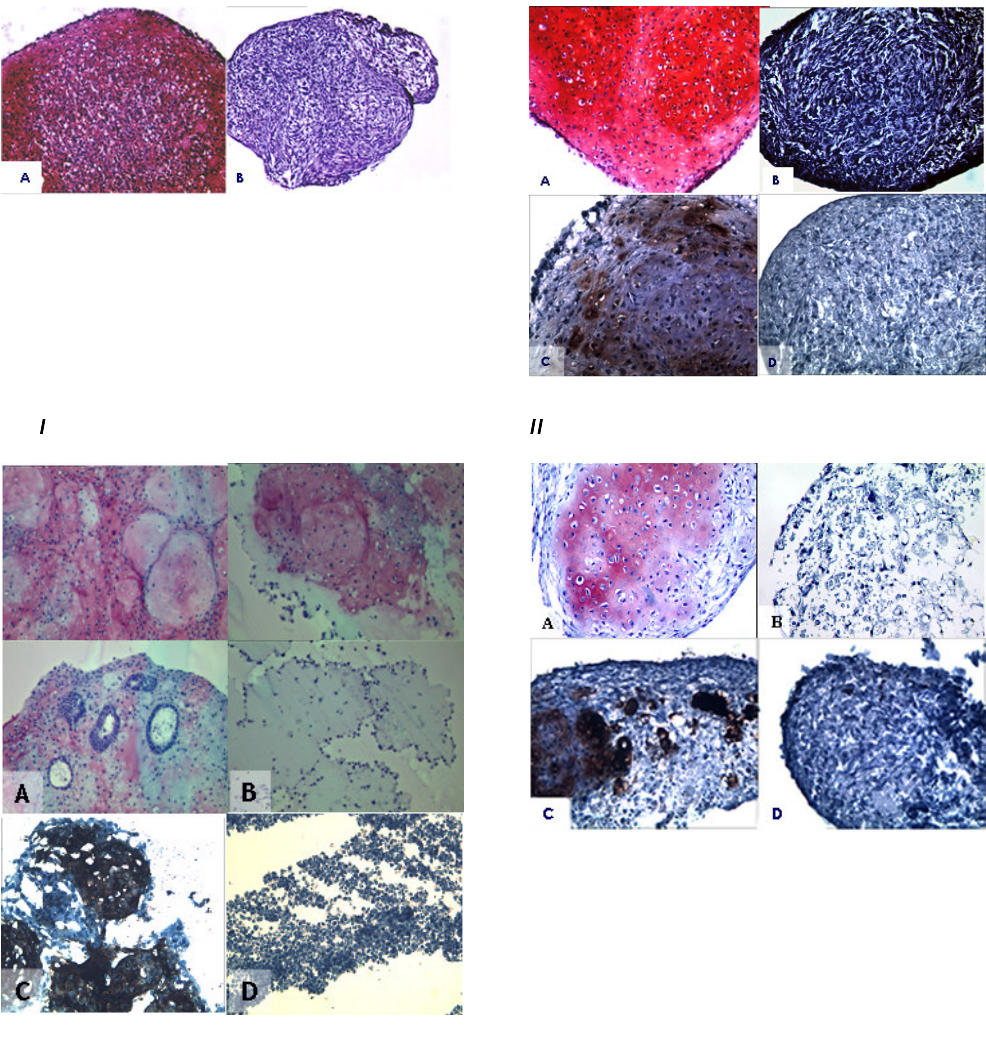


***Figure 2** Safranin O staining and type II collagen immuno staining of co-cultures combining bovine primary chondrocytes (BPC) with human expanded chondrocytes (HECs)(I), human dermal fibroblasts (HDF)(II), Mouse embryonic stem cells (MESC)(III) and Mouse 3T3 fibroblasts (M3T3)(IV).*

BPCs were co-cultured at a ratio 1:5 with HECs, HDF, MESCs and M3T3s in micromass culture for 4 weeks. Micromass cultures were analyzed for sulphated proteoglycan with safranin O staining (A,B) and type II collagen (C,D) with immunostaining. Micromass co-culture (1:5) groups are shown in A and C panels and control (0:5) in B and D panels. For co-cultures of BPCs with MESCs two pictures were shown to give a representative impression of the heterogeneity of tissues performed.

In the co-culture group with BPCs/HDFs, type II collagen was found throughout the micromass except for the outer rim (Figure 2 II-C).
In co-cultured BPCs/MESCs micromass cultures, type II collagen was preferentially found in the extensions at the periphery of the micromass (Figure 2 III-C). In the co-culture group combining BPCs and 3T3s, type II collagen was detected in intense stained patches throughout the micromass and again not in the outer rim of the micromass (Figure 2 IV-C). In the BPC/MESC co-culture group, histological analysis clearly indicated that besides cartilage, also other tissues were generated. Structures resembling vessels and columnar epithelia histological and in some areas were found together with groups of cells showeing differential organization (Figure 2 III-A). Surprisingly, these structures were not found in the control group (Figure 2 III-B). Previous data already showed the cartilaginous nature of tissue formed when BPCs were co-cultured with HECs (14).

Origin of cartilaginous extracellular matrix when BPCs are co-cultured with HDFs, M3T3s, MESCs and HECs.

In none of the 0:5 micromass control groups, type II collagen mRNA expressing cells could be found (data not shown). In BPC/HEC micromass co-cultures, 27.3 ± 11.3% of the cells were found to express type II collagen mRNA (Figure 3-IA). Type I collagen expressing cells were not present in BPC/HEC micromass co-cultures (Figure 3-IB). Interestingly, human specific antibody staining showed that expanded chondrocytes could be found throughout the micromass also in areas containing no type II collagen mRNA positive cells (Figure 3-IC).
In situ hybridization results showed that in the 1:5 co-culture group with HDFs, 24,0 ± 3,3% of the cells express type II collagen (Figure 3-IIA). Type I collagen mRNA was not expressed in BPC/HDF co-culture micromass cultures (Figure 3-IIB). Interestingly, antibody-staining specific for human cells showed co-localization of areas lacking dermal fibroblasts with intense safranin O stained areas. Thus, areas intensely stained for proteoglycans with safranin O were rich in primary bovine chondrocytes (Figure 3-IIC & E).
In Situ hybridization results of BPC/MESCs shown in Figure 3-IIIA, showed that specific areas in the micromass contain cells actively expressing type II collagen mRNA. Nearly all cells in these specific areas contribute to type II collagen production, whereas in the rest of the micromass, no type II collagen mRNA positive cells were found. In the BPC/MESC co-cultures, 30.8 % of the cells contributed to type II collagen mRNA expression. Type I collagen specific mRNA was detected in BPC/MESC micromass co-cultures also only in specific areas (Figure 3-IIIB). When BPCs were co-cultured with M3T3s, 16.3 ± 11.5 % of the cells actively contributed to type II collagen mRNA expression (Figure 3-IVA). Large standard deviation reflected that some areas contained a high number of type II collagen mRNA expressing cells and in some areas none of the cells expressed type I collagen mRNA. Results showed that type I collagen expressing cells can be found throughout the micromass but not in the outer rim and the middle of the micromass. Strikingly, the

pattern of type I collagen mRNA expressing cells in the BPC/3T3 micromass culture, creates a contrast picture of the type II collagen expression pattern (Figure 3-IVB).

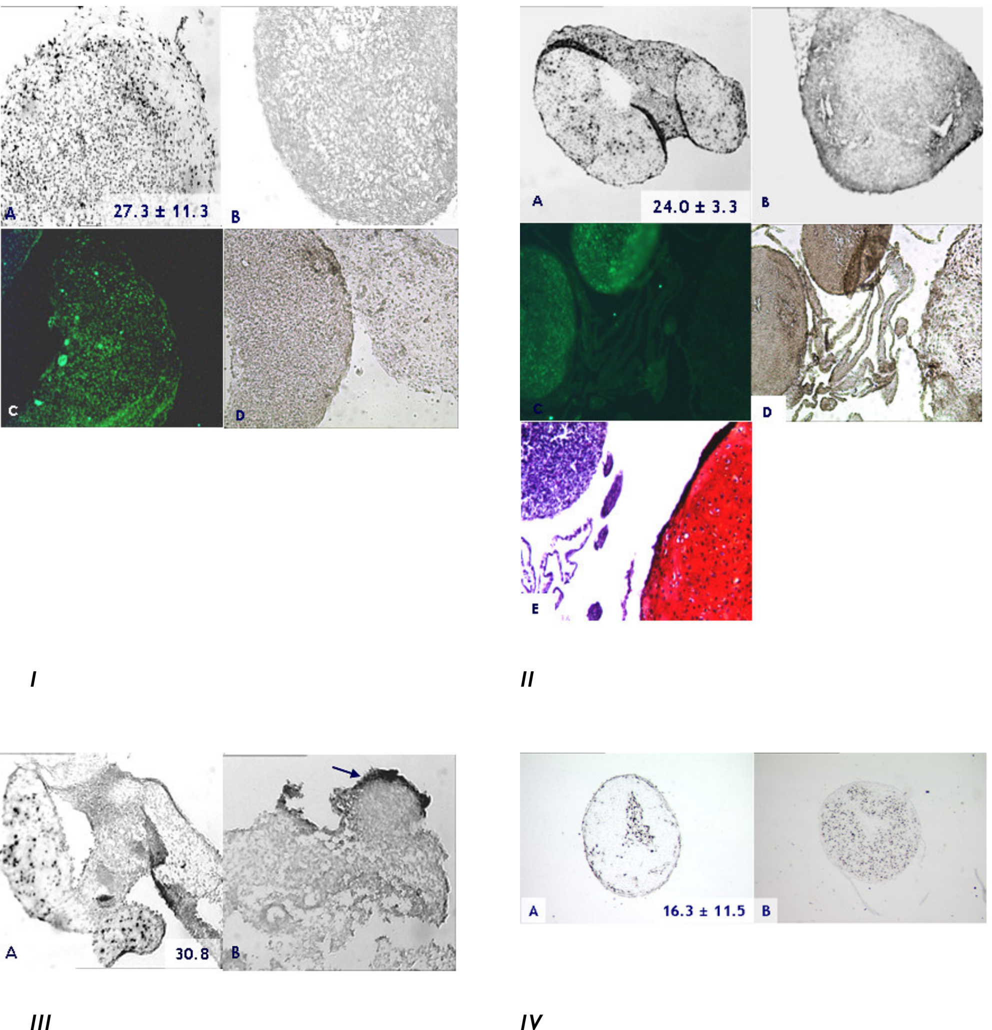


Figure 3 Type I collagen and I specific In Situ Hybridization and human cell specific antibody staining of co-cultures combining bovine primary chondrocytes (BPC) with human expanded chondrocytes (HECs)(I), human dermal fibroblasts (HDF)(II), Mouse embryonic stem cells (MESC)(III) and Mouse 3T3 fibroblasts (M3T3)(IV).

BPCs were co-cultured at a ratio 1:5 with HECs, HDF, MESCs and M3T3s in micromass culture for 4 weeks. Micromass cultures were analyzed type I collagen (A) and I (B) mRNA expressing cells with In Situ Hybridization. Micromass co-cultures containing cell types of human origin were analyzed with a fluorescent human cell specific antibody (C), corresponding light microscopy picture is shown in panels (D) and corresponding safranin O staining for BPC/HDF (II) micromass co-cultures is shown in panel (E).

Enhanced tissue formation of co-cultured BPCs with HDFs, M3T3s, MESCs and HECs

For this study, 100,000 primary chondrocytes were co-cultured with 400,000 expanded chondrocytes, dermal fibroblasts, 3T3 feeder cells and embryonic stem cells, respectively. GAG per initial primary chondrocyte enhanced 5.6, 5.0, 3.1 and 3.8 fold respectively, compared to the 100% BPC group (Figure 4A and B). Moreover, the total amount of GAG was not significantly different from the 100% primary chondrocyte group, when primary chondrocytes were co-cultured with human dermal fibroblasts and even significantly higher when co-cultured with expanded chondrocytes (Figure 4A). GAG produced in the BPC/3T3 1:5 group, was 0.6 fold the amount and the BPC/MESC was 0.8 fold of the 100% primary chondrocyte group. For all co-cultured groups, the amount of GAG is significantly higher than their respective 0:5 controls (Figure 4A). According to the DNA results, the amount of cells in the BPC/HDF, BPC/MESC and BPC/M3T3 experimental groups (1:5) was significantly higher then 0:5 control group, (Figure 4B). The amount of DNA in the HEC 0:5 control group was not significantly different from the BPC/HEC co-culture group. The amount of GAG/DNA was not significantly different in the 1:5 co-culture groups compared to the 0:5 control group for HDFs, 3T3s and MESCs. While the amount of GAG/DNA was significantly higher in the 1:5 BPC/HEC group compared to the 0:5 control group (Figure 4B).

Discussion and Conclusions

Medium conditioning

ECs were co-culture with PCs without cell-cell contact in micromass cultures while sharing their medium (Figure 1C). Expanded chondrocytes showed no cartilage tissue formation as apparent from lack of safranin O and type II collagen staining. When expanded chondrocytes were mixed with primary chondrocytes 1:1 cartilagenous tissue formation was shown by intense GAG and type II collagen staining (Figure 1D). From these results, it is evident that medium is not conditioned in a biological relevant manner as shown by the lack of cartilage tissue formation by ECs when in co-culture with primary chondrocytes in this model. Which cell type contributes to cartilage tissue formation in micromass co-cultures of primary chondrocytes with a variety of cell types was examined in following experiments.

Cartilage tissue formation when co-culturing BPCs with HDFs, M3T3s, MESCs and HECs

Results showed cartilage tissue formation, when primary chondrocytes were co-cultured with multipotent cells such as bone marrow mesenchymal stem cells or embryonic stem cells. Cartilage tissue formation was also generated, when primary chondrocytes were co-

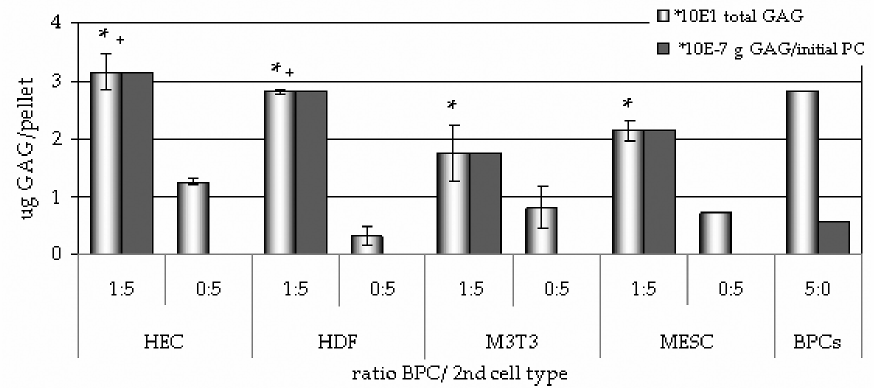
cultured with cell populations such as 3T3 feeder cells and dermal fibroblasts. Previously, the chondrogenic potential of expanded chondrocytes, embryonic stem cells as well as dermal fibroblasts has extensively been shown (16),(25),(33). Most of these experiment comprised 3D culture and some chondrogenic stimulation in the form of growth factors and/or extracellular matrix proteins. Results shown here indicate that cartilage tissue was formed when primary chondrocytes were co-cultured not only with cell types shown to have chondrogenic capacity with expanded chondrocytes, dermal fibroblasts and embryonic stem cells and 3T3 feeder cells. To the best of our knowledge 3T3 feeder cells never showed to hold chondrogenic differentiation capacity.

Previously it was shown that when bone marrow mesenchymal stem cells were co-cultured with primary cell types from different tissues, showed that mesenchymal stem cells differentiated into the same lineage as the cell type they were co-cultured with (34),(35),(36),(37). In stead, other co-culture studies with BM-MSCs and primary cells from adult tissues showed that the mesenchymal stem cells support the tissue specific physiology, possibly by release of trophoblastic factors (38),(39),(19). When embryonic stem cells were combined with differentiated cells from embryonic origin, the embryonic stem cells differentiated into the same lineage as the cell type they were co-cultured with (40). Next, in co-culture 3T3 feeder cells only have shown to support the physiology of the cell type they are co-cultured with (41),(42),(43). Finally, in co-culture experiments with dermal fibroblasts and keratinocytes for skin tissue engineering they showed to support each others physiology (23),(44),(45). In the following paragraph it was determined which cell type in micromass co-culture contributes to cartilage tissue formation.

Origin of cartilaginous extracellular matrix when BPCs are co-cultured with HDFs, M3T3s, MESCs and HECs.

In situ hybridization results showed that when bovine primary chondrocytes were co-cultured (1:5 ratio) with human dermal fibroblasts, mouse 3T3 feeder cells, mouse embryonic stem cells or human expanded chondrocytes, 24 ± 3%, 16 ± 11%, 30% and 27 ± 11% of the total amount of cells respectively, contributed to type II collagen mRNA expression (Figure 3A). The percentage of cells contributing to cartilage tissue formation is approximately the same as the initial percentage of primary chondrocytes (20%) in micromass cultures. This suggests that in all co-culture groups, only primary chondrocytes contribute to cartilage specific matrix production. Moreover, human cell specific antibody staining of BPC/HDF micromass co-cultures showed that some areas rich in glycosamino-glycans (safranin O) contained only bovine primary chondrocytes (Figure 3 II C&E). This confirms that in this co-culture model, primary chondrocytes were the cells contributing to cartilage tissue formation when combined with human dermal fibroblasts.

In the experiments described here, we did not specifically examine other forms of tissue formation. Type I collagen specific ISH result showed absence of dermal specific markers after micromass co-culture with primary chondrocytes as well as in controls.



A

Cells cocultured	Ratio cells (day 0)	Total GAG	Total DNA	(µg GAG/ initial % BPC
	%/%	µg	µg	
BPC/HDF	1:5	28.2 ± 3.1	29.40 ± 3.95	1.58
	0:5	3.1 ± 0.7	3.46 ± 0.58	
BPC/M3T3	1:5	17.5 ± 0.4	19.58 ± 2.20	1.41
	0:5	8.1 ± 1.6	13.71 ± 2.54	
BPC/MESC	1:5	21.5 ± 4.8	41.57 ± 3.74	0.88
	0:5	7.2 ± 3.5	7.45 ± 0.75	
BPC/HEC	1:5	31.5 ± 0.4	20.32 ± 4.06	1.07
	0:5	12.5 ± 1.9	22.32 ± 4.36	
BPC	5:0	28.1 ± 1.7	34.16 ± 6.88	0.28

B

Figure 4 Total GAG, DNA and GAG/initial % primary chondrocytes in micromass co-cultures combining bovine primary chondrocytes (BPC) with human expanded chondrocytes (HEC), human dermal fibroblasts (HDF), Mouse embryonic stem cells (MESC) Mouse 3T3 fibroblasts (M3T3).
BPCs were co-cultured at a ratio 1:5 with HECs, HDF, MESCs and M3T3s in micromass culture for 4 weeks. Micromass cultures were analyzed for total GAG, total DNA.
A. Graph depicts total GAG (µg) per micromass culture for all groups and ·10-7g GAG per initial percentage of primary chondrocyte for co-cultured groups (1:5 ratio).
*B. Table shows total GAG (µg), total DNA (µg) for all groups and GAG per primary chondrocytes (·10-7 g GAG/initial % PC) for co-cultured groups and BPC control group (5:0) . * = significantly different from 0:5 control, + = not significantly different from 5:0 control (100% primary chondrocytes)(p<0.05).*

in micromass co-culture group with embryonic stem cells, besides cartilage, also other tissues were formed as indicated by histology (Figure 2 III). Unexpectedly, the diversity of tissues formed in the co-culture group was not found in the control group consisting of only embryonic stem cells (Figure 2 III-B), suggesting that the co-culture conditions favor differentiation of embryonic stem cells. Co-culture of MESCs with primary chondrocytes might direct this increased differentiation ability into the cartilaginous lineage, but tissue formation into other lineages was also enhanced. Embryonic stem cells are totipotent and in culture are kept on leukaemia inhibitory factor (LIF) to maintain their undifferentiated state. When LIF was subtracted from the medium, MESCs form nodules and start differentiating into a variety of lineages (46),(47). The 0:5 MESC control group also proved to contain some cartilage specific extracellular matrix proteins as shown by the presence of sulphated proteoglycans in the embryonic stem cell Figure 4-I). However, in contrast to the safranin O results, no type II collagen protein was detected in the 0:5 control group; indicating that the tissue formed was not truly cartilaginous (Figure 4-I).
Upon BPC/HEC co-culture, ISH results suggest also that the proportion primary chondrocytes remain the same during co-culture. This is in line with findings from Tsuchiya (8) wherein they showed that in micromass co-cultures of chondrocytes with bone marrow mesenchymal stem cells the proportion of the 2 cell types remained the same during culture.

Quantification of cartilage tissue formation by primary chondrocytes in co-culture with HECs, HDFs, MESCs and M3T3s

Finally, quantitive GAG data showed that the total amount of GAG matched or was 0.6 fold the total amount of GAG produced by 100% primary chondrocyte group (5:0) in micromass co-culture groups. As suggested by ISH results and specie specific antibody staining, only primary chondrocytes contribute to cartilage tissue formation. When the amount of GAG produced by primary chondrocyte initially present is calculateds, it is apparent that GAG production of primary chondrocytes enhanced upon co-culture with expanded chondrocytes, dermal fibroblasts, embryonic stem cells and 3T3 feeder cells.
Enhanced cartilage tissue formation of primary chondrocytes possibly is mediated by HECs, HDFs, MESCs and 3T3s via direct cellular interactions or via common molecules secreted by the 2nd cell type in co-culture.

In summary, these results showed that primary chondrocytes enhance their cartilage tissue formation, when co-cultured with any cell type that can facilitate this, possibly through very similar mechanisms. In a review by Caplan A. (2006) (19), it was discussed that bone marrow mesenchymal stem cells not only are totipotent but also support proliferation, differentiation or physiology of other cell types. Results shown here suggest that mesenchymal stem cells might not be the only cell type that mediates differentiation of support cell type specific physiology via release of trophoblastic factors. We compared

the influence of cell types on each other's physiology and differentiation in many different co-culture studies. Results in these studies showed a whole range of the influence of cellular interaction during coculture, namely; 1. cell types supporting physiology or differentiation of one another, 2. cell type 1 supporting differentiation or physiology of cell type 2, 3. one cell type (trans) differentiating towards the lineage of the cell type it is co-cultured with, 4. one cell type differentiates into a tissue specific lineage which is different from the cell that initiates or enhances the differentiation or 5. cell type 1 inhibiting terminal differentiation of cell type 2 (5),(7),(12),(34),(35),(48),(49),(50),(51). No correlation could be found between these data and differentiation status of cell types, differences in species origin of cell types or wether direct cell-cell contact was established or not. Further studies are required to clarify through which mechanisms primary chondrocytes enhance their cartilage tissue formation in micromass co-culture. More generally, these results raise the following questions; 1. when is cell identity sustained in vivo and 2. when do multipotent cells adapt the identity of their surrounding cells?

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Chapter 5

Co-culture or co-implantation of primary chondrocytes with expanded chondrocytes or bone marrow mesenchymal stem cells enhances cartilage tissue formation; a powerful tool in cartilage cell therapy

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Chapter 5

5

Abstract

In this study, the potential of primary articular chondrocytes to enhance matrix production, when mixed with expanded chondrocytes or mesenchymal stem cells in vitro and in vivo was examined. Primary chondrocytes were mixed with expanded chondrocytes at different ratios and either co-cultured in a micromass model or co-implanted in nude mice after seeding onto 3D-fiber deposited PEGT/PBT block co-polymer scaffolds. Tissue generated was analyzed for glycosaminoglycans (GAG), relative amount of cells and collagen type I, II and IX. The amount of GAG produced per initially seeded primary cell was enhanced both the in vitro and in vivo studies. Surprisingly, this was further elevated as the proportion of expanded chondrocytes or mesenchymal stem cells in culture increased. GAG per initially seeded primary chondrocyte increased up to 17 fold when as little as 2% of primary chondrocytes were co-implanted with mesenchymal stem cells. Moreover, total GAG per construct matched that produced by 100% primary chondrocytes, when down to 10% of primary chondrocytes when co-cultured with mesenchymal stem cells. All histological and Immunohistochemical data on GAGs and type II collagen show a close correlation with quantitative GAG data. These results demonstrate that primary chondrocytes combined with expanded chondrocytes or mesenchymal stem cells enhance cartilage specific matrix production in micromass culture in vitro, as well as in scaffolds in vivo. These findings provide the basis for development of a novel cell therapy intended for in situ cartilage tissue engineering.

Introduction

Expanded articular chondrocytes, as well as mesenchymal stem cells from for example bone marrow (MSCs) are considered suitable for tissue engineering or cell therapy for cartilage defect repair (1-4). Clinically, only small cartilage biopsies are available for primary chondrocyte isolation. These cells can be expanded in culture in order to acquire an amount of cells suitable for tissue engineering or cell therapy (5-8). Although during expansion chondrocytes lose their chondrogenic characteristics, given the right conditions they maintain their capacity to differentiate (9),(10). Furthermore, the multipotency of MSCs includes the capacity to differentiate into the chondrogenic lineage (4),(11-13). In general, (re)differentiation of expanded chondrocytes or mesenchymal stem cells is

initiated by culturing at high cell-density in the presence of chondrogenic growth factors (CGFs) (14). Culturing and differentiating multipotent cells for clinical purposes would require large amounts of chondrogenic growth factors, which are costly. Additionally, it remains to be clarified which combination of growth factors, applied in which sequence, will lead to formation of cartilage tissue resembling the in vivo equivalent (14),(15). Interestingly, primary articular chondrocytes produce and excrete some of the cytokines, growth factors and extra cellular matrix components that stimulate chondrogenic metabolism or initiate chondrogenesis (16),(17),(18),(19). However, the limited amount of extra cellular matrix and primary cells in the surrounding tissue or in the cartilage wound site is apparently not sufficient to initiate an appropriate repair reaction (1),(23),(24),(25). Possibly the limited repair reaction could also caused by the absence of direct cell-cell interaction between cell residing in the surrounding tissue of the defect. Additionally, expanded chondrocytes as well as mesenchymal stem cells produce and excrete cytokines and growth factors involved in paracrine and autocrine signalling (20),(21),(22).

We hypothesized that, when primary chondrocytes are in direct contact with expanded chondrocytes or mesenchymal stem cells this would result in enhanced cartilage tissue formation. We examined whether the amount of primary chondrocytes necessary to initiate cartilage tissue formation could be decreased. Consequently, this would enable applying this co-culture or co-implantation model in tissue engineering of cell therapy and reduce or eliminate the need for growth factors. Firstly, cartilage matrix production by a combination of freshly isolated bovine chondrocytes (primary chondrocytes) and bovine expanded chondrocytes was studied, in a micromass co-culture model. Subsequently, human primary chondrocytes were co-cultured with human mesenchymal stem cells. Finally, cartilagenous matrix production after co-implantation of primary chondrocytes with either expanded chondrocytes or mesenchymal stem cells on poly (ethylene glycol)-terephthalate-poly (butylene terephthalate (PEGT/PBT)) scaffolds was examined. To verify the cartilagenous nature of the tissue formed, for sulphated proteoglycans using safranin O staining and collagen type-II and IX with immunohistochemistry was determined.

Materials and Methods

Cell isolation & culture

In this paper, primary chondrocytes are referred to when chondrocytes were applied immediately after isolation. For bovine chondrocytes, full thickness articular cartilage was dissected from the patellar femoral groove of adult bovine femora. After agreements with the local ethical committee, human chondrocytes were released from full thickness cartilage knee biopsies from (CARTD-K011). The cartilage biopsy used for the experiment

described in Figure 2 was derived from the femur of a 75–year old male undergoing a total knee procedure. Dissected cartilage was incubated for 20-22 hours in collagenase type II solution containing 0.15% collagenase (Worthington), Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The suspension was filtered through a 100μm mesh nylon filter (cell strainer Nucleon) and cells were washed 2 times with PBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). For expansion, chondrocytes were plated at a density of $3.5 * 10^4$ cells per square cm and cultured in culture medium 1 (CM1) containing DMEM medium supplemented with 10 % fetal bovine serum, 1x non- essential amino acids (Sigma-Aldrich), 10 mM HEPES buffer (Biowhitakker), 0.2 mM Ascorbic acid 2-phosphate (InVitrogen), 0.4 mM proline (Sigma-Aldrich) , 100U/ml penicillin (InVitrogen) and 100 μg/ml streptomycin (InVitrogen). After cells were released with trypsin-EDTA treatment, the amount of viable cells was determined with trypan blue staining and a Burker-Turk counting chamber and plated again at a density of $3.5 * 10^4$ cells/cm2. After 2-3 passages, cells were mixed with primary chondrocytes for either micromass culture or seeding onto porous scaffolds.
After informed consent was obtained from the patients and in agreement with the local ethical committee, human bone marrow was collected in heparin tubes. For the experiment described in Figure 2 a bone marrow biopsy was derived from the acetabulum of a 50-year old woman undergoing a total hip replacement. For the experiment described in Figure 4, a bone marrow biopsy was derived from the acetabulum of a 66-year old female undergoing a total hip replacement. Whole bone marrow was seeded in tissue culture flasks and cell attachment was allowed for approximately 7 days. Attached cells were subsequently cultured for 2-3 passages in culture medium 2 (CM2) containing αMEM supplemented with 10 % serum, 0.2 mM Ascorbic acid 2-phosphate (InVitrogen), 100 U/ml penicillin (InVitrogen) and 100 μg/ml streptomycin (InVitrogen).

Micromass culture

Either $5 * 10^5$ (bovine) or $2.5 * 10^5$ (human) cells were centrifuged at 500 g for 2 minutes in 3 ml of CM1 in a polypropylene Falcon centrifuge tube. Micromass cultures were cultured in CM1 and medium was refreshed every 3-5 days. Micromass cultures were cultured for 2 weeks for experiments with expanded chondrocytes and for 4 weeks with mesenchymal stem cells. This was based on previous differentiation experiments with these cell populations. Per experimental group, 9 micromass cultures were cultured separately. Micromass cultures were further processed for histology, immunohistochemistry or quantitative biochemical analysis.

Implantation in nude mice

Since availability of healthy human donor cartilage is limited, primary chondrocytes from bovine cartilage of the femoral condyle were used. Prior experiments established that

during co-culture, cells from different species interact and influence each others behavior similarly as cells from the same specie (26),(27).

Scaffold fabrication

Poly (ethylene glycol)-terephthalate-poly (butylene terephthalate (Mw PEG)/ (w/w PEGT/PBT) 300/55/45 co-polymers were deposited with 3D fiber deposition technique, yielding a porous scaffold. Molten PEGT/PBT fibres were successively layered in a 0-90 pattern from a 250um nozzle onto a computer aided x/y/z table with a layer thickness of 0.15 mm and fiber spacing of 0.6 mm as described in previous publications (28). Cylindrical scaffolds (∅4 x 4 mm) with constant pore size and 100% interconnecting pore volume were cored and sterilized in isopropyl alcohol.

Seeding & culturing on PEGT/PBT Scaffold

Cylindrical scaffolds were extensively rinsed in phosphate buffered saline (PBS), incubated overnight in CM1 and blotted dry prior to seeding. Primary and expanded chondrocytes were mixed, centrifuged at 300 g for 5 minutes and re-suspended in 54 μl of CM1 containing 300 μg/ml fibronectin. Scaffolds were seeded statically with 3 x 10^6 cells per scaffold for 1 day in CM1 and implanted subcutaneously in nude mice.

Implantation

Nude mice studies were performed after consent of the Ethical Committee for Animal studies (#DEC-GDL 102566 Utrecht, the Netherlands). Six week old nude mice (mouse strain HdCpb:NMRI-nu Harlan, the Netherlands) were anaesthetized with 0.02 ml of a 3.5 : 3 : 1 mixture of ketamine (100 μg/ml): xylazine (20 μg/ml): atropine (0.5 mg/ml). After swabbing the skin with 70% ethanol, subcutaneous pockets were made in the posterior-lateral side of the back, pockets were provided with a construct and the skin sutured. Per experimental group 9 constructs were implanted randomly implanted in the mice, with 4 implants maximal per mouse. After 4 weeks, mice were sacrificed by cervical dislocation. The constructs were then removed and further processed for histology, immunohistochemistry and biochemical analyses.

Analysis

Histology

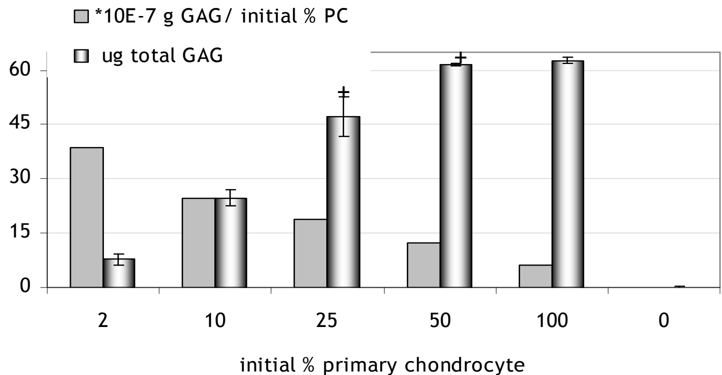
Micromass cultures (n=3) or constructs (n=3) were fixed with 1.5 % glutaraldehyde in cacodylate buffer (0,14 M / pH 7.2-7.4). Samples were washed in PBS, dehydrated and embedded in Glycol Methyl Acrylate (GMA). samples were cut with a microtome to yield 5μm thick sections. Sections were stained for sulphated Glycosaminoglycans (GAG) with safranin O and counterstained with haematoxylin (Gillnr3) and fast green respectively for nuclei and cytoplasm.

Immunohistochemistry

Micromass cultures (n=3) or constructs (n=3) were embedded and immediately frozen to –80°C in OCT compound (Tissue-Tek) for immunostaining. Sections were cut with a cryo-microtome to a thickness of 5 μm and fixated with acetone for 10 minutes. Cryo-sections were stained for Collagen type-II (1:100, DSHB II-II6B3), -IX (1:100, DSHB D1-9) and -I (1:1000, Ab-1, Calbiochem). Blocking was achieved with 10% human serum and as a secondary antibody; goat anti-mouse (1:100, DAKO) was used. Staining was visualized with 3, 3 diaminobenzidine (DAB)-solution (DAKO) for 10-20 minutes.

Quantative GAG- and DNA assay

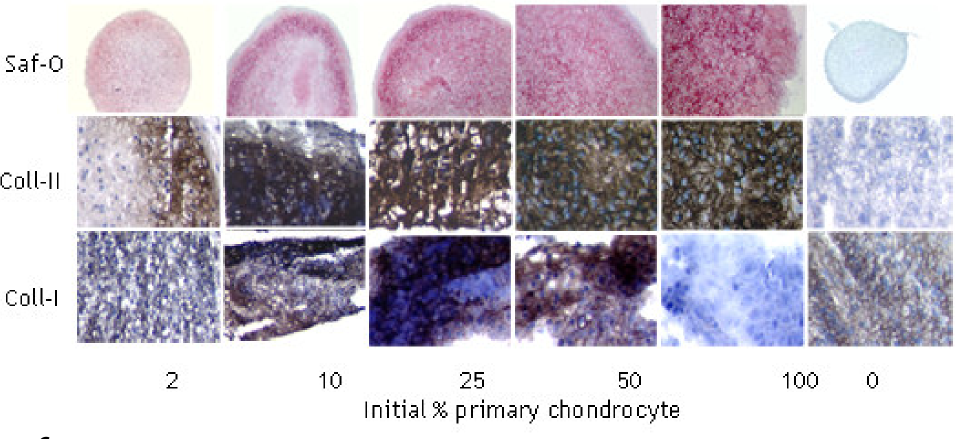
Micromass cultures (n=3) or constructs (n=6) for quantitative analysis of GAG's and cell number were washed with PBS and frozen overnight at -80°C. Subsequently they were digested with 1 mg/ml proteinase K (Sigma) in Tris/EDTA buffer (pH7.6) containing
18.5 μg/ml iodoacetamide and 1 μg/ml pepstatin A (SIGMA-Aldrich) for >16 hrs at 56°C. GAG content was spectrophotometrically determined with 1,9-dimethylmethylene blue chloride (DMMB) (Sigma-Aldrich) staining in PBE buffer (14.2 g/l Na_2HPO_4 and



A

PC / EC	total GAG	Total DNA	GAG/DNA	GAG/initial % PC
(%/%)	(μg)	(μg)	(μg/μg)	(·10^{-7} g)
0 / 100	0.00 ± 0.2	2.8 ± 0.1	-	-
2 / 98	7.7 ± 1.5	4.6 ± 0.5	1.7 ± 0.4	38.5
10 / 90	24.7 ± 2.3	4.6 ± 0.2	5.4 ± 0.9	24.7
25 / 75	47.2 ± 5.6	6.4 ± 0.1	7.4 ± 0.1	18.9
50 / 50	61.5 ± 0.4	8.6 ± 0.2	7.1 ± 0.2	12.1
100 / 0	62.7 ± 1.0	10.0 ± 0.3	6.3 ± 0.2	6.3

B



C

Figure 1 micromass co-culture of bovine primary chondrocytes (PCs) bovine expanded chondrocytes (ECs) at different ratios (percentage / percentage n/n cells).
Primary chondrocytes (PC) were mixed with expanded chondrocytes (EC) at indicated percentages and cultured for 14 days in micromass assay. (A) Graph shows total amount of GAG per micromass (μg) and amount of GAG per initial percentage primary cells ($\cdot 10^{-7}$ g GAG/initial percentage PC). (B)Table shows total amount of GAG (μg), total amount of DNA(μg), μg GAG/μg DNA and amount of GAG/ initial percentage of primary chondrocytes ($\cdot 10^{-7}$ g GAG/initial % PC) (C) pictures show Safranin O staining for sulphated GAG's (Saf O) and immunohistochemical staining for type II collagen (Coll-II) and I (Coll-I). + not significantly different from 100% PC control group $p \geq 0.06$. All groups are significantly different from 100% EC control group.

3.72 g/l Na_2EDTA, pH 6.5) using a micro plate reader (Bio-TEK instruments) at an absorbance of 520 nm with chondroïtin sulfate as a standard. Cell number was determined by quantification of total DNA using a CyQuant DNA kit, performed according to the manufacturer's instructions (Molecular probes) and a fluorescent plate reader (Perkin-Elmer). Quantitive total GAG and total DNA data were normalized for differences in wet weight of the scaffolds.

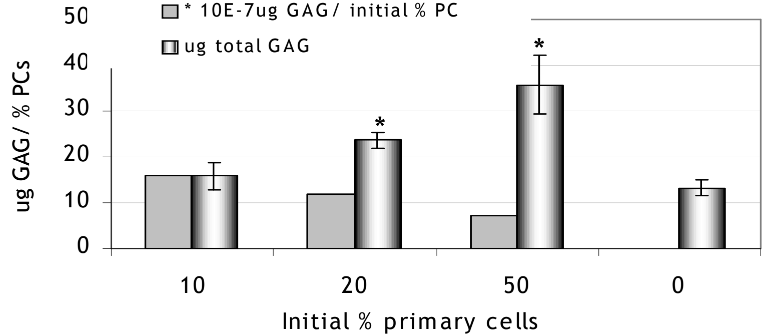
Statistical analysis

Normality of the data was analyzed by determining skewness being between -3 and 3 and showed a normal distribution of the data in all groups. Data were analyzed for differences of the means with the ANOVA test and $p < 0.05$ as the criterion for statistical difference. When statistical differences were characterized by $p \leq 0.01$, this is indicated in the legend. Additionally, the lack of significant differences compared to the 100% primary chondrocyte group is indicated in the legend with addition of the accompanying p value.

Results

Co-culture of bovine primary chondrocytes with expanded chondrocytes

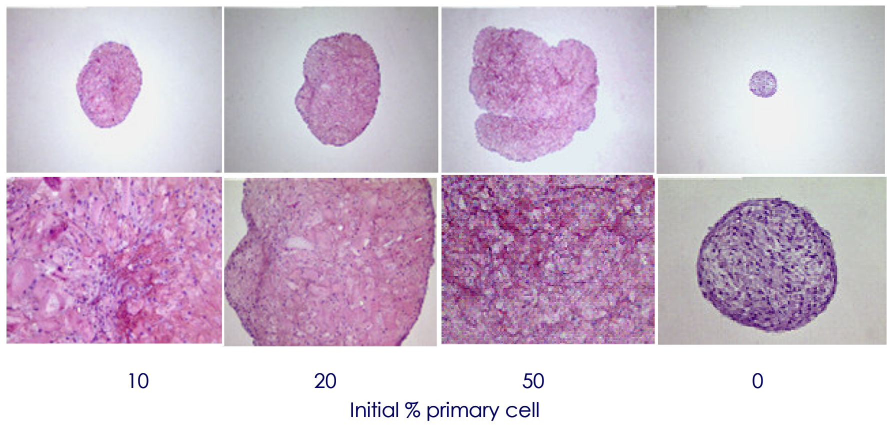
Bovine primary chondrocytes (2%, 10%, 25% and 50%) were co-cultured with bovine expanded chondrocytes for 2 weeks in micromass. The negative control groups contained 0% primary chondrocytes and thus 100% expanded chondrocytes and the positive control contained 100% primary chondrocytes. With decreasing percentages of primary chondrocytes initially present in micromass cultures, the amount of GAG per initially seeded primary chondrocyte continuously increased up to 6 fold more for the 2% group, compared to the 100% primary chondrocyte control group (Figure 1A). The total amount of GAG per micromass for experimental groups initially containing 25 % or 50 % primary chondrocytes was not significantly different from the 100% primary chondrocyte control group. Moreover, it was demonstrated that GAG/DNA in the 25% group was not significantly different and even significantly higher for the 50% group, compared to the 100% primary chondrocyte group. Total DNA increased approximately 2 fold with increasing initial percentage of primary chondrocytes (Figure 1B). All experimental groups, including the 100% primary chondrocyte group formed tissue containing sulphated proteoglycans and collagen type-II, as shown by safranin O staining and immunohisto-chemistry (Figure 1C).



A

PC / MSC	Total GAG	Total DNA	GAG/DNA	GAG/initial % PC
(%/%)	(μg)	(μg)	(μg/μg)	$(\cdot 10^{-7}$ g)
0 / 100	13.2 ± 1.6	0 ± 0.01	-	-
10 / 90	15.8 ± 2.9	0.13 ± 0.01	108.4 ± 37.7	15.8
20 / 80	23.6 ± 1.7	0.45 ± 0.07	55.4 ± 16.0	11.8
50 / 50	35.7 ± 6.5	0.70 ± 0.09	50.5 ± 1.2	7.1

B



C

Figure 2 ***micromass co-cultures of human primary chondrocytes (PCs) and human mesenchymal stem cells from bone marrow(MSCs) different percentages (% / %) cells.***
Primary chondrocytes (PCs) were mixed with mesenchymal stem cells (MSCs) at indicated percentages and cultured for 28 days in micromass assay. (A) Graph shows total amount of GAG (μg) and amount of GAG per initial percentage of primary cells ($\cdot 10^{-7}$ g). (B) Table shows total amount of GAG(μg), total amount of DNA (μg), amount of GAG/DNA(μg/μg) and GAG per initial percentage of primary chondr-ocytes($\cdot 10^{-7}$ g). (C) Pictures show Safranin O staining for sulphated GAG's. Pictures are an overview of whole micromass cultures (20x) and detailed (200x).
** significantly different from 100% MSC control group $p < 0.01$.*

Histology of micromass cultures showed cartilagenous morphology indicated by lacunae formation around the cells and cells adapted a spherical morphology (Figure 1C). The control group with 100% expanded chondrocytes lacked proteoglycans and type II collagen and contained collagen type I. Experimental groups stained positive for collagen type I, however the intensity decreased with decreasing amounts of primary chondrocytes (Figure1).

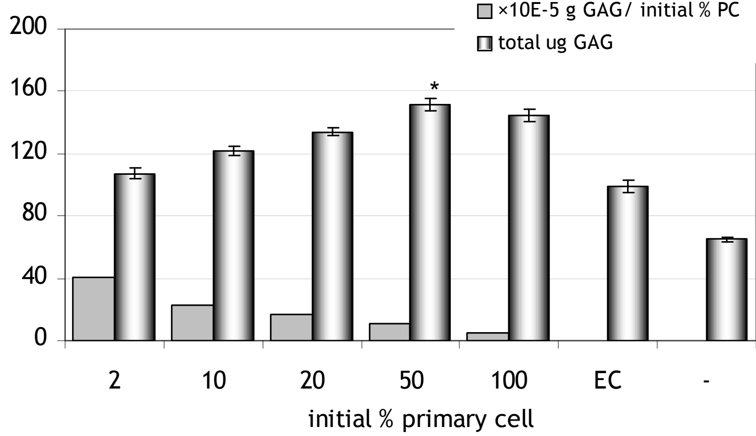
Co-culture of human primary chondrocytes with bone marrow mesenchymal stem cells

The limited amount of primary chondrocytes that can be made available from a single human cartilage biopsy restricted the design of this experiment. Therefore, it was not possible to include a primary chondrocyte control group. In this experiment, 10%, 25% and 50% human primary chondrocytes were co-cultured with human bone marrow mesenchymal stem cells in micromass cultures for 4 weeks. The control group contained 0% primary chondrocytes and thus 100% mesenchymal stem cells. Again, a trend was shown for GAG per initially percentage of primary chondrocytes to increase with decreasing percentages of initial primary chondrocyte (Figure 2A). micromass cultures with the 100 % mesenchymal stem cell group did not yield a signal with the DNA assay, whereas in all experimental groups, DNA could be measured. However, from histological

analysis, it was clear that in the 100% MSC group, a small micromass was formed containing viable cells. Whereas all experimental groups showed, abundant safranin O staining, indicating cartilage specific GAG formation, in the 100% MSC group no sulphated proteoglycans detected with safranin O staining (Figure 2C).

Co-implantation of bovine primary chondrocytes with expanded chondrocytes in nude mice.

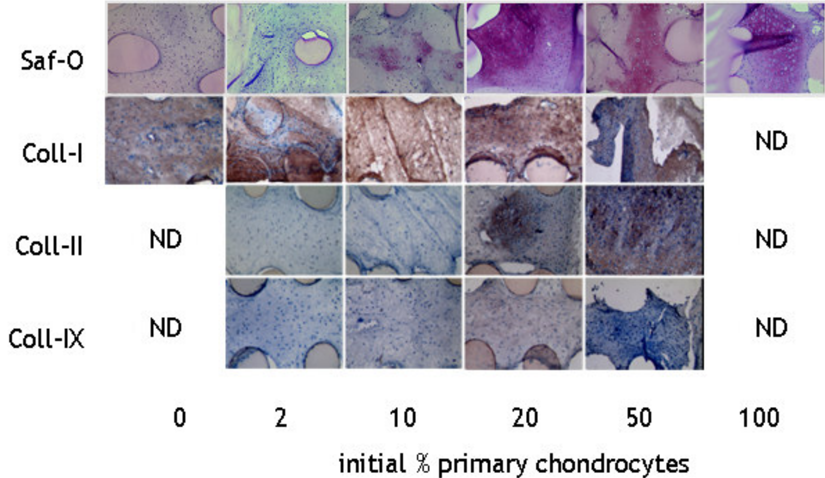
Bovine primary chondrocytes (2%, 10%, 20% and 50%) were combined with bovine expanded chondrocytes, aggregated with fibronectin, and seeded on 3D porous PEGT/PBT scaffolds. The resulting constructs were implanted subcutaneously in nude mice. The amount of GAG per initial percentage primary chondrocytes increased up to 9 fold with decreasing percentages of initial primary chondrocytes (Figure 3A). Although total GAG also appeared to increase with increasing initial percentage of primary chondrocytes, only the 50 % group was significantly different from the 100 % expanded chondrocyte control group (Figure 3B).



A

PCs / ECs	Total GAG	Total DNA	GAG/DNA	GAG/initial % PC
(%/%)	(µg)	(µg)	(µg/µg)	(·10-5 g)
0 / 100	99.2 ± 39.1	12.2 ± 3.2	10.4 ± 3.4	-
2 / 98	107.4 ± 39.0	18.0 ± 5.8	12.3 ± 2.7	40.9
10 / 90	121.9 ± 27.6	17.5 ± 4.7	12.3 ± 1.2	22.7
20 / 80	133.8 ± 24.1	19.6 ± 2.5	12.9 ± 1.2	17.3
50 / 50	151.4 ± 38.8	19.1 ± 2.4	13.8 ± 3.8	10.5
100 / 0	144.3 ± 40.3	20.2 ± 5.8	13.2 ± 3.2	4.5
-	65.0 ± 16.5	6.8 ± 1.8	10.5 ± 1.1	-

B



C

Figure 3 Co-implantation of bovine primary chondrocytes (PCs) mixed with bovine expanded chondrocytes (ECs) on PEGT/PBT 300/55/45 scaffolds in nude mouse model (8 wks).
*Bovine primary chondrocytes were mixed with bovine expanded chondrocytes at indicated percentage's, seeded onto 3D printed PEGT/PBT 300/55/45 scaffolds and implanted subcutaneously in nude mice for 8 weeks. A. μg GAG/ initial percentage primary cells, B. safranin O staining C collagen type I, II and IX immunostaining. All data are NOT significantly different from 100% PC group $p \geq 0.2$, * significantly different from 100% EC control group $p<0.05$.*

Safranin O staining indicated the presence of sulphated proteoglycans for all experimental groups but not for the 2% primary chondrocytes, the 100% expanded chondrocyte control group and the empty scaffold control group. Safranin O staining clearly showed that those experimental groups initially containing 20% or 50% of seeded primary chondrocytes have similar intensity in proteoglycan staining to the 100% primary chondrocyte control group. Increased cartilagenous tissue formation in these experimental groups was further exemplified by the abundant type II collagen staining and subtle type IX collagen staining. Furthermore, in proteoglycan stained areas, cells show morphological features of cartilage such as a spherical morphology and lacunae around the cells. Type I collagen staining showed that at 8 weeks of implantation, constructs of all experimental groups still contained cells which were not fully differentiated into the cartilagenous lineage (Figure 3C).

Co-implantation of bovine primary chondrocytes with human mesenchymal stem cells in nude mice.

As with the previous experiment, availability of healthy human cartilage was scarce, therefore primary chondrocytes were derived from adult bovine cartilage. For this experiment, 2%, 10%, 20% and 50% bovine primary chondrocytes were mixed with human bone marrow mesenchymal stem cells, aggregated with fibronectin and seeded onto 3D

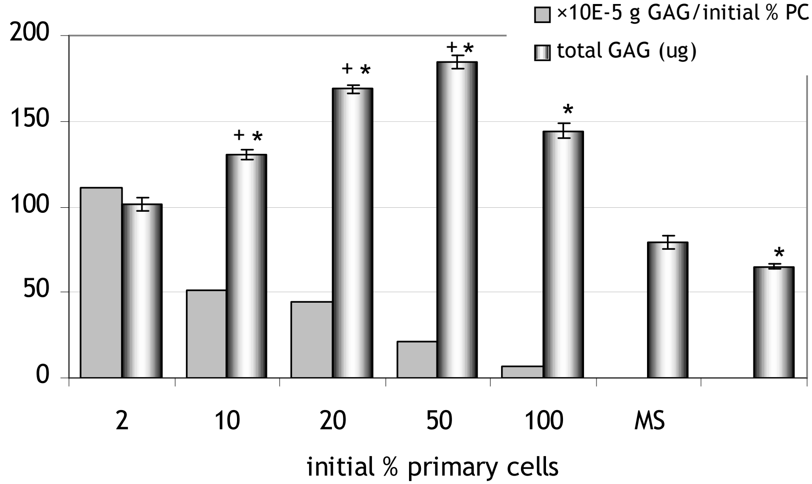
porous PEGT/PBT scaffolds. Resulting constructs were implanted in a nude mouse model. The amount of GAG per initially seeded primary chondrocyte again continuously increased up to 17-fold more with decreasing percentage of primary chondrocytes initially seeded (Figure 4A). The total amount of GAG was significantly higher for experimental groups initially containing 10%, 20% or 50% of primary chondrocytes compared to the 100% mesenchymal stem cell control group and more importantly, not significantly different from the 100% primary chondrocyte control group (Figure 4B). The latter was also reflected in safranin O stained sections, since from the 20% primary chondrocyte group upwards, similar intensity in staining of and number of positively stained areas were found compared to the primary chondrocyte control group. Type II collagen immunohistochemistry was subtle in the 10% primary chondrocyte group but again abundant for the 20% and 50% primary chondrocyte experimental groups. Type I collagen was present in all experimental groups including the mesenchymal stem cell control group (Figure 4C). Safranin O stained areas in constructs of all experimental groups showed cells which morphologically resembled chondrocyte morphology and around these cells, lacunae were found. Histological examination revealed that no other tissue than hyaline or fibrocartilage like tissue was present in these constructs. DNA content for all experimental groups was not significantly different from the mesenchymal stem cell control group (Figure 4B). In vivo data supported in vitro data showing an upward trend in cartilage tissue formation with decreasing percentages of seeded primary chondrocytes.

Discussion

Co-culture primary chondrocytes with expanded chondrocytes or bone marrow mesenchymal stem cells

For tissue engineering or cell therapy purposes, the number of primary chondrocytes that can be isolated from a small biopsy is considered insufficient for immediate clinical application (5-8). However, for in vitro or in vivo differentiation experiments, primary chondrocytes can be looked at as the “golden” standard. In this study, cartilagenous tissue formation was examined when decreasing percentages of primary chondrocytes were combined with expanded chondrocytes or mesenchymal stem cells and compared to cartilagenous tissue generated by 100% primary chondrocytes, 100% expanded chondrocytes or 100% mesenchymal stem cells. Next to total μg GAG produced per construct; the data was presented as μg GAG/initial % primary chondrocytes (Figure 1A, 2A, 3A and 4A). This shows the correlation between the initial amounts of primary chondrocytes with the amount of cartilagenous tissue formation eventually formed. Decreasing the initial amount of primary chondrocytes is crucial for application of the co-culture or co-implantation model for tissue engineering or cell therapy. The need for expansion of cells or the use of growth factors can be reduced or even eliminated if a

limited amount of primary chondrocytes, together with expanded chondrocytes or mesenchymal stem cells, leads to enhanced cartilage tissue formation.
GAG produced per initial percentage of primary chondrocytes increased 6 fold when 2% bovine primary chondrocytes were co-cultured with expanded chondrocytes in vitro (Figure 1A and B). Moreover, μg total GAG was not significantly different in micromass cultures with initially 25% or 50% primary chondrocyte compared to μg total GAG formed by 100% primary chondrocytes. Similar chondrogenic induction was found when human primary chondrocytes were co-cultured with human mesenchymal stem cells (Figure 2A).

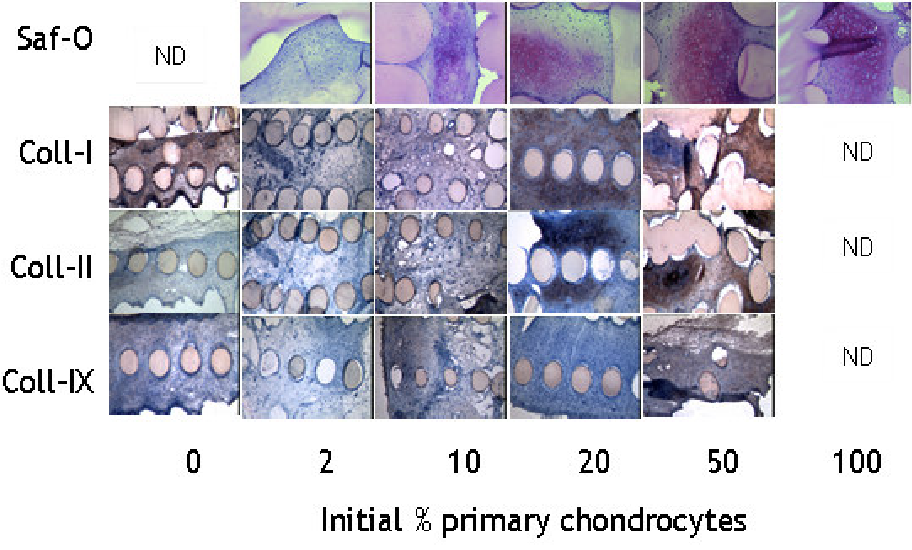


A

PCs / MSCs	total GAG	total DNA	GAG/DNA	GAG/initial % PC
(%/%)	μg	μg	μg/μg	(·10-5 g)
0 / 100	79.2 ± 11.6	12.6 ± 4.8	7.3 ± 3.5	-
2 / 98	101.5 ± 31.5	13.8 ± 4.1	6.1 ± 2.4	111.4
10 / 90	130.8 ± 44.3	11.4 ± 7.4	9.3 ± 1.5	51.5
20 / 80	168.7 ± 29.9	14.3 ± 3.0	9.4 ± 2.4	44.7
50 / 50	184.3 ± 54.8	19.2 ± 7.9	10.3 ± 3.9	21.0
100 / 0	144.3 ± 40.3	20.2 ± 5.8	13.2 ± 3.2	6.5
-	65.0 ± 16.5	6.8 ± 1.8	10.5 ± 1.1	-

B

5



C

Figure 4 Co-implantation of bovine primary chondrocytes (PCs) with human mesenchymal stem cells (MSCs) on PEGT/PBT 300/55/45 scaffolds in nude mice (8 wks).
*Bovine primary chondrocytes were mixed with mesenchymal stem cells from bone marrow at indicated percentage's, seeded onto 3D printed PEGT/PBT 300/55/45 scaffolds and implanted subcutaneously in nude mice for 8 weeks. A. μg GAG/ initial percentage primary cells B. safranin O staining for sulphated GAG's and C. collagentype-I, II and IX staining with immunohistochemistry. Arrows indicate scaffold material. + NOT significantly different from 100% PC group $p \geq 0.1$, * significantly different from 100% MSC group $p \leq 0.01$.*

The GAG/DNA ranged from 2 to 7 μg/μg for bovine (2 wks) and 50-110 μg/μg for human cells (4 wks) and was similar or higher to that produced in micromass culture experiments performed by others, in which growth factors were applied (Figure 1B). That is, others found with expanded chondrocytes or mesenchymal stem cells, GAG/DNA ranging from 0.9 to 13 μg/μg after 2wks, depending on the concentration and combination of the chondrogenic growth factors applied (table 1) (29),(30),(31),(14),(32). From these data, together with consistent data on proteoglycan staining (Figure 1C and 2C), type II collagen and IX immunostaining (Figure 1C), it was evident that co-culturing a limited amount of primary chondrocytes with expanded chondrocytes or mesenchymal stem cells increased the formation of cartilage specific tissue and was as effective as 100% primary chondrocytes. Additionally, at this follow up time, GAG levels in co-cultured experimental groups were similar to GAG levels produced by growth factor stimulated human expanded chondrocytes or human mesenchymal stem cells (Table 1).
Type I collagen is typically produced by de-differentiated chondrocytes and was detected in all experimental groups containing expanded chondrocytes (22). Expanded chondrocytes and bone marrow mesenchymal stem cells have previously been shown to continue type I

collagen production in micromass culture (33). Type I collagen thus indicated that chondrogenic differentiation at these follow up times was not at the same level as in native cartilage. Type II collagen produced by differentiated chondrocytes was detected in all groups containing primary chondrocytes (Figure 1C). The latter, together with type I collagen data could suggest that both cell populations contribute to the tissue formation in micromass cultures, at least to some extent. However, when in co-culture, primary chondrocytes, as well as expanded chondrocytes or mesenchymal stem cells, might proliferate and/or differentiate. Furthermore, each cell population might influence the behaviour of the other and thus stimulate its proliferation or differentiation. Regardless of the underlying mechanism, an increase in cartilaginous tissue formation was clearly shown when primary chondrocytes were co-cultured with expanded chondrocytes or mesenchymal stem cells.

Cell source	GAG/DNA	Culture method	Origin cells	Initial cell density	reference
	ug/ug			* 10E6	
HBM-MSCs	3-7	Pellet+T	Human bone marrow/ Percoll gradient/ p1	0.2	(31)
HEACs	2-6	Pellets + dex + TPF	Ear, nasal and rib cartilage	0.5	(30)
HEACs	2-4.5	Pellets +TD	Human cartilage expanded to p2	0.5	(29)
HEACs	3-10	Pellets +TD	Human cartilage expanded in TFP to p2	0.5	(29)
HEACs	0.9	Pellets	Human cartilage expanded to p2	0.5	(14)
HEACs	6.5	Pellets	Human cartilage expanded in TFP to p2	0.5	(14)
HEACs	13	Pellets + PGE2	Human cartilage expanded in TFP to p2	0.5	(32)

Table 1 Literature survey on GAG per DNA produced by cells in pellet culture.
GAG/DNA ($\mu g/\mu g$) metabolized by human mesenchymal stem cells from bone marrow (HBM-MSCs) or human expanded chondrocytes (HEACs) in pellet culture (2wks).
T = TGFβ1, F = bFGF, P = PDGF-BB, D = dexamethasone, PGE2 = ProstaglandinE_2.

Finally, Tsuchiya K et al (2004) (34) was the first to co-culture chondrocytes with bone marrow mesenchymal stem cells in micromass cultures. Results showed intensified safranin O staining with increasing initial percentages of chondrocytes (passage 2). Nevertheless, to our understanding, in the Tsuchiya study, micromass cultures were cultured in medium containing dexamethasone and TGFβ3, which in itself might be responsible for differentiation of the expanded chondrocytes present in the micromass cultures. Their type II collage /I percentage data, which showed an increase equivalent to the initial percentage of expanded chondrocytes, support this. Since they also showed

that the percentage of expanded chondrocytes: mesenchymal stem cells remained unchanged, it is likely in our opinion that the type II collage/I percentage is also equivalent to the amount of expanded chondrocytes after 4 weeks of culture.

Co-implantion of primary chondrocytes with expanded chondrocytes or bone marrow mesenchymal stem cells in nude mice

The combination of a low percentage of primary chondrocytes with expanded chondrocytes or bone marrow mesenchymal stem cells enhanced cartilage tissue formation beyond the initial percentage of primary chondrocytes present. Mechanistic questions influencing cell behaviour during co-culture remain to be resolved. However, we first sought to investigate whether the synergistic effect of combining expanded chondrocytes or mesenchymal stem cells with primary chondrocytes was sustained when implanted in an in vivo model.

When primary chondrocytes were co-implanted with expanded chondrocytes, a similar trend was observed in that GAG per initial percentage of primary chondrocytes increased with decreasing percentage primary chondrocytes. However, only the experimental group containing 50% primary chondrocytes was significantly different from the 100% expanded chondrocytes control group. With the GAG assay applied in this study and commonly used in cartilage research, GAGs present in skin, lung, muscle and cartilage tissue for example, can be measured (35). In the nude mouse implantation model, cartilagenous tissue formation can be studied. However, it can not be excluded that the multipotent cells in the construct also start producing other extra cellular matrix proteins because of the influx of host cells or cytokines/growth factors from the host. Thus, quantitive GAG assay was always supported with cartilage specific safranin O staining. We concluded that the basic level of GAG measured in constructs with 100% MSCs, expanded chondrocytes or empty constructs were not cartilage specific, as shown by negative safranin O staining. More importantly, data show that the increase in GAG measured with the GAG assay corresponded with an increase in safranin O staining intensity. Therefore, we conclude that the increase in GAG is responsible for the increase in cartilage tissue formation. This was further exemplified by an increase in intensity of cartilage specific type II collagen and IX immunostaining.

When 20% primary chondrocytes were co-implanted with expanded chondrocytes or mesenchymal stem cells, the cartilagenous tissue produced was similar to that produced by 100% primary chondrocytes. Co-implantation of 10% primary chondrocytes with either expanded chondrocytes or mesenchymal stem cells already showed safranin O staining, as confirmed by elevated GAG. That the tissue formed was of cartilagenous nature was further supported by type II collagen immunostaining. Furthermore, constructs implanted in this study were sampled at 8 weeks, in which time the cartilage specific ECM protein plateau phase was possibly not reached. Thus, experimental groups with low initial % primary chondrocytes might reach the same total GAG amount as primary chondrocytes,

but at a later time point. This was supported by results of a kinetic study of Mauck et al (2006) (36), where they showed in vitro that the plateau phase for total GAG was reached earlier for mesenchymal stem cells compared to expanded chondrocytes. This possibly also explains why co-implantation of primary chondrocytes with mesenchymal stem cells at 8 weeks, induced formation of tissue with a higher total amount of GAGs compared to when primary chondrocytes were co-implanted with expanded chondrocytes. Furthermore, upon co-implantation of primary chondrocytes with mesenchymal stem cells, more intense proteoglycan staining with safranin O and type II collagen and IX staining in more areas throughout the scaffold were found to be present (Figure 4).

To our knowledge, this is the first time that such an increase in cartilage-like matrix production was shown to be induced by mixing two different cell populations without the addition of growth or other chondrogenic factors. We suggest the following hypotheses that might explain this phenomenon; I. Primary chondrocytes induce differentiation of expanded chondrocytes or bone marrow mesenchymal stem cells, II. Primary chondrocytes are stimulated to produce and secrete more GAGS under the influence of expanded chondrocytes or mesenchymal stem cells or III. Both mechanisms occur simultaneously.

Finally, co-implantation of expanded chondrocytes with primary chondrocytes might provide an important improvement to Autologous Chondrocytes Implantation (ACI) without major additional health risks for the patient. Furthermore, results shown here might provide the basis for developing a novel cell therapy using the synergy of mesenchymal stem cells together with primary chondrocytes for cartilage repair.

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Chapter 6

Fibronectin aggregation-mediated seeding enhances cartilage tissue formation by articular chondrocytes on porous scaffolds

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Chapter 6

Abstract

The objective of this study was to increase seeding efficiency and subsequent cartilage tissue formation of chondrocytes on porous scaffolds through aggregation-mediated seeding compared to attachment mediated seeding in vivo and in vitro.

Primary and expanded chondrocytes were mixed (1:5 ratio) and seeded either with fibronectin(Fn)-attachment or with Fn-aggregation onto hydrophobic scaffolds and implanted in a nude mouse model for 4 weeks. Next, the influence of aggregation-mediated seeding on cartilagenous tissue formation of primary and expanded chondrocytes 1:5, was compared to primary or expanded chondrocytes alone. Finally, the influence of aggregation-mediated seeding and subsequent cartilage tissue formation on hydrophilic scaffolds compared to hydrophobic ones was examined in vitro. Glycosaminoglycans and DNA were determined quantitatively, type II collagen and IX with immunohistochemistry and qualitative glycosaminoglycans with safranin O staining. Cell viability was determined with Life/Dead assay and cell morphology with scanning electron microscopy. Differentiation of the primary/expanded chondrocyte mixture was enhanced by Fn-aggregation seeding as demonstrated by glycosaminoglycans, histological appearance and type II collagen and IX staining. In vivo, Fn-aggregation-mediated seeding showed to enhance GAG formation more efficient with primary and expanded chondrocyte mix compared to primary or expanded chondrocytes alone. In vitro results showed that Fn-aggregation-mediated seeding is 3-fold more efficient for primary chondrocytes compared to expanded chondrocytes on hydrophilic as well as hydrophobic scaffolds. This is also reflected in the amount of GAG detected after 4 weeks of in vitro culture. Fn-aggregation-mediated seeding stimulated cartilagenous tissue formation of the primary/expanded chondrocytes mixture in vivo compared to attachment mediated seeding. In addition, this was more so for the primary expanded chondrocyte mixture than for primary or expanded chondrocytes alone. Furthermore, it was shown that the influence of Fn-mediated aggregation on differentiation is depending on initial differentiation status and/or morphology of chondrocytes.

Introduction

For cartilage tissue engineering and cell therapy, isolated chondrocytes from a small biopsy need to be expanded in vitro. During expansion chondrocytes lose their chondrogenic phenotype but maintain their capacity to differentiate given the proper

conditions (1),(2),(3). Previously it was shown that cartilagenous tissue formation was enhanced when primary chondrocytes were co-cultured with expanded chondrocytes (1:5) in micromass (4). For tissue engineering purposes it is interesting to examine if the synergistic effect of combining primary chondrocytes with expanded chondrocytes on cartilage tissue formation is sustained when seeded onto porous scaffolds. As initial cell density proved to be important for efficient cartilagenous tissue formation, seeding efficiency is essential (5),(6),(7),(8). Previously, several approaches to enhance seeding efficiency onto porous biomaterials were applied: dynamic seeding in bioreactors, improvement of cell attachment properties of scaffolds surface (9),(10),(11) or applying gels as a delivery vehicle (12),(13). Aggregation-mediated seeding of cells in porous scaffolds would combine the advantages of dynamic seeding and in-gel seeding and at the same eliminating some of its disadvantages. Advantages of dynamic seeding and in-gel seeding are high seeding efficiency and allowing the cells to preserve or retain a spherical morphology. Disadvantages of dynamic seeding are low reproducibility, prolonged seeding time is necessary for efficient seeding and cells are often inhomogeneously distributed through the scaffold. For some of the disadvantages of in-gel seeding: the gel is preventing direct cell-cell interactions and is causing increased nutrient limitation subsequent to seeding. The latter results in inhibited tissue formation homogenous ly throughout the scaffold. With aggregation seeding, cellular interaction and cartilage tissue formation is stimulated, cells maintain or regain a spherical morphology, are exposed to medium directly and is not time-consuming (2),(14),(15).

When aggregation of cells was stimulated, fibronectin facilitates initial cell-cell interactions and plays an important role in enhancing intercellular cohesion in the aggregates (16),(17). Furthermore, fibronectin and fibronectin fragments play a crucial role in cartilage metabolism, and a specific fibronectin splice variant is uniquely expressed in cartilage in vivo (18),(19),(20),(21). Nevertheless fibronectin also showed to mediate cell attachment on materials and extra cellular matrices via $\alpha5\beta1$ integrin and concomitantly correlated with dedifferentiation of chondrocytes (22). Moreover when chondrocytes are cultured on fibronectin, this enhanced proliferation and was negatively correlated with redifferentiation capacity of chondrocytes (23).

In general, redifferentiation of chondrocytes is initiated in a 3D-environment (24),(25),(26). Biomaterials can provide such a 3D-environment and additionally can provide instructive signals (27),(28). In previous work, a relationship is apparent between cell seeding on hydrophilic surfaces like poly (ethylene glycol)-terephthalate-poly (butylene terephthalate (PEGT/PBT) 1000/70/30 and chondrocyte aggregation, acquiring a spherical morphology and enhancing their cartilagenous metabolism. In contrast, on hydrophobic surfaces like PEGT/PBT 300/55/45 enhanced cell attachment correlated with a spread chondrocyte morphology, elevated expression of fibronectin (FN) integrin receptor and concomitant reduced differentiation capacity (22).

This led us to investigate the influence of Fn-mediated aggregation of chondrocytes on seeding efficiency and chondrogenic metabolism in hydrophobic (300/55/45) and hydrophilic (1000/70/30) PEGT/PBT scaffolds. We hypothesized that chondrocytes respond differently to Fn-mediated seeding depending on their cellular context and this is directly correlated with cell morphology and initial differentiation status.

Materials and Methods

Cell isolation & culture

For bovine chondrocytes full thickness articular cartilage was dissected from the patellar femoral groove of adult bovine femur. Dissected cartilage was incubated for 20-22 hrs in collagenase type II solution containing 0.15% collagenase (Worthington), Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml)(Sigma-Aldrich). Suspension was filtered through a 100 μm mesh nylon filter (cell strainer Nucleon) and cells were washed 2 times with phosphate buffered saline (PBS) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). For expansion chondrocytes were plated at a density of 3.5 * 104 cells/cm2 and cultured in proliferation medium (PM) containing dulbecco's minimal essential medium (DMEM) supplemented with 10 % fetal bovine serum, 1x non- essential amino acids (Sigma-Aldrich), 10 mM HEPES buffer (Biowhitakker), 0.2 mM ascorbic acid 2-phosphate (InVitrogen), 0.4 mM proline (Sigma-Aldrich), 100 U/ml penicillin (InVitrogen) and 100 μg/ml streptomycin (InVitrogen). After 2-3 passages cells were mixed with primary chondrocytes and seeded onto a porous scaffold.

Scaffold fabrication

Three-dimensional fiber deposited (3DF) scaffolds were manufactured from the polymers with a Bioplotter device (Envisiontec GmbH, Germany), essentially an XYZ plotter device as previously described (Moroni et al, J Biomed Mater Res 2005). Few modifications were done to extrude highly viscous polymeric fibers. The polymers were put in a stainless steel syringe and heated at T = 200 °C through a thermoset cartridge unit, fixed on the "X"-mobile arm of the apparatus. When the molten phase was attained, a nitrogen pressure of 5 Bars was applied to the syringe through a pressurized cap. Rectangular block models were loaded on the Bioplotter CAM software and deposited layer by layer, through the extrusion of the polymeric system on a stage as a fiber. The deposition speed varied between 230 mm/min and 300 mm/min, whether 300/55/45 or 1000/70/30 was used respectively. Scaffolds were then characterized by the fiber diameter (through the nozzle diameter), the spacing between fibers in the same layer, and the layer thickness. A stainless steel Luer Lock needle with internal diameter (ID) of 250 µm shortened to a length of 16.2 mm was used as a nozzle. The fiber spacing was set to 600 µm, while the

layer thickness to 150 µm. Scaffolds were fabricated by depositing fibers with 90º angle steps between successive extruded layers (0-90 architecture).

Seeding & culturing on PEGT/PBT Scaffold

Cylindrical scaffolds were rinsed in phosphate buffered saline (PBS) extensively, incubated overnight in PM and blotted dry prior to seeding. Primary and expanded chondrocytes were mixed, centrifuged at 300 g for 5 min and re-suspended in 54 μl of PM containing 300μg/ml fibronectin. Cells were agitated for 10 minutes and subsequently aspirated on the scaffold to fill. Seeded scaffolds were left undisturbed for 1 hour and subsequently culture medium was carefully added to the well. Scaffolds were seeded statically with 3×10^6 cells per scaffold for 1 day in PM and either cultured in vitro or implanted subcutaneously in nude mice.

For in vitro studies 9 constructs were cultured statically in 6 wells plate in 3 ml PM. Medium was refreshed every 3 to 5 days and after 4 weeks constructs were harvested and further processed for analysis. In vitro experiments were repeated 3 times and results were similar to data presented here.

Implantation in nude mouse model (in vivo)

Nude mice studies were performed after consent of the Ethical Committee for Animal studies (DEC-GDL Utrecht, the Netherlands). 6-week old nude mice (HdCpb:NMRI-nu Harlan, the Netherlands) were anaesthetized with 0.02 ml of a 3,5 : 3 : 1 mixture of ketamine (100μg/ml): xylazine (20 μg/ml): atropine (0.5 mg/ml). 6 constructs per experimental group were randomly implanted subcutaneously (4 constructs per mouse). After swapping the skin with 70% EtOH subcutaneous pockets were made in the posterior-lateral side of the back, pockets were provided with a construct and skin sutured. After 4 weeks mice were sacrificed with cervical dislocation and after release, construct were further processed for analysis.

Analysis

Histology

Constructs implanted in nude mice (n=3) or constructs culture in vitro (n=3) were fixated with 1.5% glutaraldehyde in cacodylate buffer (0,14 M / pH 7.2-7.4). In vitro experiments were repeated 3 times and data of the other two were similar to the data presented here. Samples were washed in PBS, dehydrated and embedded in Glycol Methyl Acrylate (GMA). Sections were cut with a microtome to a thickness of 5 μm, stained for sulphated Glycosaminoglycans (GAG) with safraninO and counterstained with haematoxyline (Gillnr3) and fast green respectively for nuclei and cytoplasm.

Immunohistochemistry

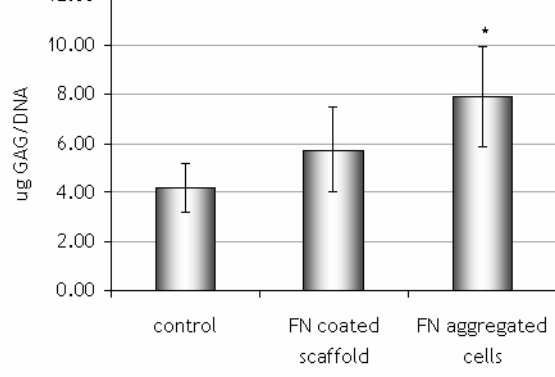
Constructs implanted in nude mice (n=3) or constructs culture in vitro (n=3) were embedded and immediately frozen to –80°C in OCT compound (Tissue-Tek) for immuno-staining. Sections were cut with a cryo-microtome to a thickness of 5 μm and fixated with acetone for 10 min. Cryo-sections were stained for Type II (1:100, DSHB II-II6B3) and IX collagen (1:100, DSHB D1-9. Blocking was done with 10% human serum and as a secondary antibody goat anti-mouse (1:100, DAKO) was used. Staining was visualized with DAB-solution (DAKO) for 10-20 minutes.

Quantative GAG- and DNA assay

Constructs implanted in nude mice (n=6) or constructs culture in vitro (n=3) for quantitative analysis of GAG's and cell number were washed with PBS and frozen o/n at -80°C. Subsequently they were digested with 1 mg/ml proteinase K (Sigma) in Tris/EDTA buffer (pH7.6) containing 18.5 μg/ml iodoacetamide and 1 μg/ml pepstatin A (Sigma-Aldrich) for at least 16 hrs at 56°C. GAG content was spectrophotometrically determined with 9-dimethylmethylene blue chloride (DMMB)(Sigma-Aldrich) staining in PBE buffer (14.2 g/l Na_2HPO_4 and 3.72 g/l Na_2EDTA, pH 6.5) with a micro plate reader (Bio-TEK instruments) at an absorbance of 520 nm. Cell number was determined via quantification of total DNA with CyQuant DNA kit according to the manufacturer description (Molecular probes) and fluorescent plate reader (Perkin-Elmer).

Scanning Electron Microscopy (SEM) Analysis

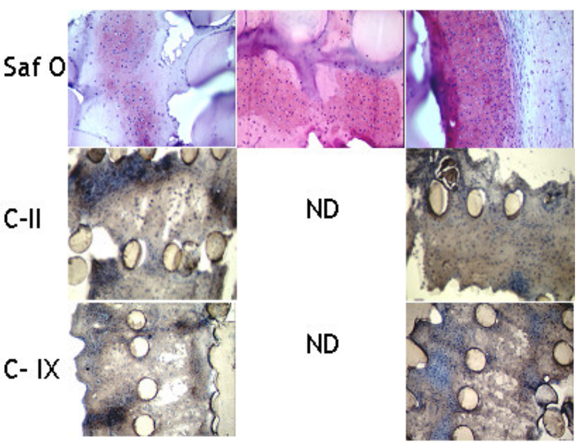
Tissue constructs were also analyzed by SEM. Samples were fixed overnight in 0.14 M cacodylate buffer (pH = 7.2 – 7.4) containing 0.25% glutaraldehyde (Merck). Scaffolds were subsequently dehydrated in sequential ethanol series and critical point dried from liquid carbon dioxide using a Balzers CPD 030 machine. Specimens were then gold sputtered (Cressington) and studied under the SEM.



A

Seeding	Total GAG	Cells	GAG/DNA
	(μg)	(* 10^7)	(μg/μg)
Control	212.7 ± 75.9	1.3 ± 0.2	4.2 ± 1.0
Fn-coated	222.0 ± 45.9	1.0 ± 0.1	5.7 ± 1.8
Fn-aggregated	279.0 ± 37.8	1.0 ± 0.2	7.9 ± 2.0

B



C

Figure 1 Influence of Fn aggregation and Fn-attachment mediated seeding on cartilagenous tissue formation in 300/55/45 constructs after in vivo implantation in a nude mouse model (4wks).
Primary and expanded chondrocytes were mixed (1:1) were seeded into PEGT/PBT 300/55/45 scaffolds for 1 day and implanted subcutaneously in nude mice. Cells were either seeded on untreated scaffolds, fibronectin pre-coated scaffolds (300μg/ml) (FN coated) or cells were mixed with fibronectin (300μg/ml)(Fn-aggregated) prior to seeding and seeded on untreated scaffolds. Graph shows amount of GAG/DNA (μg/μg) (A), Table shows total GAG (μg) per scaffold, total amount of DNA μg/ scaffold and GAG/DNA (μg/μg) (B) and sections of control group(A), Fn-coated group(B) or Fn-aggregated group (C) stained for GAGs with safranin O (SO) and sections of control group(A) and Fn-aggregated group (C) stained for type II collagen (C-II) or type IX collagen (C-IX). ND = not done.

Statistical analysis

Significance of differences between data sets was analyzed with a 2-tailed student t-test with two sample equal variances using $p < 0.05$ as the criterion.

Results

Infuence of Fn-aggregation-mediated seeding compared to Fn-attachment-mediated seeding on cartilage tissue formation on 300/55/45 PEGT/PBT scaffolds. Aggregation-

mediated seeding compared to attachment mediated seeding of co-implanted primary/expanded chondrocytes on 300/55/45 PEGT/PBT scaffolds.

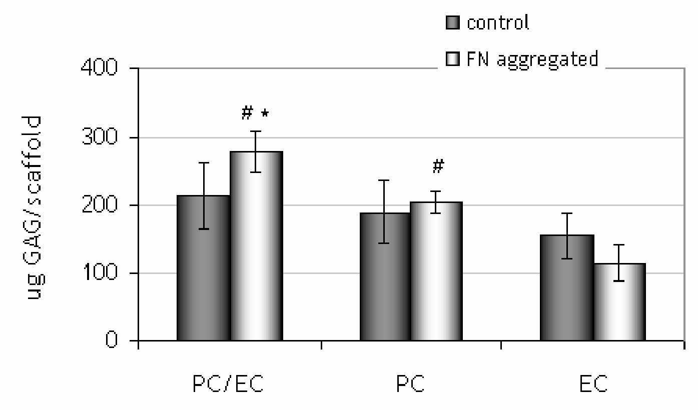
Cartilagenous tissue formation was examined after implantation of primary/expanded chondrocytes when seeded through Fn-aggregation, Fn-attachment and control. After 4 weeks of implantation, GAG was approximately 30% higher on 300/55/45 scaffolds seeded through Fn-aggregation compared to control and Fn-coated scaffolds. Interestingly, the amount of cells was lower on scaffolds seeded through Fn-mediation compared to control scaffolds (Figure 1A). GAG/DNA results again showed that cartilagenous tissue formation is higher when cells were seeded through Fn-aggregation compared to Fn-coating or control. Safranin O, type II collagen and IX confirmed that tissue formed was cartilagenous on both control and Fn-aggregation seeded scaffolds (Figure 1B &C).

Fn-aggregation-mediated seeding of co-implanted primary/expanded chondrocytes compared to primary chondrocyte or expanded chondrocytes in vivo.

Experiments were performed to examine the influence of Fn-aggregation-mediated seeding on proliferation and cartilage tissue formation of 1. co-implanted primary and expanded chondrocytes, 2. primary and 3. expanded chondrocytes. Upon Fn-aggregated seeding, GAG formation was significantly higher when primary chondrocytes were co-implanted with expanded chondrocytes compared to primary chondrocytes or expanded chondrocytes alone (Figure 2A). GAG formation was 40% higher, when the combination of primary and expanded chondrocytes were Fn-aggregated prior to implantation compared to primary chondrocytes alone (Figure 2A). The amount of cells after 4 weeks of implantation was significantly lower in scaffolds seeded with only primary or only expanded chondrocytes compared to the primary-expanded co-implantation group. While the amount of GAG/DNA is 74% lower when expanded chondrocytes were aggregated compared to the control, GAG/DNA was 71% higher when primary/expanded chondrocytes were aggregated (Figure 2B).

Fn-aggregation-mediated seeding of primary and expanded chondrocytes on hydrophilic and hydrophobic PEGT/PBT scaffolds in vitro.

The aim of this experiment was to examine 1st the efficiency of Fn aggregation-mediated seeding of primary chondrocytes and expanded chondrocytes. Second, cell-scaffold interaction was examined on hydrophobic (300/55/45) or hydrophilic (1000/70/30) PEGT/PBT scaffolds. Third, the cell viability of chondrocytes upon Fn-aggregation-mediated seeding was determined. Finally, the influence of Fn-aggregation-mediated seeding on cartilagenous tissue formation was examined in vitro. Fn-aggregation-mediated seeding resulted in a 3-fold higher amount of primary chondrocytes compared to expanded chondrocytes on 300/55/45 scaffolds and a 6 fold higher amount on 1000/70/30 scaffolds at day 1.



A

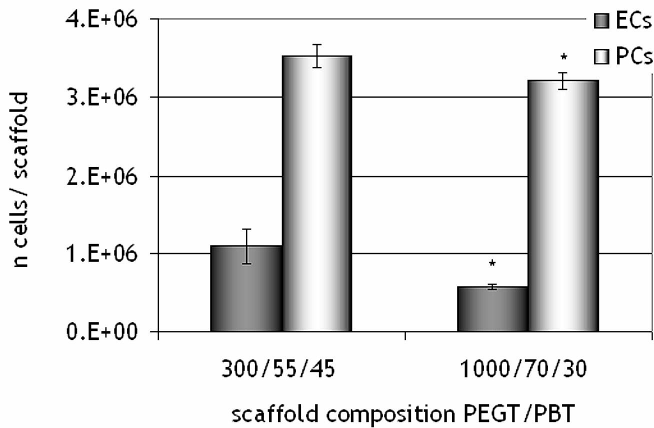
Cell population	seeding	Total GAG	cells	GAG/DNA
		(μg)	(* 107)	(μg/μg)
P/E	Control	212.7 ± 50.0	1.3 ± 0.2	4.2 ± 1.0
P/E	Fn-aggregated	279.0 ± 30.0	1.0 ± 0.2	7.9 ± 2.0
PC	Control	189.2 ± 47.2	1.1 ± 0.1&	5.9 ± 1.6
PC	Fn-aggregated	204.0 ± 16.2	1.0 ± 0.3	5.4 ± 2.3
EC	Control	153.8 ± 34.9	0.9 ± 0.1&	4.6 ± 1.5
EC	Fn-aggregated	112.8 ± 26.4	1.1 ± 0.1	2.7 ± 0.7

B

Figure 2 Aamount of GAG, cells and GAG/DNA in 300/55/45 constructs after in vivo implantation in a nude mouse model (4wks).
*Primary (PC) and expanded (EC) chondrocytes were either mixed (1:1 (PC/EC)) or separately seeded into 300/55/45 scaffolds and subcutaneously implanted in nude mice for 4 weeks. Seeding was done either through aggregated with fibronectin (Fn-aggregated) prior to seeding or cells were seeded immediately upon isolation (control). Graph shows the total amount of GAG per scaffold (μg) (A) table shows total amount of GAG (μg), number of cells and GAG/DNA (μg/μg)(B). * = significant different from PC, # = significant different from EC, & significant different from P/E ($p<0.05$).*

Seeding efficiency for both primary and expanded chondrocytes was significantly higher on 300/55/45 compared to 1000/70/30 (Figure 3A). For the 2nd purpose morphology of cells was examined with scanning electron microscopy (SEM). Morphology remained spherical for primary chondrocytes on both scaffold compositions and for expanded chondrocytes on 1000/70/30 after one day of seeding. In contrast a substantial part of the expanded chondrocytes attached and took a fibroblastic morphology on 300/55/45 scaffolds (Figure

3B). Third, live-dead assay results showed no apparent difference in amount of dead (red) cells between Fn-aggregated versus not treated chondrocytes and more importantly the majority of the cells showed to be alive (green) (Figure 3C).



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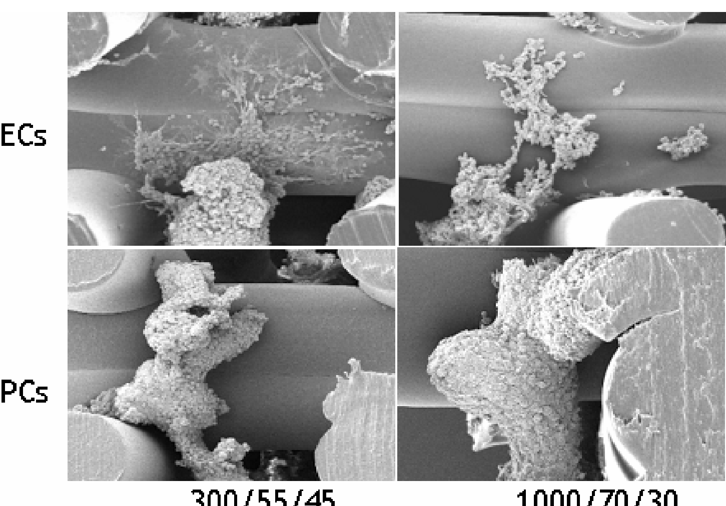

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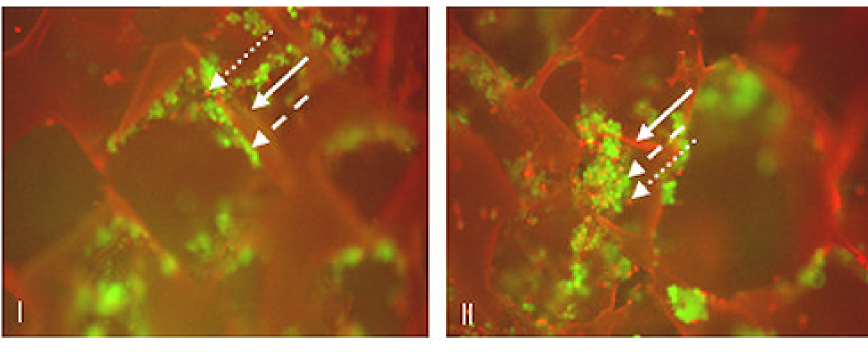


C

Figure 3 Seeding efficiency, morphology and viability of primary (PC) and expanded (EC) chondrocytes on PEGT/PBT scaffolds (day 1).
*Either primary or expanded chondrocytes were seeded through FN-aggregation into hydrophobic PEGT/PBT 300/55/45 or hydrophilic PEGT/PBT 1000/70/30 scaffolds. Constructs were analyzed for seeding efficiency, morphology and viability after 1 day of seeding. Graph shows the amount of cells on the construct (cell/scaffold), ESEM pictures show the morphology of groups of cells (B) and Fluorescent pictures show viability of cells (green = viable) (C) either upon Fn aggregation (II) or in control (i) in constructs.Arrows indicate: Scaffold; solid (red), life cell; dashed(green), dead cell; dotted(red). * = significantly different from PEGT/PBT 300/55/45 constructs*

Next, we studied proliferation and cartilage tissue formation of primary and expanded chondrocytes after Fn-aggregation-mediated seeding on hydrophobic and hydrophilic scaffolds. After 4 weeks of static culture, amount of GAG was 2 to 3-fold higher on constructs seeded with primary chondrocytes compared to expanded chondrocytes independent of scaffold composition (Figure 4). However, primary chondrocyte seeded constructs contained 3 to 5-fold more DNA compared to expanded chondrocytes.
Finally, results showed that the total amount of GAG per scaffolds seeded with primary chondrocytes was significantly higher on 1000/70/30 scaffold compared to 300/55/45 while with expanded chondrocytes no significant (Figure 4A).

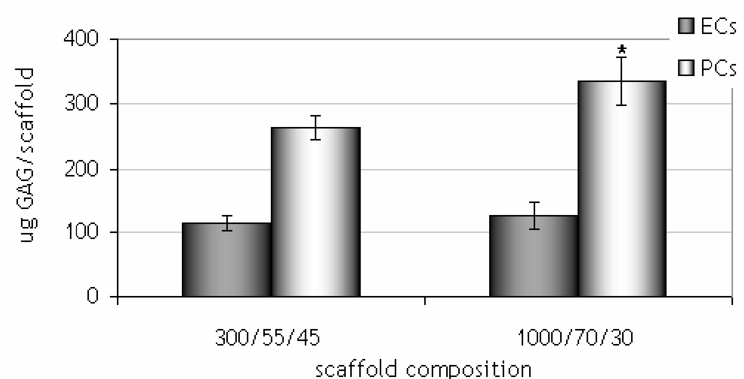
Discussion

It was examined if Fn-aggregation-mediated seeding influenced cartilage tissue formation of chondrocytes compared to Fn-attachment mediated seeding or control (no additional Fn) in a serum containing environment. After 4 weeks of co-implantation GAG formation of chondrocytes was similar on scaffolds coated with Fn and on untreated scaffolds but significantly higher when chondrocytes were Fn-aggregated prior to seeding. Tissue formed in all experimental groups was cartilagenous, as shown by proteoglycans staining with safranin O and type II collagen and IX immunostaining. These results showed that upon Fn-aggregation-mediated seeding the amount of cells on the scaffold at day 1 was 3 fold more for primary chondrocytes compared to expanded chondrocytes. After 28 days of

culture in vitro, GAG was approximately 3 fold higher for primary chondrocytes compared for expanded chondrocytes on both scaffold compositions. Finally, no significant difference was found in cartilagenous tissue formation of co-implanted primary and expanded chondrocytes by Fn-attachment mediated seeding compared to the control group or cartilage tissue formation was enhanced upon Fn-aggregation-mediated seeding.

Previous publications correlate full length fibronectin with survival of chondrocytes, supporting cell compaction and chondrogenesis (16),(22),(29),(30-33). Besides the involvement of in cell attachment to surfaces it is also involved in strengthening cell-cell attachment in aggregates as shown by Robinson et al. (17). The results shown here, suggest that the influence of fibronectin on articular chondrocyte is dependent on differentiation status and/or morphology. In the model shown in Figure 5 we propose the cell morphology and organization of chondrocytes upon Fn-aggregation or –attachment mediated seeding and upon seeding on hydrophilic and hydrophobic scaffolds. In vitro results showed that Fn-aggregation-mediated seeding was approximately 3-6 fold more efficient with primary chondrocyte compared to expanded chondrocytes dependent of scaffold composition. This resulted in approximately 100% seeding efficiency for primary chondrocytes (3 million/scaffold) on both hydrophilic (100/70/30) and hydrophobic (300/55/45) scaffolds. For expanded chondrocytes seeding efficiency was respectively 30% on 300/55/45 and 15% on 1000/70/30 PEGT/PBT scaffolds (Figure 3B). A difference between primary and expanded chondrocytes is their morphology prior to Fn-aggregation. While expanded chondrocytes attach and take a fibroblastic appearance during culture, primary chondrocytes maintain their spherical morphology. We hypothesize that primary chondrocytes form 3D cellular networks more efficiently upon Fn-aggregation because of preserved spherical morphology. However, another difference between primary and expanded chondrocytes is their initial differentiation status and related level of fibronectin receptor $\alpha5\beta1$ integrin expression. In some studies attachment of cells and/or expansion of chondrocytes is correlated with an up regulation of fibronectin ($\alpha5\beta1$-integrin) receptor (34),(35). Whereas, in other studies aggregation and related to this cell-cell communication, has shown to be associated with $\alpha5\beta1$ integrin-fibronectin interaction (20).

In the study described here, it was examined if Fn-aggregation of primary or expanded chondrocytes influences their differentiation on hydrophilic or hydrophobic biomaterials. Chondrocytes underwent approximately two population doublings after 4 weeks of culture independent of initial differentiation status, initial seeding efficiency or material properties. While the total amount of GAG produced by primary chondrocytes was 2.6 fold higher compared to expanded chondrocytes, the amount of GAG produced per cell was approximately 1.4 fold more for expanded chondrocytes compared to primary chondrocytes.



A

Scaffolds composition	Cell population	Total GAG	Total cells	GAG/DNA
(Mw PEG/PEGT /PBT ratio)		μg/scaffold	million/scaffold	μg/μg
300/55/45	EC	113.8 ± 11.2	2.3 ± 0.5	6.3 ± 0.8
300/55/45	PC	263.2 ± 18.9	7.2 ± 0.6	4.8 ± 0.7
1000/70/30	EC	124.7 ± 21.2	1.5 ± 0.3	10.7 ± 2.8
1000/70/30	PC	334.6 ± 37.4 *	6.5 ± 0.6	6.7 ± 1.9

B

Figure 4 Amount of GAG, cells and GAG/ DNA in 300/55/45 and 1000/70/30 construct seeded with either expanded (EC) or primary chondrocytes (PC) through Fn-aggregation and cultured in vitro for 4 weeks.
*Primary (PCs) or expanded (ECs) chondrocytes were seeded onto PEGT/PBT 300/55/45 or 1000/70/30 scaffolds through Fn-aggregation. Constructs were cultured statically in PM for 4 weeks. Graph shows the total amount of GAG (μg/scaffold)(A) and Table shows total amount of GAG (μg/scaffold), total amount of cells (n million/scaffold) and GAG/DNA (μg/μg). * = significantly different from 300/55/45.*

6

Thus, we conclude that primary chondrocytes produced more GAG compared to expanded chondrocytes because of high seeding efficiency and at the same time, expanded chondrocytes are triggered to produce more GAG per cell than primary chondrocytes do. Primary chondrocytes produced significantly more GAG on 1000/70/30 compared to 300/55/45 (Figure 4A). These data showed, that 1000/70/30 stimulates cartilage tissue formation of primary chondrocytes while cartilage tissue formation of expanded chondrocytes was not influenced by scaffold composition.

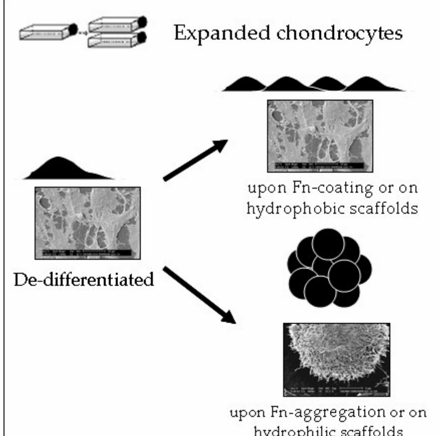


Figure 5 ***Proposed model of cell morphology and organization of a population primary and expanded chondrocytes upon isolation, culture seeding on hydrophobic (300/55/45) and hydrophilic (1000/70/30) scaffolds or upon Fn-attachment or Fn-aggregation mediated seeding.***

Conclusions

This study showed that in a co-implantation or co-culture model combining primary chondrocytes with expanded chondrocytes, aggregation-mediated seeding with fibronectin not only enhanced seeding efficiency but also enhances cartilage specific tissue formation. It remains to be seen if enhanced cartilage tissue formation in this model, is the results of the higher seeding efficiency or enhanced cellular interaction upon aggregation of the cells. Subsequently the difference in primary and expanded chondrocyte response on Fn-aggregated seeding, suggests that the influence of fibronectin on cartilage tissue formation was differentially altered by either cell morphology and/or initial differentiation status. Finally results substantiate, that upon Fn-aggregation-mediated seeding primary chondrocytes still interact with the scaffold material they were cultured on.

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Chapter 7

Three dimensional fibre deposited scaffolds with enhanced cell entrapment capacity and matching physico-chemical properties favor cartilage regeneration.

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Chapter 7

Abstract

An important tenet in designing scaffolds for tissue engineering application or cell therapy is that they should mimic the biomechanical properties of the tissues to be replaced. We hypothesized that this can be achieved by controlling scaffold's pore architecture and volume with 3D fibre deposition (3DF). In addition to mimicking mechanical properties one would also like to determine the contribution of tissue formation to the mechanical properties of a construct during culture and its dependence on scaffold composition and structure. It has been shown that depending on the material, matching the scaffolds mechanical properties to cartilage can compromise the porosity, which hampers tissue formation. Therefore, the aim of this investigation was to assess whether 3DF scaffolds with controlled pore volume and at the same time matching mechanical properties, are indeed effective to support cartilage tissue formation. Next to this, we studied mechanical stability of the constructs in time during culture. Previously it was established that co-culturing primary chondrocytes with expanded chondrocytes in micromass culture and in vivo enhanced cartilage tissue formation. In this study primary and expanded chondrocytes (1:5) were cultured on scaffolds in vitro for 4 weeks. DNA, glycosamino-glycans (GAG) and dynamic stiffness of the constructs were measured at day 1, 7 and 28 to asses the relative amount of cells, the cartilage specific matrix build up and the constructs stability. Cell morphology and matrix distribution was analyzed by scanning electron microscopy (SEM). A higher amount of cartilage specific matrix (ECM) was formed on lower pore volume scaffolds after 28 days of culture. Furthermore, a less protein adhesive composition supported chondrocytes rounded morphology, which contributed to cartilagenous differentiation. Interestingly, the dynamic stiffness of a hybrid construct remained approximately at the same value after culture, suggesting a comparable kinetics of tissue formation and scaffold degradation in low pore volume scaffolds. These results imply that 3DF scaffolds with low pore volume and appropriate physico-chemical properties support chondrocyte differentiation. Both formed tissue and scaffold were shown to contribute to the dynamic stiffness of the constructs during culture.

Introduction

In tissue engineering, scaffolds have been shown to deliver an essential contribution to treatments of chondral defects. These constructs have to posses sufficient biocompa-tibility, to be possibly biodegradable, to provide mechanical stability and to constitute an

appropriate substrate for cells to direct these into the proper lineage (1-5). They can also act as a delivery vehicle for cells and it has been shown that some scaffolds deliver instructive signals to enhance chondrogenesis of cells (6-8). Polyethyleneoxide-terephtalate (PEOT)/polybutylene-terephtelate (PBT) copolymers are interesting candidates to make scaffolds for tissue engineering as they comply at least partially with the above mentioned criteria. These polyether-ester multi-block copolymers are thermoplastic elastomers which display satisfactory physical properties like elasticity, toughness and strength in combination with easy processability (9-12).

Previous results from our group showed that both architecture and composition of the PEOT/PBT scaffolds influence cartilage specific matrix formation of chondrocytes (13-16). On two-dimensional more protein adhesive surfaces like 300/55/45 (a/b/c, where a is the molecular weight of PEG units used in the copolymerization, while b and c are the PEOT and PBT weight fractions in the final copolymer) a direct relationship between a spread chondrocyte morphology and reduced differentiation capacity has been shown (15). In contrast, on less protein adhesive surfaces (e.g. 1000/70/30) chondrocytes take a spherical morphology, aggregate and enhanced differentiation is apparent (16). When 3D scaffolds are fabricated with conventional techniques, like compression moulding and salt leaching, the achievement of mechanically matching structures to cartilage infers a low pore volume. The pore network of these scaffolds is often tortuous and not completely interconnected, resulting in a scarce nutrient perfusion. This can be overcome by a rapid prototyping technique such as 3D fibre deposition (3DF), as the resulting scaffolds have a completely interconnected pore network and scaffold's pore architecture and volume can be controlled (9),(13).

Human articular cartilage thickness has been shown to range between 0.5 and 7.1 mm at various locations in the knee. For in vitro or in vivo studies in cartilage tissue engineering, scaffold thickness ranged typically between 1-5 mm (17). In general, cells from an immature animal model are used to study correlation between limiting factors such as nutrient gradients, pore volume and interconnectivity of a scaffold and homogenous cartilage tissue formation (18). However, matching scaffolds's thickness for cartilage repair with chondrocytes from adult model origin might not be just a matter of scaling up. Oxygen tension has shown to decrease throughout such clinically relevant size scaffolds (19). Albeit no difference in oxygen tension was shown between 3DF and compression moulded (CM) scaffolds with the same porosity (80%), tissue formation with chondrocytes from immature origin showed to be enhanced in 3DF scaffolds compared to CM scaffolds. So far, with CM techniques manufactured 1000/70/30 scaffolds with matching mechanical properties of cartilage hampered porosity and pore interconnectivity to such an extent that cartilage tissue formation was obstructed. Therefore, the aim of this study was to examine the influence of scaffold's pore volume and polymeric composition on matrix build up by mature articular chondrocytes. Next to this we examined the influence of extra cellular matrix build up in chorus with scaffold degradation on mechanical stability

of scaffolds. For this purposes, 3D scaffolds were fabricated by a rapid prototyping technique such as 3DF, since with this method it is possible to achieve scaffolds with low pore volume and high pore interconnectivity.
Recently, we found that co-culturing of primary chondrocytes with expanded chondrocytes enhanced cartilage tissue formation (20). This co-culture model was applied to study the influence of cell-biomaterial interaction on cartilagenous tissue formation on PEOT/PBT scaffolds. PEOT/PBT 3DF scaffolds with 100% interconnectivity were fabricated with different porosity, pore volume, and copolymer composition. Specifically, 300/55/45 and 1000/70/30 were considered for their difference in physico-chemical properties and the differential influence this showed to have on cartilagenous tissue formation.

Materials and Methods

Scaffolds fabrication

Poly(ethylene oxide – terephthalate)/poly(butylene terephtalate) (PEOT/PBT) copolymers were obtained from IsoTis S.A. (Bilthoven, The Netherlands). The composition used in this study were 300/55/45 and 1000/70/30 where, following an aPEOTbPBTc nomenclature, a is the molecular weight in g/mol of the starting PEG blocks used in the copolymerization, while b and c are the weight fractions of the PEOT and PBT blocks, respectively.
3DF scaffolds were produced with a Bioplotter device (Envisiontec GmbH, Germany), which is basically an XYZ plotting machine as previously described by Landers et al (21), (22) and by our group (23),(24). To make the extrusion of highly viscous PEOT/PBT fibres possible few modifications were done. The polymer was placed in a stainless steel syringe and heated at T = 200-210 °C through a thermo-coupled cartridge unit, fixed on the “X”-mobile arm of the apparatus. When the polymer reached a molten phase, a nitrogen pressure of 4.5-5 Bars was applied to the syringe through a pressurized cap. Rectangular block models were deposited, layer by layer, through the extrusion of the polymer on a stage as a fibre in a CAD/CAM controlled manner. Scaffolds were then characterized by varying the fibre diameter (through the nozzle diameter or the deposition speed), the spacing between fibres in the same layer, the layer thickness and the configuration of the deposited fibres within the whole architecture. In particular, stainless steel Luer Lock needles with an internal diameter (ID) of 200 µm shortened to a length of 16 mm was used to extrude the polymeric filaments in this study. The deposition speed was set to 230 mm/min for 300/55/45 and to 280 mm/min for 1000/70/30. This resulted in extruded fibres with a diameter d1 of approximately 170 µm. Three scaffold types were fabricated based on previous studies on the mechanical properties of PEOT/PBT 3DF scaffolds: 300/55/45 and 1000/70/30 (porosity = 74%) with similar, but not matching (NM) dynamic stiffness (E’=1 MPa), as compared to articular bovine cartilage, and 1000/70/30 (porosity = 56%) mechanically matching (M) articular cartilage (E’=10 MPa). The rationale behind

the scaffolds' design was first to compare 3D scaffolds with different physico-chemical properties, but identical structure, resulting in approximately similar dynamic stiffness. This aimed at assessing the net contribution of the polymers' physico-chemical properties on cartilage formation in a 3D scaffold model. At the same time, the influence of high pore volume and low stiffness on cartilaginous tissue build up was studied. Next, a 3DF scaffold with low pore volume, resulting in matching the articular cartilage stiffness, was fabricated with 1000/70/30 for its potential capacity to support cell round morphology. This also allowed to compare the influence of low or high pore volume , that is matching or non-matching dynamic stiffness, on extra cellular matrix formation and scaffold degradation, and thus on the construct stability. For NM 300/55/45 and 1000/70/30 scaffolds the fibre spacing d2 was set to 600 μm, the layer thickness d3 was set to 150 μm, and the layer configuration was changed by 90º every two deposited layers (00-9090 configuration). For M 1000/70/30 scaffolds the fibre spacing d2 was fixed to 370 μm, the layer thickness was fixed to 140 μm, while the layer configuration was changed by 90º every single deposited layer (0-90 configuration).

Scaffolds Characterization

Cylindrical samples of 4 mm in diameter by 4 mm in height were cored out in the "Z-direction" from the rectangular 3D fabricated blocks. Scaffolds geometry and architecture was characterized by environmental scanning electron microscopy (ESEM) analysis with a Philips XL 30 ESEM-FEG. The porosity of 3DF scaffolds was calculated as (21),(24):

$$P = 1 - \frac{V_{scaffold}}{V_{cube}} = 1 - \frac{\pi}{4} \cdot \frac{1}{\frac{d_2}{d_1}} \cdot \frac{1}{\frac{d_3}{d_1}}$$

(1)

where P is the scaffold porosity, d1 the fibre diameter, d2 the fibre spacing and d3 the layer thickness, within each different structure.

Cell Isolation and Culture

Primary chondrocytes are referred to when chondrocytes were applied immediately after isolation. For chondrocytes isolation, full thickness articular cartilage was dissected from the patellar femoral groove of adult bovine. Dissected cartilage was incubated for 20-22 hrs in collagenase type II solution containing 0.15% collagenase (Worthington), Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Suspension was filtered through a 100 μm mesh nylon filter (cell strainer Nucleon) and cells were washed 2 times with PBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). For expansion chondrocytes were plated at a density of 3.5 * 104 cells/cm2 and cultured in proliferation medium (PM) containing Dulbecco's minimal essential medium (DMEM) supplemented with 10 % fetal bovine serum, 1x non- essential amino acids (Sigma-Aldrich), 10 mM HEPES buffer (Biowhitaker), 0.2 mM

ascorbic acid 2-phosphate (InVitrogen), 0.4 mM proline (Sigma-Aldrich), 100 U/ml penicillin (InVitrogen) and 100μg/ml streptomycin (Sigma-Aldrich). Medium was refreshed every 3-5 days and cells were cultured in an incubator at 37°C and 5% CO2. After 2-3 passages (9-12 population doublings), expanded cells were mixed with primary chondrocytes for seeding onto a porous scaffold.

Cell seeding and culturing on 3DF scaffolds

Cylindrical scaffolds were sterilized in isopropanol for 4 hours, rinsed in PBS extensively, incubated overnight in PM and blotted dry prior to seeding. Primary and expanded chondrocytes were mixed at a 1:5 ratio, centrifuged at 300 g for 5 min and re-suspended in 54 μl of PM containing 300 μg/ml fibronectin. Scaffolds were seeded with 54 μl of cell suspension containing 3 x 106 cells for 1 hr, after which medium was carefully added. Construct were cultured statically in PM for 4 weeks in an incubator at 37°C and 5% CO2.

Biochemical Analysis

Constructs (n=3) for quantitative analysis of sulphated glycosaminoglycans (GAGs) and cell number were washed with PBS and frozen overnight at -80°C. Subsequently they were digested with 1 mg/ml proteinase K (SIGMA) in Tris/EDTA buffer (pH7.6) containing 18.5 μg/ml iodoacetamide and 1 μg/ml pepstatin A (SIGMA-Aldrich) for >16 hrs at 56°C. GAG content was spectrophotometrically determined with 9-dimethylmethylene blue chloride (DMMB)(Sigma-Aldrich) staining in PBE buffer (14.2 g/l Na2HPO4 and 3.72 g/l Na_2EDTA, pH 6.5) with a micro plate reader (Bio-TEK instruments) at an absorbance of 520 nm with chondroïtin sulfate as a standard.

Cell number was determined via quantification of total DNA with CyQuant DNA kit according to the manufacturer's description (Molecular probes) and fluorescent plate reader (Perkin-Elmer). Quantitive total GAG and total DNA data were normalized for differences in wet weight of the scaffolds.

Scanning Electron microscopy (SEM) Analysis

Tissue constructs were also analyzed by SEM. Samples were fixed overnight in 0.14 M cacodylate buffer (pH = 7.2 – 7.4) containing 0.25% glutaraldehyde (Merck). Scaffolds were subsequently dehydrated in sequential ethanol series and critical point dried from liquid carbon dioxide using a Balzers CPD 030 machine. Specimens were then gold sputtered (Cressington) and studied under the SEM.

Mechanical Characterization

A dynamic mechanical analysis (DMA) instrument (Perkin Elmer 7e) was used to measure the dynamic stiffness of the 3DF scaffolds before and after culturing (n=6). Cylindrical fixtures were chosen to test the specimens and evaluate their behaviour as a whole structure along their compression axis, in the "Z-direction".

Scaffolds were loaded with a dynamic force varying from 100 mN to 150 mN in a dynamic stress experiment. More specifically, a starting force of 100 mN was applied and then continuously increased to 150 mN with a sinusoidal loading ramp of 5 mN/min, at a constant frequency of 1Hz. The dynamic stiffness was calculated by the instrument software (Pyris). The tests were conducted under wet conditions: a synovium-like fluid was prepared as a 3% (w/v) solution of poly(vinyl pyrrolidone) (PVP) in de-ionized water. This was done to simulate the physiological environment of articular cartilage. Scaffolds were soaked in PVP-solution, before loading. The temperature was 37 ºC during all of the experiments.

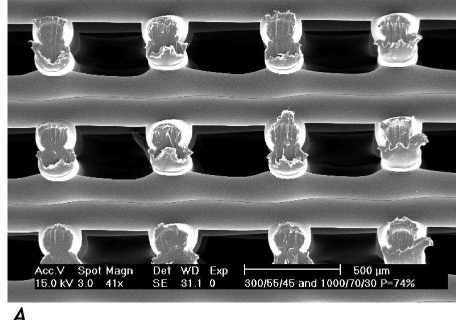
Statistical Analysis

Statistical Analysis was performed using a two-tailed Student's t-test with two sample equal variance, where the confidence level (p) was set to 0.05 for statistical significance, unless otherwise specified. Values in this study are reported as mean and standard deviation.

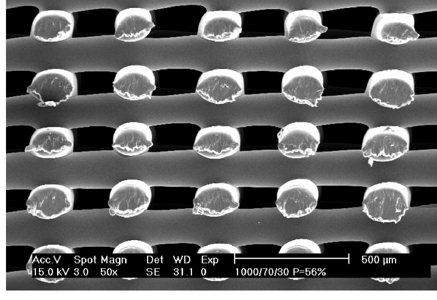
Results

Scaffolds Characterization

SEM analysis revealed a fibre diameter d1 of 170 ± 15 µm, a fibre spacing d2 of 605 ± 12 µm for the NM scaffolds and of 377 ± 3 µm for the M scaffold, and a layer thickness d3 of 148 ± 10 µm for the NM scaffolds and of 140 ± 7 µm for the M scaffold. This corresponded to rectangular pores of 435 ± 2 µm in the X-Y plane by 148 ± 10 µm in the Z-plane for the NM scaffolds, and of 202 ± 7 µm in the X-Y plane by 140 ± 7 µm in the Z-plane for the M scaffold (Figure 1). The porosity of the NM scaffolds was 74 ± 2%, while the porosity of the M scaffold was 56 ± 4%.



A

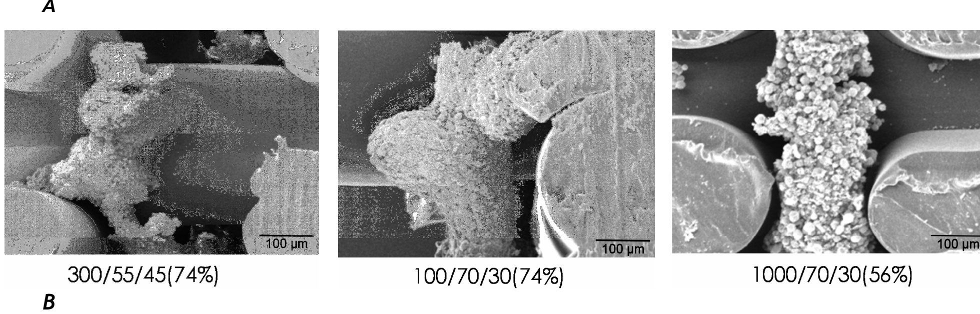


B

***Figure 1** SEM micrographs of scaffolds architecture in terms of pore volume for (a) 300/55/45 and 1000/70/30 NM with a porosity of 74% and a starting stiffness of 1 Mpa, and for (b) 1000/70/30 M with a porosity of 56% and a starting stiffness of 10 MPa.*

μg DNA/scaffold
30
20
10
0
300/55/45 (74%)
1000/70/30 (74%)
1000/70/30 (56%)
Mw PEG/ PEGT/PBT ratio (porosity)

A



B

Figure 2 Seeding efficiency (a) and ESEM pictures (b) of 3DF PEGT/PBT scaffolds seeded with chondrocytes (day 1).
*(a) Quantative DNA assay results representing the relative amount of cells after 1 day of static seeding. Cells seeded are 1:5 primary/expanded chondrocytes. Scaffold composition is described as Mw PEG/ ratio PEOT/PBT (porosity %). * = significantly different from 300/55/45 (74%), + = significantly different from 1000/70/30 (74%). (b) ESEM micrographs of the 3DF constructs showing a maintained or recovered rounded morphology in all the scaffolds due to fibronectin aggregation. Scale bar: 100 μm*

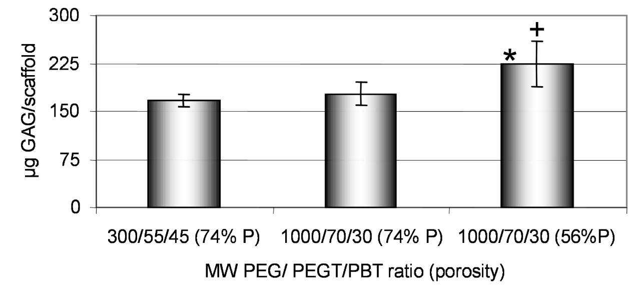


Figure 3 Total glycosaminoglycans (GAG) of in vitro cultured PEOT/PBT constructs (4 wks).
*Composition scaffold is described as Mw PEG/ PEOT/PBT ratio (porosity). Constructs were seeded with 20% primary chondrocytes and 80% expanded chondrocytes with fibronectin aggregation and cultured for 4 weeks. * = significantly different from 300/55/45 (74%), + = significantly different from 1000/70/30 (74%).*

scaffold composition	total GAG	Total DNA	GAG/DNA
	μg	μg	μg/μg
300/55/45 (74%)	166.3 ± 9.1	41.5 ± 4.3	4.0 ± 0.5
1000/70/30 (74%)	176.6 ± 18.2	34.0 ± 3.9 *	5.2 ± 0.8
1000/70/30 (56%)	223.3 ± 35.6	36.9 ± 3.9	6.1 ± 1.2

Table 1 Total glycosaminoglycans (GAG), total DNA and GAG/DNA of in vitro cultured PEOT/PBT constructs (4 wks).

Tissue Formation

Seeding efficiency results at day 1 showed that on NM 300/55/45 (74%) and M 1000/70/30 (56%) the amount of DNA was significantly 25% higher compared to NM 1000/70/30 (74%) (Figure 2A). SEM evaluation showed that at day 1, chondrocytes regained or maintained a spherical morphology after fibronectin mediated aggregation seeding on all scaffolds (figure 2B). Subsequently, seeded cells were distributed more homogeneously in these two types of scaffolds, than in the NM 1000/70/30. Interestingly, after 4 weeks of culture tissue formed contained significantly 35% more GAG on M 1000/70/30 (56%) compared to NM 300/55/45 (74%) and NM 1000/70/30 (74%) (Figure 3). The amount of DNA in the constructs was significantly lower only on NM 1000/70/30 (74%) compared to NM 300/55/45 (74%) and M 1000/70/30. Consequently, GAG/DNA was highest in tissue formed on M 1000/70/30 (56%). At this time point, while on 300/55/45 cells and tissue appeared as stretched structures which align with the scaffold surface and form long fibres, in 1000/70/30 (NM and M) cells still appeared to be spherical and tissue formed is condensed without any extrusions (Figures 4g, 4h, 4i). Additionally, it seems that ECM formed is filling the pores to a higher degree in the M 1000/70/30 scaffold as compared to NM 300/55/45 (Figure 5). This together with the quantitive GAG data indicates more cartilaginous tissue formation in M 1000/70/30 scaffolds.

Cartilage Construct Mechanical properties

The dynamic stiffness of the constructs and of empty scaffolds soaked and incubated in medium was measured at different time points to examine the contribution of tissue formation and of scaffold degradation during culture. The dynamic stiffness of NM 300/55/45 tissue constructs increased from 1.01 ± 0.13 MPa prior to culture, to 1.41 ± 0.23 MPa at day 1, and to 1.68 ± 0.09 MPa at day 28 (Figure 6). No significant change in the stiffness was measured for NM 300/55/45 empty scaffolds left in culture media at 37°C during the whole culture period. Similarly, the dynamic stiffness of NM 1000/70/30 cartilage constructs increased from 1.06 ± 0.13 MPa before culturing, to 1.4 ± 0.2 MPa at day 1, and to 2.75 ± 0.2 MPa at day 28. A slight decrease in stiffness of NM 1000/70/30

empty scaffolds was measured as 0.92 ± 0.18 MPa after 28 days. Interestingly, the dynamic stiffness of M 1000/70/30 cartilage constructs increased from 10.18 ± 1.04 MPa prior to culture, to 13.40 ± 1.3 MPa at day 1, and to 13.51 ± 2.56 MPa at day 28. A consistent decrease in the stiffness of M 1000/70/30 empty scaffolds was also measured as 7.75 ± 2.15 MPa after 28 days. Since the dynamic stiffness of this construct remained constant over during culture, it can be inferred that a similar speed of tissue formation and of scaffold degradation occurs for the matching 1000/70/30 architecture. In line with the previous findings, tissue formed in the M 1000/70/30 scaffold contributed more to dynamic stiffness of the construct, compared tissue formed onto NM configurations (Figure 7). Specifically, the net tissue formed stiffness increased from 0.4 ± 0.18 MPa to 0.54 ± 0.06 MPa for NM 300/55/45, from 0.34 ± 0.06 MPa to 1.47 ± 0.15 MPa for NM 1000/70/30, and from 3.22 ± 0.29 MPa to 5.06 ± 1.74 MPa for M 1000/70/30.

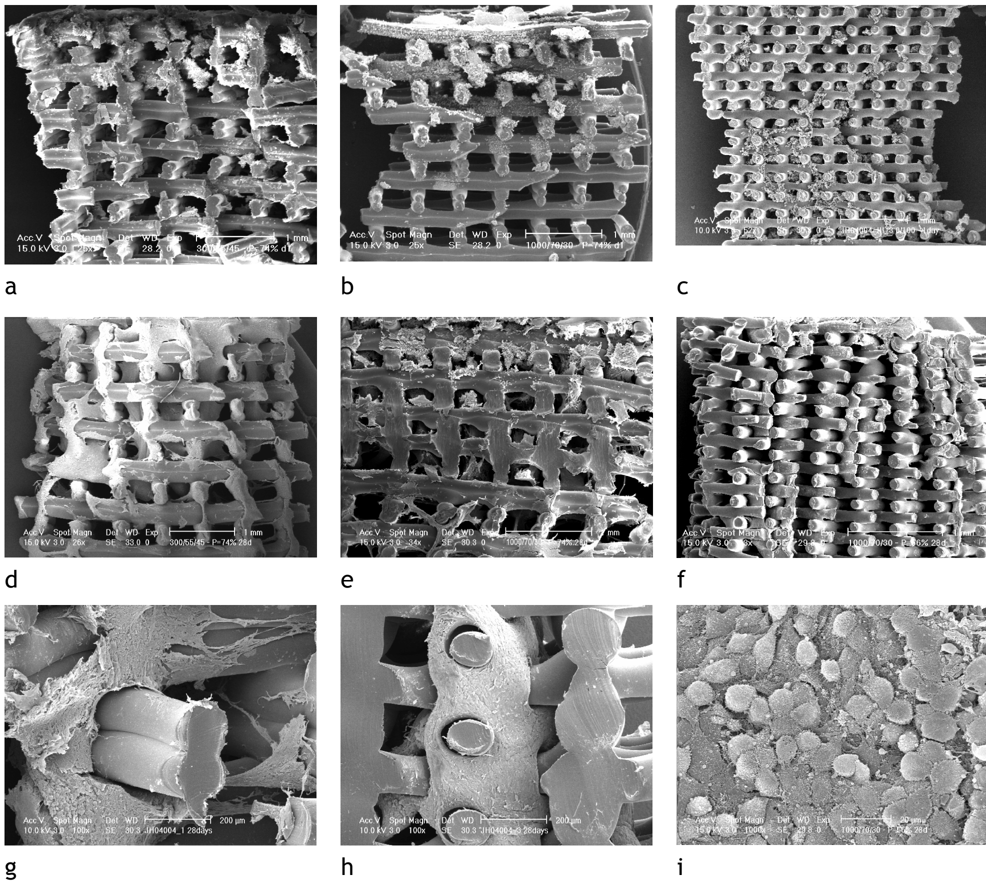
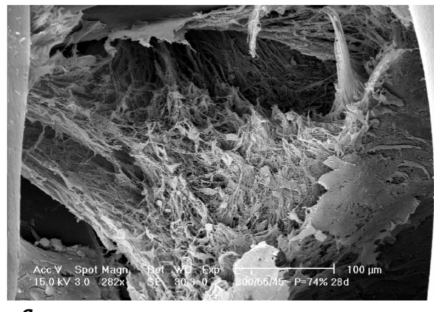
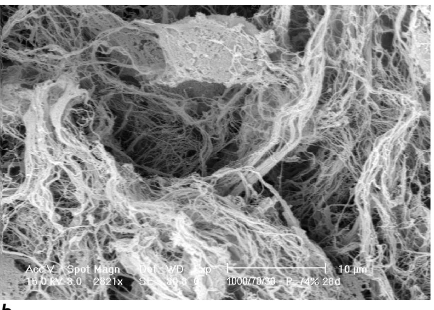


Figure 4 SEM analysis of cell distribution and matrix formation on NM (a, b, d, e) and M (c, f) constructs at day 1 (a, b, c) and at day 28 (d, e, f). *Cells attach better to 300/55/45 NM (d) but dedifferentiate (g), while they are better entrapped and more homogeneously distributed in 1000/70/30 NM and M (e, f), where they maintain their phenotype (h, i). Scale bar: (a - f) 1 mm; (g, h) 200 µm; (i) 20 µm.*



a



b

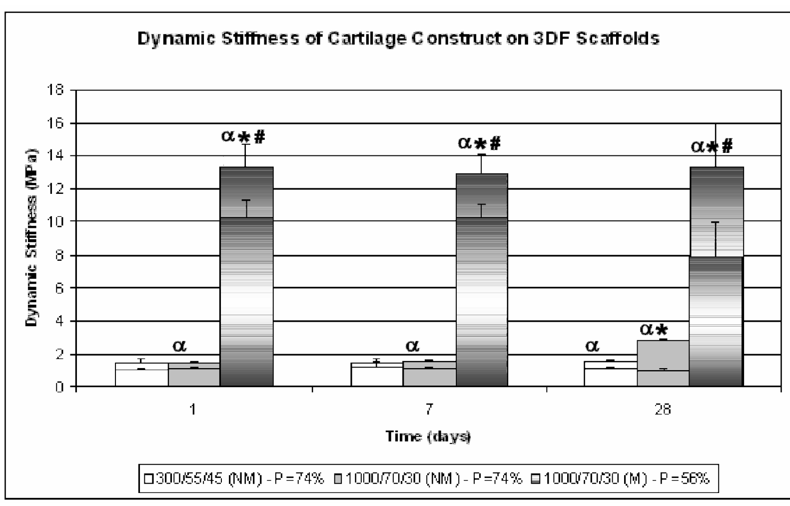
Figure 5 SEM micrographs show extra cellular matrix (ECM) formation after 28 days both in 300/55/45 NM (a) and 1000/70/30 NM and M (b), respectively. Scale bar: (a) 100 µm; (b) 10 µm.

Discussion and Conclusions

Previous work from our group showed that the oxygen tension rapidly decreases from 1 to 2 mm of scaffold depth during chondrocyte culture in 3DF scaffolds as well as compression molded CM scaffolds (both 80% porosity) (19). However, cell distribution and matrix deposition of articular chondrocytes from immature origin was still enhanced in 3DF scaffolds (80% porosity) compared to CM scaffold. In the here described study the difference in tissue formation of chondrocytes from mature origin in 3DF scaffolds with a porosity of either 74% or 56% was examined. If porosity does not hamper homogenous cartilage tissue formation it is possible to manufacture 1000/70/30 scaffolds, having favourable surface properties, made matching mechanical properties of cartilage.

Therefore, 3D fibre deposited PEOT/PBT scaffolds with different physico-chemical properties, but similar mechanical properties resulted from high pore volume architecture were first fabricated to evaluate the influence of the polymer composition on tissue formation. Next, 3DF scaffolds with favourable surface properties and with matching mechanical properties of cartilage resulted from a low pore volume architecture were also fabricated to assess the influence of pore volume and architecture on cartilage regeneration. While porosity and pore volume decreased, interconnectivity of pores was not impaired (Figure 1) resulting in increased mechanical stability of the M 1000/70/30 scaffold compared to the NM 1000/70/30. In this study, we showed that at low porosity (56%) in M 1000/70/30 cartilage tissue formed by mature bovine chondrocytes homogenously distributed throughout the scaffold and the amount of GAG is significantly enhanced compared to higher porous NM 300/55/45 or NM 1000/70/30 scaffolds. Two factors appear to play an important role with increased cartilagenous tissue formation on low porosity M 1000/70/30 scaffolds: seeding efficiency and pore interconnectivity. Seeding efficiency on more protein adhesive surfaces like 300/55/45 is depending on cell attachment, while on less protein adhesive surfaces like 1000/70/30 chondrocytes have

shown to maintain a spherical morphology and do not attach. Previously, chondrocytes treated with fibronectin prior to seeding showed to aggregate and enhance cartilage tissue formation (25),(26). In the study here described, it was shown that fibronectin cell aggregation increased seeding efficiency on M 1000/70/30 with a lower porosity compared to NM 1000/70/30 with a higher porosity. From this, we concluded that more aggregates were captured and sustained in M 1000/70/30 as a result of the lower porosity of this scaffold. Second, pores were fully interconnected for all 3 scaffolds. Tissue formation of chondrocytes from an immature model is not hampered at low porosity in clinical relevant size scaffolds as previously mentioned (18). From the results shown here, we conclude that low porosity does not hinder cartilage tissue formation by chondrocytes originating from a mature model throughout a scaffold. We conclude that the high interconnectivity of the pores allows for a decreased porosity thus preventing detrimental nutrient or oxygen limitations in the scaffold. Next, we studied the net result of tissue formation and scaffold degradation in vitro on dynamic stiffness of 1000/70/30 and 300/55/45 (NM) and 1000/70/30 (M). We showed that the dynamic stiffness of 300/55/45 scaffolds without cells remained unchanged over 28 days. In contrast, dynamic stiffness of both NM and M 1000/70/30 showed to decrease respectively of 15 and 24% when incubated in medium for 28 days. This is in agreement with what has been previously shown by Deschamps et al. (10), where degradation of scaffolds with a high water uptake like 1000/70/30 occurred faster due to more rapid hydrolysis compared to scaffolds like 300/55/45 that take up less water.



7

Figure 6 Dynamic Stiffness of cartilage constructs (higher bars) and of empty scaffolds (lower bars) at day 1, 7, and 28.
(a) indicate significant differences of the cartilage constructs with respect to the empty scaffolds; (*) point at significant differences with respect to 300/55/45 NM; (#) show significant differences as compared to 1000/70/30 NM.

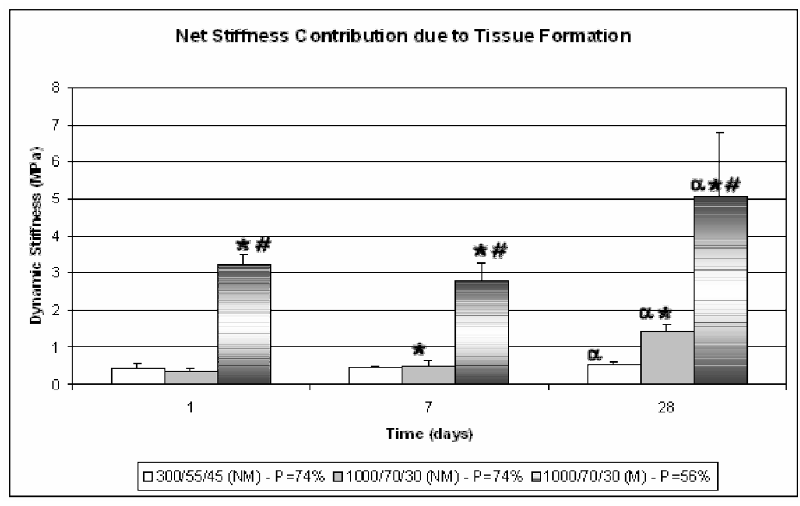


Figure 7 Net stiffness of the Tissue formed on the scaffolds, calculated as the difference between the stiffness of the constructs and the stiffness of empty scaffolds maintained in cartilage medium at physiological values for the same culture period.
(a) indicate significant differences during culture time; (*) show significant differences from 300/55/45 NM; (#) point at significant differences from 1000/70/30 NM.

Surprisingly, the dynamic stiffness already increased of 0.4, 0.34, and 3.22 MPa when NM300/55/45, NM1000/70/30 and M 1000/70/30 were seeded with cells, respectively. Recently it has been shown that the shear modulus of a single mammalian cell (the shear modulus can be assumed to correlate with the stiffness by a factor varying between 0.5 and 1, depending on the Poisson's ratio) ranges between 20 and 60 Pa (27),(28). In an ideal situation all the seeded cells are distributed homogenously throughout a scaffold. If we consider cells as a biological "material", this would result in a corresponding homogeneous stress distribution on loading. Thus, if we consider the Voigt-Reuss model to calculate the modulus of the scaffold-cell composite (29),(30), the theoretical contribution of the seeded cells to the modulus of the construct would be negligible, considering that cells fill the pores of the scaffolds. However, in this case cells were aggregated with fibronectin prior seeding. It might be that fibronectin and aggregated cells contributed to the stiffness of the construct at an early stage. Welsh and Tirrell showed that the stiffness of protein engineered fibronectin varies with its molecular weight from 0.1 MPa to 0.321 MPa (31). Furthermore, few researchers showed that when cells are exposed to fibronectin or fibronectin coated surfaces their intrinsic stiffness is significantly higher (32),(33). This might explain the contribution of cells to the dynamic stiffness of the NM constructs here investigated. However, it does not explain the higher contribution of cells in M 1000/70/30 scaffolds with a higher initial dynamic stiffness

compared to the other scaffolds with a lower initial dynamic stiffness. From quantitative data shown in Figure 2 it is clear that the amount of cells in M 1000/70/30 is not significantly different from the amount of cells in NM 300/55/45. However, the increase in dynamic stiffness after seeding is approximately 10-fold more for the first construct compared to the latter. One of the major differences between these scaffolds besides the stiffness is their porosity. From a pure material point of view, if we again consider cells as biological “materials”, we can expect a higher contribution to the dynamic stiffness by a given number of cells seeded and entrapped into a scaffold with low porosity as compared to a scaffold with high porosity (34),(35). This is due to the power (34) or exponential (36) relationship that links the static or dynamic stiffness, respectively, to the porosity of a cellular solid. For a scaffold with high porosity, a reduction in porosity results in a small increase of the scaffold’s stiffness. For scaffolds with a low porosity, a small decrease may cause a significant increase in stiffness. This suggests that the contribution of the cells to the mechanical properties in M 1000/70/30 with 56% porosity is much higher than in NM 300/55/45 or NM1000/70/30 with 74% porosity. Moreover, since in the case of M 1000/70/30 the dynamic stiffness of the cartilagenous construct remains substantially constant during time and the stiffness of empty scaffolds decreases in time, a similar speed of tissue formation and scaffold degradation rate could be inferred.

Finally, results showed that the dynamic stiffness of both M and NM 1000/70/30 constructs significantly increased as compared to NM 300/55/45 constructs. When taking into account that both M and NM 1000/70/30 degraded in medium, it is clear that scaffolds with this physicochemical composition support cartilaginous tissue formation with extra cellular matrix composition contributing substantially more to the mechanical properties of a construct. Taken together, these findings suggest that by accurately controlling the pore volume and the total porosity of a 3D scaffold with a completely interconnected pore network, it is possible to improve cell aggregated seeding efficiency and cartilage tissue formation. In the specific case of 1000/70/30 this was associated to appropriate physicochemical properties for sustaining chondrocyte rounded morphology and to a dynamic stiffness matching articular cartilage. 3DF scaffolds with low pore volume and low porosity made with 1000PEOT70PBT30 could, therefore, be interesting candidate to regenerate articular cartilage in long-term applications.

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Chapter 8

Conclusions & future perspectives

Chapter 8

Tissue engineering research

In adult tissues, continuous cellular interactions between different cell types and extracellular matrix proteins and (growth) factors maintain the form, spatial organization and physiological function of these cells. Repair and regeneration of these tissues is multifaceted and requires an ordered, integrated use of processes including intercellular communication (via extracellular and intracellular signalling), gene expression and morphogenetic signals. When damage to tissues is beyond their regeneration capacity this irreversibly leads to tissue's erosion and decreased functionality. In many species, regeneration of tissues is possible, but this capacity is has been largely lost in mammals.
The cellular interaction chondrocytes engage in cartilage is depending on their location in the cartilage zones. The spatial organization of articular cartilage recognizes four distinctive regions; the superficial zone, transitional zone, radial zone and the tight zone (Chapter1, Figure1). Chondrocytes committed to these distinctive zones maintain their differentiated status through specific cellular interactions depending on their localization. Chondrocytes in the superficial zone interact and maintain their phenotype and physiology through cellular interactions with synovial cells and factors present in the synovial fluid. Cellular interactions in the transitional zone and radial zone are mainly taking place between chondrocytes and extracellular matrix or factors embedded in the extracellular matrix. Whereas, chondrocytes found in the tight zone, at the cartilage-bone border also interact with osteoblast from the subchondral bone and solid bone matrix. When a defect is inflicted on cartilage, the lack of blood vessels and nerves results in the lack of a suitable cartilage repair or regeneration reaction. In cartilage tissue engineering this lack of regeneration capacity in vivo is attempted to be overcome by regenerating cartilage in vitro with patient own cells. It is very unlikely that complete cartilage regeneration can be initiated by the delivery of one growth factor applied at an aspecific (over)dose at one time point or during an specific duration of time. Nor that delivery of one (or two) growth factors to a defect, can trigger a whole cascade of cellular events necessary for regeneration into normal shaped and functional cartilage tissue. Primary articular chondrocytes have shown to produce and secrete some of the cytokines, growth factors and extra cellular matrix components that stimulate chondrogenic metabolism or initiate chondrogenesis (1),(2),(3),(4). In addition, it has been shown that expanded chondrocytes as well as mesenchymal stem cells, produce and secrete extracellular matrix proteins, cytokines or growth factors (5),(6),(7),(8). In cartilage defects the presence of extra cellular matrix and primary cells in the surrounding tissue or access to bone marrow from

the subchondral bone was shown not be sufficient to initiate an proper repair or regeneration reaction (9-11),(12),(13),(14). Prior to investigation on which and how cellular interactions can influence cartilage regeneration in vitro, the following question was formed:

"Can cellular interactions between chondrocytes and other cell types influence proliferation of chondrocytes and/or formation of cartilage tissue?"

Results in chapter 3 show that stratified culture of chondrocytes allows them to continue exponential proliferation and overcome nutrient limitation in culture. Since chondrocytes are in close contact during stratified culture, it is very likely that tolerance to nutrient limitation is the result of cellular interactions between chondrocytes. Furthermore, findings described in chapter 4 and 5 suggest that primary chondrocytes enhance their cartilage tissue when co-cultured or co-implantation in a nude mouse model together with other cell types. Besides cell-cell interaction between primary chondrocytes and other cell types, enhanced cartilage tissue formation could also be the result of a "dilution" effect. This hypothesis is based on continues cellular interaction of primary chondrocytes with their environment. In micromass co-culture, the initial absence of cartilage specific extracellular matrix can be "sensed" by primary chondrocytes through membrane receptors involved in cell-ECM interactions (15-17). At the same time, the 3D environment supports chondrogenic differentiation rather than primary chondrocyte proliferation. The hypothesis was that with decreasing primary chondrocytes concentrations, cartilage specific physiology is stimulated. Culture in hydrogels dilutes chondrocytes and provides them with a 3D environment. Several hydrogels have been shown to support cartilage tissue formation by chondrocytes (chapter 1). Nevertheless, several studies culturing chondrocytes in different hydrogels showed a correlation between high cell density and increased cartilagenous tissue formation (18),(19).These data suggest that upon dilution of chondrocytes, cartilage tissue formation is not enhanced. Therefore most likely, enhanced cartilage tissue formation by primary chondrocytes in co-culture or upon co-implantation (chapter 4-7), is the results of cellular interactions between two cell types. These findings, together with findings in coculture studies of others combining chondrocytes with other cell types, showed that the co-culture and co-implantation are suitable models to evaluate cellular interactions playing a role in tissue regeneration.

In this thesis, research described and discussed in chapters 3-7 show that cellular interactions support proliferation and cartilage tissue formation of articular chondrocytes. In the remaining part, the possible influence of these outcomes on future tissue engineering research and clinical application is discussed. Identification of cell types, cellular interactions and factors involved in successful regeneration of a tissue could circumvent failing tissue generation in critical size defects. For cartilage at this moment,

no suitable model is available which could give clues on cellular interaction cascades and factors that are involved in normal cartilage regeneration, or so it seems. Cartilage regeneration studies in animals capable of regeneration could help to unravel cell types involved in required cell-cell interactions during natural regeneration of cartilage. In contrast to for example urodeles, mammals have lost the ability to regenerate largely. Urodele amphibians can regenerate fully functional multi-tissue structures of a tail or limb including cartilage by transdifferentiation of notochord cells from the spine (20). Regeneration of tail cartilage in for example axolotl is accomplished by cellular interactions and involves transdifferentiation of differentiated notochord cells from embryonic ectodermal origin to seemingly unrelated chondrocytes from embryonic mesodermal origin. In contrast, it seems that tail regeneration of Xenopus leavis tadpoles does not involve metaplasia or transdifferentiation. Regeneration of tissues in the tail of these tadpoles is found to be similar to normal growth and cell turnover in mammals (21). Thus, tail regeneration of Xenopus tadpoles might provide a suitable model to study cartilage regeneration. However, in some cases also in mammals regeneration can be revived. By coincidence, the regenerative capacity of a mouse strain was discovered. This mouse strain (MRL/MpJ) is an inbred strain generated as a control mouse for a mouse strain with a Fas mutation. As does the Fas mutant strain, the MRL/MpJ strain exhibits autoimmune disorders, but symptoms are manifested much later in life. Upon through-and-through ear punches creating holes, which in other mouse models are used for identification, the MRL mouse is capable of multi-tissue regeneration including cartilage (22). Microarray analysis and SELDI Protein Chip analysis of MRL mice compared to a mouse strain not capable of regeneration, have identified changes in expression patterns suggesting that in MRL mice there is less of an inflammatory response and an earlier transition into tissue repair than is seen in other mice strains (23) (24). Interestingly, in all of the models mentioned, regeneration it is not limited to one tissue, but extends to cartilage, skin, blood vessel and hart tissue (23),(25). Apparently, the differences between animal models that have lost their regeneration capacity and animal models that maintained their regeneration capacity are common denominators, important for tissue regeneration of all tissues (20),(26). This emphasizes that in cartilage tissue engineering, studying cartilage regeneration and cellular interaction involved has more potential to engineer a functional tissue, than studying cartilage repair.

While differences in cellular response to defects are examined in regenerating animal models compared to animal models not capable of regeneration, a model to investigate the relevance of these finding for human cartilage regeneration was lacking. Findings described in this thesis on the influence of chondrocyte co-cultured or co-implanted with different cell types, indicate these are promising models to study cellular interaction involved in tissue regeneration with clinically relevant cells.

In tissue engineering, studying cellular interactions between different cell types and its influence on tissue formation is gaining interest. Understanding and being able to direct

cellular interactions between different cell types, cells and extracellular matrix proteins and scaffolds is crucial for the engineering of (multi)cellular tissues and organs. Co-culture provides a suitable model to study these cellular interactions. For future studies on cellular interaction of different cell types in co-culture and their influence on tissue formation, it is of the utmost importance to localize cell types after co-culture, their specific physiology and differentiation status. In the meantime, there is an increasing need in the clinical for regenerative medicine in cartilage repair. Patients with a cartilage defect now do not have the luxury of time to wait until the perfect solution in tissue engineering is available. Such patients can be supported relatively soon by translating knowledge currently available in cartilage research to safe and effective clinical therapies.

Clinical applications in cartilage repair

Autologous chondrocyte implantation

Findings described in this thesis show that cellular interaction between primary chondrocytes and expanded chondrocytes in addition to a variety of other cell type's results in enhanced cartilage tissue formation (chapters 4 and 5). This was shown in vitro with micromass culture and in a nude mouse model in vivo. The new culture method described in chapter 3, allows for application in a truly autologous cell therapy, and is very time and resource efficient. Expansion of chondrocyte can be promoted with only a limited amount of serum required and the chondrogenic capacity of resulting cells remains unchanged or even enhanced upon expansion. Stratified culture for efficient expansion of chondrocytes followed by combining primary chondrocytes with expanded chondrocytes just prior to implantation, possibly would offer a substantial improvement to autologous chondrocyte implantation (ACI) which is currently available in the clinic. However, before application in the clinic, animal studies in a suitable animal model should provide data about safety of the proposed model.

Single surgery for cartilage repair

For the development of the next generation treatments for cartilage repair, enhanced cartilage tissue formation of primary chondrocytes in co-culture or upon co-implantation with for example cultured bone marrow cells or dermal fibroblasts, provides a model for the development of a single surgery cell therapy.

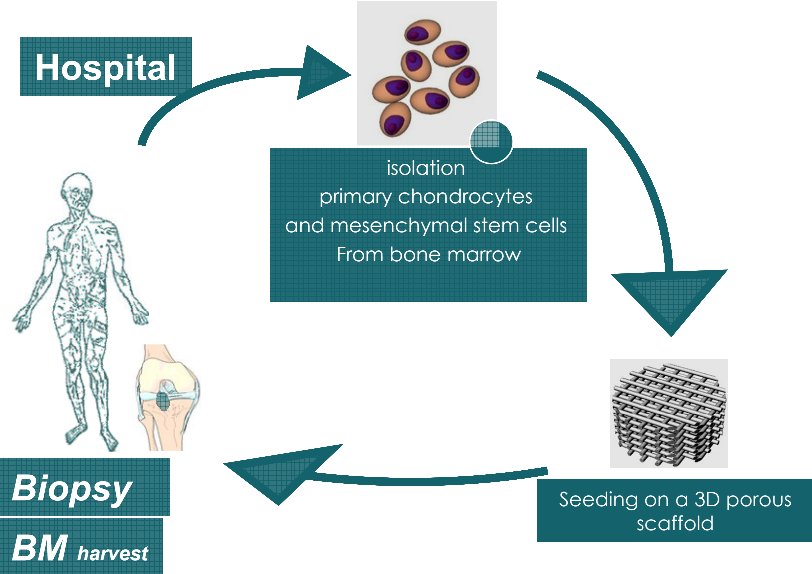

Figure 1 ***Schematic representation of a single surgery model for co-implantation of primary chondrocytes with mesenchymal stem cells from bone marrow.***

The model for single surgery cell therapy commences, during for example knee surgery, by the decision that cell therapy is the most favourable treatment for a specific cartilage defect. Subsequently, primary chondrocytes and a secondary cell type, for example mesenchymal stem cells from bone marrow or fat or dermal fibroblasts from biopsies, are isolated. This is followed by combining the isolated cell types and efficient seeding into a porous scaffold with mechanical properties matching cartilage (chapters 4-7). Implantation of the construct into the (osteo) chondral defect of the patient finalizes the procedure. Developing such a model, which proved to be successful in the laboratory, towards a clinically applicable procedure requires several subsequent assessments. Initially, it is essential to establish cellular interactions between mesenchymal stem cells or dermal fibroblasts that are isolated and, without further expansion, mixed with primary chondrocytes, enhance cartilage tissue formation similarly as described in this thesis with culture cell types (chapters 4-7). Furthermore, single surgery for cartilage repair would require a fast and efficient way to isolate primary chondrocytes. Before applying co-culture for tissue engineering in the clinic, several requirements still need to be addressed, among which are the sustainable influence of co-cultured or co-implanted cell types in a cartilage defect. Animal studies can provide crucial information about efficacy of repair mechanism and safety issues involved upon implantation of scaffolds and/or cells

(27). However, efficacy of single surgery therapies for cartilage repair remains to be shown in the clinic.
Enabling single surgery for cartilage repair in the clinic would circumvent the disadvantage of conventional tissue engineering approaches. Conventional tissue engineering requires, two surgical procedures, complicated logistics, a controlled culture environment time and dedicated personnel and are therefore are time and resource consuming. Due to time and resource efficiency of single surgery cell therapy, a major cost reduction can be made compared to tissue engineering approaches currently available. This would enable an increasing population of patient's, access to an autologous cell therapy for cartilage defects.

The studies in this thesis strongly promote the use of autologous single surgery cell therapy as a new solution in cartilage tissue repair in the way to ultimately, develop a regenerative treatment for cartilage defects.

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Summary

Tissues are complex 3-dimensional structures with a highly organized architecture made up of cells and matrix. The cells and matrix in a tissue are continuously interacting with each other and (cells from) their surrounding tissues to maintain their form and function. Interactions of cells with their surrounding cells and matrix are equally important for a successful repair reaction. While in tissue engineering significant progress has been made, still a number of challenges need to be overcome for the development of clinically applicable treatments. One of the challenges in cartilage tissue engineering is to direct cellular interactions involved in either engineering of a repair tissue outside of the body or an appropriate repair reaction in situ. Cellular (inter)actions can be influenced indirectly via scaffolds or directly by supplying the cells with growth factors, extracellular matrix proteins or other cells. This thesis reviews and discusses research data and model systems related to the influence of cellular interactions between chondrocytes and extracellular matrix proteins, scaffolds and other cell types on cartilage tissue formation. With this in mind, in chapter 3 the growth behavior of human articular chondrocytes during stratified culture as opposed to routine culture to confluency was studied and described. Depending on donor variation and factors supplemented to the medium, stratified culture increased the cell yield tremendously compared to conventional culture. Furthermore, stratified cultured cells maintained their differentiation capacity with or without growth factors (bFGF, TGFβ and PDGF), but not when cultured with high levels of AsAP and NEAA. In contrast to other 3D culture techniques like micro-carrier or suspension culture, nutrient consumption during stratified culture remained equal to during conventional expansion. Possibly cellular interactions between chondrocytes occuring during stratified culture increases their tolerance for nutrient limitation.
For biotechnological research in vitro in general and tissue engineering specifically, it is essential to mimic the natural conditions of the cellular environment as much as possible. Most tissues in the body consist of more than one cell type. In a co-culture system, two or more cell types are brought together in the same culture environment enabling them to interact and communicate. Co-culture proved to be a powerful tool in unravelling the importance of cellular interactions during physiology, communication and homeostasis. In chapter 2 the history of co-culture studies and more specifically the role it plays in cartilage tissue engineering is described and discussed. In the study described in chapter 4, the influence of cellular interactions on cartilage tissue formation when primary chondrocytes are co-cultured with expanded chondrocytes, dermal fibroblasts, embryonic stem cells or 3T3-eeder cells was studied. Cell type(s) contributing to cartilage tissue formation when in co-cultures were identified. Results shown, suggest that primary chondrocytes enhance their cartilage tissue formation when co-cultured with expanded

chondrocyte, dermal fibroblasts, embryonic stem cells or 3T3-feeder cells. Cartilage tissue formation enhanced 3 to 5-fold when primary chondrocytes were co-cultured with expanded chondrocytes, dermal fibroblasts, embryonic stem cells or 3T3-feeder cells. It was hypothesized that cellular interactions between the two cell types in co-culture, stimulated primary chondrocytes to enhance their cartilage matrix formation, possibly through trophoblastic factors.

These vitro results showed that cartilage tissue formation was enhanced when 20% primary chondrocytes were co-cultured with other cell types. In chapter 5 the potential of primary articular chondrocytes to enhance matrix production, when mixed in different ratios with expanded chondrocytes or mesenchymal stem cells in vitro and in vivo, was examined. Primary chondrocytes were mixed with expanded chondrocytes at different ratios and, either co-cultured in a micromass model, or co-implanted in nude mice after seeding onto 3D-fiber, deposited PEGT/PBT block co-polymer scaffolds. The results demonstrated that primary chondrocytes combined with expanded chondrocytes or mesenchymal stem cells enhance cartilage specific matrix production in micromass culture in vitro, as well as in scaffolds in vivo. The results emphasize that cellular interaction between two cell types in co-culture might promote cartilage tissue formation. Thus, providing the basis for development of a novel cell therapy intended for in situ cartilage tissue engineering. In tissue, engineering cells are combined with biomaterials to provide to the cartilage defect with immediate mechanical stability and possibly cues to guide specific tissue formation. Efficient seeding of cells into a porous scaffold showed to be important for tissue engineering. In chapter 6 the influence of cell-fibronectin interactions on seeding efficiency and subsequent cartilage tissue formation of chondrocytes was examined in vivo and in vitro. Fibronectin-aggregation mediated seeding on porous scaffolds was compared to fibronectin-attachment mediated seeding. Fibronectin-aggregation-mediated seeding stimulated cartilagenous tissue formation of the co-implanted primary and expanded chondrocytes, compared to fibronectin-attachment-mediated seeding. This was even more so for the co-implanted primary and expanded chondrocytes than for primary or expanded chondrocytes alone. These data support that the influence of fibronectin-mediated aggregation on differentiation is depending on initial differentiation status and/or morphology of chondrocytes. The aim of the investigation described in chapter 7 was to assess whether 3DF scaffolds with controlled pore volume and at the same time matching mechanical properties, are indeed effective to support cartilage tissue formation. Next to this, the mechanical stability of the constructs in time during culture was studied. The results imply that 3DF scaffolds with low pore volume and appropriate physico-chemical properties support chondrocyte differentiation. Both formed tissue, and scaffold were shown to contribute to the dynamic stiffness of the constructs during culture. In chapter 8, outcomes presented in the previous chapters are further discussed and future perspectives are elaborated on.

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Jeanine

List of publications

This thesis was based on the following publications:

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Jeanine A.A. Hendriks, Ewart de Bruijn, Roka Schotel, Clemens A. van Blitterswijk and Jens Riesle. Co-culture or Co-implantation of Primary Chondrocytes with Expanded Chondrocytes or Bone marrow Mesenchymal Stem Cells Enhances Cartilage Tissue Formation; A Powerful tool in Cartilage Cell therapy.
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Jeanine A.A. Hendriks, Razvan Miclea , Marcel Karperien , Jens Riesle and Clemens A. van Blitterswijk. Primary chondrocytes enhanced their cartilage tissue formation when co-cultured with dermal fibroblasts, 3T3 fibroblasts and embryonic stem cells. Manuscript in preparation.

Hendriks J.A.A. and Moroni L., Riesle J., de Wijn J.R. and Blitterswijk van C.A.
Three Dimensional Fiber Deposited Scaffolds with Enhanced Cell Entrapment Capacity and Matching Physico-Chemical Properties Favor Cartilage Regeneration. Manuscript in preparation.

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Publication related to this thesis:

L.Moroni, **J.A.A.Hendriks,** R.Schotel, J de Wijn and CA van Blitterswijk
Design of Biphasic polymeric 3D fiber deposited scaffolds for cartilage tissue engineering applications
Submitted to Tissue engineering

Selected Abstracts

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Curriculum Vitea

Jeanine Hendriks werd op 28 april 1968 geboren te Spaubeek. Na opgegroeid te zijn in Sittard behaalde zij het diploma MAVO in 1984 aan het Nic Beckers college te Sittard. Vervolgens behaalde ze achtereen volgens het KMLO diploma in 1988 aan de Zuidlimburgse laboratorium school te Sittard, het MLO diploma in 1992 aan het MBO college te Arnhem en het HLO diploma in 1997 aan de Hogeschool Utrecht. Vanaf 1992 werkt zij aan de Landbouw Universiteit Wageningen als onderzoeksanalist aan de "regeneration and transformation of Vigna unguiculata" bij Dr. T.Sijen in de groep van Prof.Dr. A. van Kammen. Vanaf 1994 werkte ze achtereenvolgens bij het Hubrecht Laboratorium (Nederlands Instituut voor Ontwikkelings Biologie) als onderzoeksanalist aan "anterior-posterior axis formation of Xenopus leavis" bij Prof.Dr. A.Durston en vervolgens als hoofanalist aan "the role of Parathyroid hormone related peptide and its receptor in EM transitions" bij Dr.B.Defize in de groep van Prof.Dr. C.Mummery. Vanaf 1999 werkte zij bij IsoTis BV als hoofdanalist in de kraakbeen onderzoeksgroep van Dr.J.Riesle en vervolgens als manager product development cartilage tissue engineering. Zij begon haar promotie onderzoek in 2003 aan de Universiteit Twente bij de groep van Prof. Dr. Clemens van Blitterswijk. In vanaf 2004 werd zij verder ondersteund bij haar promotie onderzoek door haar co-promotor Dr. Jens Riesle en was ze mede-oprichter van CellCoTec B.V. Vanaf Augustus 2006 is zij werkzaam als product development manager bij CellCoTec BV.