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**Fine-tuning Cartilage Tissue Engineering
by Applying Principles from Embryonic Development**

**Verbeterde controle over kraakbeen tissue engineering
door toepassen van principes uit de embryonale ontwikkeling**

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List of definitions

Autologous	transfer of material (cells, tissue) from a donor to him or herself
Autograft	transplant from a donor to him or herself
Allograft	transplant from genetically non-identical donor of same species
Chondrocyte	cell embedded within the cartilage matrix
Chondrogenesis	cartilage development/differentiation
Collagen II	main collagen type in cartilage
Collagen X	collagen type that is produced by hypertrophic chondrocytes in endochondral ossification, in this thesis it is used as a marker for terminal differentiation or hypertrophy
Differentiation	process by which a cell becomes a more specialized cell type (e.g. chondrocyte)
Elastin	protein, main component of elastic fiber, present in elastic cartilage
Endochondral ossification	formation of bone through a template of cartilage
Expansion	proliferation (=cell divisions) of cells in vitro (to obtain sufficient number of cells)
Gene expression	conversion of the information encoded in a gene first into messenger RNA and later to a protein
Growth factor	protein that regulates cell growth, proliferation or differentiation
Growth factor receptor	a protein embedded in cell membrane that when bound to a growth factor initiates an intracellular signaling pathway
Hypertrophy	increase in cell size, often used as synonym for terminal differentiation of chondrocytes as cells become larger during this process
In vitro	“within glass”, experiment in laboratory controlled environment
In vivo	“within the living”, experiment in living organism
Matrix	extracellular molecules surrounding the cells and providing structural support to form the tissue
Medium	fluid in which cells grow or differentiate in vitro
Mesenchymal stem cell	undifferentiated cell (for instance in bone-marrow), that can differentiate in mesenchymal lineage (i.e. connective tissue)
Nude mouse	laboratory mouse with absent thymus, resulting in inhibited immune response
Osteoblast	bone-forming cell
Osteogenesis	bone development/differentiation
Passage	cycle in in-vitro expansion
Perichondrium	membrane lining cartilage, supplying cartilage with nutrients and containing progenitor cells important for growth and repair of the cartilage
Pellet	construct of cells spinned down in a tube

Progenitor cell	undifferentiated precursor cell, only capable of differentiation into its "target" cell
Proteoglycan	a glycosaminoglycan linked to a protein forming a hydrophilic matrix molecule providing compression strength to cartilage
Scaffold	porous biomaterial on which cells can be seeded, provides form and biomechanical properties even if no extracellular matrix is yet formed by the cells
Stem cell	undifferentiated cell, capable of unlimited self-renewal and differentiation into any specialized cell type
Terminal differentiation	final stage in chondrogenesis, characterized by a.o. collagen X, also called hypertrophic differentiation
Tissue engineering	an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ

List of abbreviations

BGP	β -glycerophosphate
BMSC	bone-marrow derived mesenchymal stem cell
ECM	extracellular matrix
GAG	glycosaminoglycan (an extracellular matrix molecule)
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
MMP13	matrix metallo preteinase 13, matrix degradation enzyme
TGF- β	transforming growth factor- β
VEGF	vascular endothelial growth factor



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GENERAL INTRODUCTION

Cartilage has a very poor capacity for regeneration *in vivo*. In head and neck surgery cartilage defects are usually reconstructed with autologous cartilage from for instance the external ear or the ribs. Cartilage tissue engineering may be a promising alternative to supply tissue for cartilage reconstructions in otorhinolaryngology as well as in plastic surgery and orthopaedics. The aim of this thesis is to find new tools by which cartilage tissue engineering can be better controlled. In head and neck surgery it is important that tissue engineered cartilage is stable and does not mineralize when used for cartilage reconstructions. Therefore, in this thesis our primary focus was the generation of cartilage of a hyaline phenotype that does not mineralize when implanted *in vivo*. For this purpose we studied expanded chondrocytes and adult bone-marrow derived mesenchymal stem cells (BMSC). We hypothesize that new insights for cartilage tissue engineering can be gained by studying *in-vivo* cartilage development during embryonic development. As these processes may involve similar pathways, understanding these common pathways may lead to advances in cartilage tissue engineering. We concentrated on two growth factor signaling pathways known to be important for cartilage development: TGF- β and FGF.

As a background for the research in this thesis we will discuss the following topics in this general introduction: cartilage, cartilage defects in head and neck surgery, cartilage tissue engineering, embryonic chondrogenesis, and growth factors influencing chondrogenesis. We will conclude the general introduction with the aim and outline of this thesis.

Cartilage

Cartilage is a tissue with unique properties. It is avascular, aneural and alymphatic (1) and consists of only one cell type: the chondrocyte. These chondrocytes make up only 1-5% of the wet weight of cartilage, as they are embedded in a large amount of extracellular matrix (ECM). Nutritional supply of the chondrocytes is provided by the perichondrium, a fibrous membrane lining the cartilage, by diffusion through the ECM. The most abundant protein in the ECM is collagen II, which provides the tissue with tensile-strength (2). Proteoglycan is the second major class of matrix molecules, of which aggrecan is the most common. The large, hydrophilic proteoglycan molecules provides the tissue with compressive strength, by attracting large amounts of water to the cartilage matrix (2). In fact, water makes up 60-80% of the wet weight of cartilage (3). The ECM not only supplies the cartilage its biomechanical properties, but also represents the microenvironment of the chondrocytes. Chondrocytes are continuously in contact with the ECM through multiple receptors, and thus the ECM influences chondrocyte differentiation and function.

Classically, cartilage is subdivided in three subtypes based on differential molecular characteristics of the ECM: hyaline, elastic and fibrous cartilage. Hyaline cartilage has the highest collagen II content, and is found in the nasal septum, larynx, trachea, ventral sites of the ribs and the joints. Elastic cartilage, characterized by the presence of elastic fibers in the ECM, is found in the auricle and in the epiglottis. Fibrous cartilage contains a large amount of collagen I and is located in the menisci and the intervertebral disks.

A fourth cartilage subtype could also be specified, although its presence is by definition transient: terminally differentiated or hypertrophic cartilage. It is found during the process of endochondral ossification, in which a cartilage template precedes the formation of bone (4; 5). The skeletal bones as well as the ribs and the perpendicular plate of the nasal septum are formed this way. Chondrocytes in this cartilage template become terminally differentiated and express specific markers such as collagen X, MMP13, VEGF and alkaline phosphatase. This results in mineralization and degradation of the terminally differentiated cartilage, and attraction of vasculature. This vasculature carries bone progenitor cells to the mineralized cartilage which results in replacement of the cartilage by bone (4; 5).

This classical subdivision based on matrix composition (the presence of collagen I, II, X or elastin) is arbitrary. Because of site-specific differences, for instance in mechanical stress load and nutritional properties, cartilage from different locations have adjusted to their specific physiological requirements. For instance, hyaline cartilage in the joints is built up of four zones with distinct characteristics, while hyaline cartilage in the nasal septum is more symmetrical.

Interestingly in this classical classification of cartilage subtypes an important difference is not taken into account: whether or not a perichondrium is present. While articular hyaline cartilage has no perichondrium, both hyaline nasoseptal and elastic auricular cartilage are lined by perichondrium on both sides. This presence of a perichondrium is important as it is known that in this perichondrium undifferentiated progenitor cells reside that form new cartilage (6; 7; 8; 9).

An important problem for cartilage tissue is that despite these progenitor cells it has a very limited spontaneous healing capacity (10; 11; 12). As chondrocytes are embedded in extracellular matrix they are limited in migration to the defect. Furthermore, classic wound healing starts with an inflammatory phase in which proteolytic enzymes are produced to remove damaged extracellular matrix components so that newly formed tissue can fill in the defect. In contrast, in cartilage repair this phase is restricted as no matrix-degrading enzymes are produced by death chondrocytes present in the wound edges (13). As a consequence cartilage defects are usually repaired by fibrocartilage scar tissue, leading to permanent loss of the original structure and function.

Cartilage defects in head and neck

Cartilage provides flexibility and support to the surrounding tissue. In the head and neck area the major function of cartilage is to support the airway and therefore loss of cartilage may be a major clinical problem. Furthermore, cartilage is esthetically important as it defines the appearance of the face by offering support to the nose and external ear. Cartilaginous defects in the head and neck region may be due to congenital malformations, trauma or tumor resection. Cartilage has a limited intrinsic regeneration capacity in vivo and therefore implants are needed for reconstruction of this tissue.

Biomaterial implants are subdivided in autografts and allografts (14). Autografts are obtained from the patient and include cartilage or bone. Allografts or homologous grafts are derived from tissues donated by members of the same species and include irradiated

cartilage. Another option is alloplastic implants, which are synthetic biocompatible polymers (15). Autologous cartilage grafts have relatively high biocompatibility and low risk of infection and extrusion as compared with homografts and alloplastic implants and are therefore preferred (14; 15; 16). In head and neck surgery cartilage defects, for instance of the nose, auricle or larynx, are usually reconstructed with autologous cartilage from donor sites, such as the auricle and the ribs. A major disadvantage of autologous cartilage is its limited availability, especially in large reconstructions or after multiple surgeries. Moreover, donor-site morbidity, such as pain and contour defects in costal cartilage (17) and hypoesthesia of the skin in auricular cartilage (18), is likely to increase when more cartilage is harvested. An extra source of autologous cartilage would be desired. Therefore, autologous tissue-engineered cartilage for reconstructions is a potential alternative.

Cartilage tissue engineering

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or prove tissue function or a whole organ. As cartilage has a very limited spontaneous healing capacity (10; 11; 12) and autologous cartilage supply is limited, cartilage has become an important target in tissue-engineering research. As cartilage consists of only one cell type and does not contain blood vessels or neural innervations, it seems an easy tissue to engineer.

In orthopaedic surgery, cartilage tissue engineering has already been used clinically since the late 1980's to treat focal joint defects with autologous chondrocyte implantation (19). In otorhinolaryngology and head and neck surgery, cartilage tissue engineering may offer a promising alternative for the current use of auricular or costal cartilage to reconstruct cartilage defects. Unfortunately, less research has been performed on cartilage tissue engineering in this specialty (20; 21). However, recent developments such as the first patient receiving a tissue-engineered trachea (22) underline the future possibilities of cartilage regenerative medicine.

A major drawback in head and neck surgery is the well-vascularized subcutaneous transplant site, which, in contrast to the immune-privileged region of the joint, poses the risk of a strong inflammatory response and resorption of the bioartificial cartilage (21). Therefore, not surprisingly, the use of (degradable) scaffolds to create a tissue-engineered cartilage construct has proved unsuccessful in the head and neck region (23; 24). It seems necessary to tissue engineer a construct of cartilage in vitro that consists of only cells and their ECM that has sufficient mechanical properties to support the surrounding tissues from the moment of implantation.

Different cell sources could be used to engineer cartilage. The most obvious choice for an autologous cell source is cartilage itself. Chondrocytes are isolated from the cartilage by degrading the matrix, and can then be expanded in vitro until a sufficient cell number is obtained. Chondrocytes are known to lose their cartilage phenotype during expansion, a process called dedifferentiation (25). However, these cells can be redifferentiated with growth factors, even after extensive cell-population doublings (26; 27; 28; 29). For research

purposes chondrocytes can be harvested as left-over material from patients undergoing surgery (for instance total knee replacement, protruding ear reconstruction or nose reconstruction). In this way chondrocytes isolated from for instance hyaline articular cartilage, hyaline nasoseptal cartilage or elastic auricular cartilage can be studied.

Another popular candidate cell source is adult mesenchymal stem cells (MSC), which can be isolated from different tissues such as bone marrow and adipose tissue (30; 31; 32). In this thesis we studied bone-marrow derived mesenchymal stem cells (BMSC). These cells are isolated from bone-marrow biopsies performed on patients undergoing total hip replacement. Like chondrocytes they can be expanded in vitro until a sufficient number is obtained. BMSC can undergo a lot more population doublings than chondrocytes without losing their chondrogenic potential. BMSC can be differentiated towards many other tissues besides cartilage (such as bone or fat) depending on which medium and growth factors are used. Finally, BMSC can be harvested without creating a new (cartilaginous) defect. Therefore, BMSC are generally considered a popular cell source for tissue engineering.

An important requirement for tissue-engineered cartilage is that it is stable and will not mineralize when implanted in the body for reconstruction. Therefore, recent studies comparing different cell sources or culture systems did not only evaluate chondrogenic capacity, but also took the stability of the tissue-engineered cartilage in consideration. These studies demonstrated that, while articular chondrocytes produced hyaline cartilage that did not mineralize (33; 34), BMSC displayed signs of chondrocyte hypertrophy such as production of collagen X (33; 35; 36). Furthermore, cartilage that was produced by BMSCs mineralized in vivo (33; 37). Therefore, more research is warranted to tissue engineer hyaline cartilage from BMSC that does not mineralize when implanted in vivo.

Though articular chondrocytes produce hyaline cartilage, they are not a likely cell source for cartilage reconstructions in the head and neck. Chondrocytes isolated from auricular or nasal septum cartilage on the other hand have not been studied as extensively as articular chondrocytes, in particular it has not been investigated whether the cartilage produced by these chondrocytes mineralizes. In contrast to articular cartilage, auricular and nasoseptal cartilage is lined by a perichondrium. The inner layer of this perichondrium (the cambium) contains progenitor cells and is difficult to remove from the cartilage. As these progenitor cells are thus likely to be isolated alongside auricular and nasoseptal chondrocytes, one should not simply translate results from studies with articular chondrocytes to expanded auricular and nasoseptal chondrocytes.

Embryonic chondrogenesis

In vivo, cartilage is only formed during embryology. As cartilage tissue engineering and in-vivo cartilage development during embryology may involve similar pathways, understanding these common pathways may lead to advances in cartilage tissue engineering. Embryonic cartilage development is primarily studied during the development of the limb (endochondral ossification). This means that the development of terminally differentiated cartilage is studied mostly.

One important characteristic of embryonic chondrogenesis is that it is regulated through different stages. The first stage in embryonic limb development is the formation of a central mesenchymal condensation. This mesenchymal condensation results from an active cell movement towards the centre of the outgrowing limb bud and is characterized by expression of adhesion molecules, such as N-cadherin (38). As the cells in this condensation differentiate to chondrocytes, expression of the cell adhesion molecules is downregulated, while collagen II and SOX9 expression is upregulated and a cartilage template is formed that consists of a proteoglycan- and collagen II-rich matrix. Next, this cartilage becomes hypertrophic, characterized by a collagen X production, and ultimately it ossifies (5; 39).

Tissue engineering and embryonic development may involve similar signaling pathways. Therefore, combining these different fields in research and understanding common signaling pathways may lead to advances in tissue engineering. However, there are also fundamental differences in these models. Chondrogenically differentiating cells in the embryonic limb bud get signals from other cell types surrounding these cells, such as in the future perichondrium and the loose mesenchyme. The signals to these cells are hugely complex and involve pathways such as TGF- β , BMP, FGF, IHH, PTHrP and Wnt/ β -catenin (4). In cartilage tissue engineering on the other hand, generally only one cell population is present and the exogenous signals provided to these cells through the medium are simplified compared to the signaling in embryonic development. However, studying embryonic chondrogenesis may shed light into the possibilities to enhance the exogenous signal to better control chondrogenic differentiation.

Growth factors influencing chondrogenesis

In cartilage tissue engineering the exogenous signals provided to the cells through the medium are simplified compared to the signaling in embryonic cartilage development. For the redifferentiation of chondrocytes as well as for the differentiation of BMSC growth factors are used. Growth factors bind to receptors on the cell membrane and thereby start intracellular signaling pathways resulting in a change in gene expression of the cell: information encoded in the gene is converted into messenger RNA and then into a protein. In cartilage tissue engineering growth factors are generally added to the chondrogenic medium throughout the culture period to study their effects. In embryonic chondrogenesis on the other hand a broad array of signaling pathways is produced by the surrounding cells depending on the stage of development. Still, studying embryonic chondrogenesis may shed light into the possibilities to enhance the exogenous signal in cartilage tissue engineering.

Transforming Growth Factor- β (TGF- β) is commonly used for chondrogenic differentiation of expanded chondrocytes and BMSC (40; 41), and the standard chondrogenic differentiation medium used in this thesis also contains TGF- β . Other growth factors are known for an additive effect on chondrogenic differentiation and cartilage matrix production, such as BMP and IGF (42). But growth factors are also important in regulating terminal differentiation of cartilage. As in cartilage tissue engineering, TGF- β plays an essential role in different stages of embryonic chondrogenesis (4).

Fibroblast Growth Factor (FGF) signaling is also important for embryonic cartilage development as mutations in the receptors for these growth factors cause syndromes with abnormal skeletal development (43). Moreover, FGF2 is added during expansion of BMSC to delay loss of chondrogenic potential (44). Therefore, in this thesis we decided to focus on TGF- β and FGF signaling pathways.

Aim and outline of this thesis

The aim of this thesis is to find new tools by which cartilage tissue engineering can be better controlled. We hypothesize that new insights to control cartilage tissue engineering can be gained by studying in-vivo chondrogenesis during embryonic development.

In **chapter 2** we studied the potential of expanded chondrocytes isolated from the external ear and the nasal septum as cell sources for cartilage tissue engineering. We investigated the general performance of these cells, focusing on subtype and stability of tissue-engineered constructs. We also performed a microarray study on expanded auricular and nasoseptal chondrocytes from the same donors in a first attempt to characterize these cells in more detail to evaluate differences as well as potential similarities in phenotype after expansion.

In the next chapters we focused on the potential of BMSC for cartilage tissue engineering. It is known that chondrogenically differentiated BMSC display signs of chondrocyte hypertrophy and mineralize in vivo and it has been said that this process resembles in-vivo endochondral ossification of the limbs. Therefore, in **chapter 3** we compared chondrogenesis of in-vitro differentiated BMSC with embryonic limb development. First, we studied whether in-vitro chondrogenic differentiation of BMSC consist of the same three developmental stages as in-vivo embryonic chondrogenesis: condensation, differentiation and hypertrophy. As Fibroblast Growth Factor Receptors (FGFR) are known to play an important role in chondrogenic differentiation in embryonic limb development, we next investigated and compared FGFR expression in in-vivo embryonic limb development and in-vitro chondrogenesis of BMSC. Finally, to evaluate stage-specific modulation of chondrogenic differentiation of BMSC with different subtypes of FGF, we studied the effect of addition of FGF2 and FGF9 to the chondrogenic medium during different stages in the culture process.

Another important growth factor in chondrogenesis is TGF- β . TGF- β is commonly used to induce chondrogenic differentiation of BMSCs. Intra-cellular signaling of members of the TGF- β superfamily involves phosphorylation of Smads. In **chapter 4** we investigated whether Smad2/3 and/or Smad1/5/8 are phosphorylated in hyaline and terminally differentiated cartilage both in vivo and in vitro. Next, we evaluated the effect of blocking Smad2/3 or Smad1/5/8 phosphorylation on chondrogenic and terminal differentiation, as well as on mineralization by addition of blocking agents during different stages of chondrogenic differentiation of BMSC.

Three mammalian subtypes of TGF- β (TGF- β 1, - β 2, - β 3) exist and all are commonly used in different laboratories. More importantly, results with one TGF- β subtype are often extrapolated to the other subtypes. In **chapter 5** we studied the chondrogenic differentiation

induced by all three TGF- β subtypes, focusing on the amount of cartilage produced as well as the subtype and stability of the cartilage produced.

The results of chapter 3-6 indicated that chondrogenic differentiation of BMSC results in the formation of hypertrophic cartilage. Furthermore, it seemed that inhibition of hypertrophy was possible to a small extent but did not result in a truly hyaline phenotype. Therefore, in **chapter 6**, we aimed to review literature supplemented with own data to answer the question whether it is possible to generate stable hyaline cartilage from adult mesenchymal stem cells. Furthermore, knowledge from developmental biology about the differential development of hyaline and terminally differentiated cartilage was reviewed and studied for implications for cartilage tissue engineering.

The terminal differentiation and mineralization seen in chondrogenically differentiated BMSC is a problem for clinical translation of cartilage tissue engineering. However, as large bones are formed through endochondral ossification during in-vivo development, the terminal differentiation of BMSC may offer a new route to engineer bone. Standard practice to tissue engineer bone is stimulate BMSC in vitro toward the osteogenic lineage, thereby aiming at intramembranous bone formation. However, results of this approach have been disappointing due to core degradation and necrosis as a result of lack of vascularization. Therefore, in **chapter 7** we studied the possibility to tissue engineer bone through endochondral ossification as an alternative approach. We tested how in-vitro differentiation of BMSCs along the osteogenic and chondrogenic lineages influences survival and osteogenesis in vivo.

Chapters 8-10 summarize and discuss the findings of this thesis. Some directions are given for further research with an emphasis on possibilities and difficulties of translating cartilage tissue engineering from research to clinical practice.



2

DIFFERENCES IN CARTILAGE FORMING CAPACITY OF EXPANDED HUMAN CHONDROCYTES FROM EAR AND NOSE AND THEIR GENE EXPRESSION PROFILES

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ABSTRACT

The aim of this study was to evaluate the potential of culture-expanded human auricular and nasoseptal chondrocytes as cell source for regeneration of stable cartilage and to analyze the differences in gene-expression profile of expanded chondrocytes from these specific locations. Auricular chondrocytes in monolayer proliferated less and more slowly (two passages took 26.7 ± 2.1 days and were reached in 4.37 ± 0.30 population doublings) than nasoseptal chondrocytes (19.3 ± 2.5 days; 5.45 ± 0.20 population doublings). However, auricular chondrocytes produced larger pellets with more cartilage-like matrix than nasoseptal chondrocytes (2.2 ± 0.71 vs 1.7 ± 0.13 mm in diameter after 35 days of culture). Although the matrix formed by auricular and nasoseptal chondrocytes contained collagen X, it did not mineralize in an in-vitro model, nor after in-vivo subcutaneous implantation. A DNA-microarray study on expanded auricular and nasoseptal chondrocytes from the same donors revealed 1090 differentially expressed genes. No difference was observed in the expression of known markers of chondrogenic capacity (e.g. collagen II, FGFR3, BMP2 and ALK1). The most striking differences were that the auricular chondrocytes had a higher expression of anabolic growth factors BMP5 and IGF1, while matrix-degrading enzymes MMP13 and ADAMTS5 were higher expressed in nasoseptal chondrocytes. This might offer a possible explanation for the observed higher matrix production by auricular chondrocytes. Moreover, chondrocytes isolated from auricular or nasoseptal cartilage had specific gene expression profiles even after expansion. These differently expressed genes were not restricted to known characterization of donor-site subtype (e.g. elastic), but were also related to developmental processes.

INTRODUCTION

Developments in regenerative medicine offer promise for the reconstruction of damaged tissues and organs including cartilage (45). In orthopaedics, cartilage regenerative medicine is being used clinically to treat focal joint defects with autologous chondrocyte implantation (ACI) since the first study in patients in 1994 (46). For this procedure, expanded articular chondrocytes are used. In otorhinolaryngology and head and neck surgery, cartilage regenerative medicine may offer a promising alternative to the current use of auricular or costal cartilage to reconstruct cartilage defects. Unfortunately, less research has been performed on cartilage regenerative medicine in this specialty (20; 21). However, recent developments such as the first patient receiving a tissue-engineered trachea (47) and the clinical application of cultured autologous auricular chondrocytes to treat craniofacial or nasal augmentation (48) and microtia (49) underline the future possibilities of cartilage regenerative medicine. Naturally, in otorhinolaryngology and head and neck surgery chondrocyte sources other than articular cartilage might be a better choice.

To select a cartilage donor source for cell therapy, one should consider at least three important things: the amount of cartilage that can be harvested without doing harm to the function of the cartilage structure at the donor site, the invasiveness of the biopsy procedure and the difference in chondrogenic phenotype (e.g. hyaline vs. elastic) of the original tissue. From the first two standpoints nasoseptal and especially auricular cartilage should be considered as favorable chondrocytes sources. Although articular chondrocytes have been extensively characterized and studied for their chondrogenic regeneration potential, overall fewer studies using alternative cell sources have been published (29; 50; 51; 52).

Auricular cartilage is elastic from origin and expanded auricular chondrocytes may therefore produce a different cartilage matrix than expanded articular chondrocytes. Though harvesting nasoseptal cartilage would not be as minimally invasive as auricular cartilage, it does have the potential benefit to originate from hyaline cartilage. However, both donor cell sources differ from articular cartilage in one other major aspect: the presence of a perichondrium.

An important requirement for tissue-engineered cartilage is that it is stable and will not mineralize upon implantation for reconstruction. Therefore, recent studies comparing different cell sources or culture systems did not only evaluate chondrogenic capacity, but took the stability of the tissue-engineered cartilage in consideration; while articular chondrocytes produced stable hyaline cartilage that did not mineralize (33; 34), bone-marrow derived mesenchymal stem cells (BMSC) displayed signs of chondrocyte hypertrophy, i.e. production of collagen X (33; 35; 36; 53; 54) and mineralized *in vivo* (33; 55). In these comparisons auricular and nasoseptal chondrocytes were not taken into account.

Results from studies on expanded articular chondrocytes cannot be translated to expanded auricular or nasoseptal chondrocytes as auricular cartilage is elastic and auricular and nasoseptal cartilage differ from the former by their perichondrium. While the outer fibrous perichondrium can be easily removed from cartilage, this is very difficult for the inner

layer (i.e. cambium) of the perichondrium. Undifferentiated progenitor cells in this cambium layer are responsible for new cartilage formation (6; 7; 8; 9), but it is possible that culturing progenitor cells from the cambium layer gives rise to rather unstable cartilage-like constructs, similar to those arising from BMSC. Therefore, it is essential to evaluate the mineralization potential of auricular and nasoseptal chondrocytes when considering them for reconstructions. To our knowledge this was never done before.

As expanded articular chondrocytes at the moment are clinically used for ACI, recent studies focus on better characterization of the molecular phenotype of the cells (56; 57). In fact, some markers are defined that are predictive of the capacity of these expanded chondrocytes to form stable cartilage (57; 58). Characterization might lead to more insight into the capacity of the cell sources. Furthermore, these types of characterizations before use can be expected to become more important in regenerative medicine. If culture-expanded auricular or nasoseptal chondrocytes are to be used in the future clinically, they should also be better characterized.

In this study, we demonstrate the potential of human auricular and nasoseptal chondrocytes as cell source for cartilage regenerative medicine. We investigate the general performance of these cells, focusing on subtype and stability of tissue-engineered constructs. Finally, we performed a DNA microarray study on expanded auricular and nasoseptal chondrocytes from the same donors in a first attempt to characterize these cells in more detail to evaluate differences as well as potential similarities in phenotype after expansion.

MATERIALS AND METHODS

Cell sources

From 4 donors (age 18, 26, 28, 47) undergoing rhinoplasty using ear cartilage, both ear and nasoseptal cartilage were obtained (MEC-2005-359). Additionally, auricular cartilage was obtained from in total 4 donors (age 5, 7, 9, 13) undergoing protruding ear reconstruction (MEC-2006-186). Nasoseptal cartilage was obtained as leftover material from 2 donors (age 33, 65) undergoing rhinoplasty (MEC-2005-359).

The first aim of this study was to evaluate the potential of culture-expanded human auricular and nasoseptal chondrocytes as cell source for regeneration of stable cartilage. Because it is known from previous studies that articular chondrocytes form stable hyaline cartilage, without collagen X expression and mineralization (33; 34), we used these cells as negative controls for hypertrophic differentiation. Healthy, articular cartilage was obtained as leftover material from 2 donors (age 3, 8) undergoing triple arthrodesis to treat clubfoot deformity (MEC-2007-032). Because BMSC are known to become hypertrophically differentiated and mineralize in vivo (33; 35; 36; 54; 55) these cells were used as a positive control. BMSC were isolated from a femoral shaft biopsy of 4 donors (age 30, 42, 51, 60) undergoing total hip replacement, after informed consent had been obtained in accordance with the local ethical committee (MEC-2004-142).

Cell isolation and expansion

For isolation of chondrocytes, cartilage slices were rinsed with saline after careful resection of the (fibrous) perichondrium, and subsequently digested through incubation for 2 hours at 37°C with 2 mg/ml Pronase E (Sigma-Aldrich, St. Louis, MO, USA), followed by overnight incubation with 1.5 mg/ml Collagenase B (Boehringer Mannheim, Germany) in DMEM containing Glutamax (Gibco, Carlsbad, CA, USA) and 10% fetal calf serum (FCS) (Gibco). Next, cell suspensions were filtered through a 100 µm filter, centrifuged and washed with saline. For expansion, chondrocytes were seeded at a density of 7,500 cells/cm² in “chondrocyte-expansion medium” (DMEM containing glutamax, with 10% FCS and 1.5 µg/ml fungizone (Gibco) and 50 µg/ml gentamicin (Gibco)). At subconfluency cells were trypsinized and further expanded. Chondrocytes from passage (P) 2 were used for the experiments. Small pieces of auricular and nasoseptal cartilage were kept aside for immunohistochemical characterization.

For isolation of BMSC the heparinized bone-marrow aspirate was seeded at a density of 2-5 x 10⁵ cells/cm² in “BMSC-expansion medium” (DMEM-LG (Gibco), supplemented with 10% FCS, 1 ng/ml FGF2 (AbD Serotec, Kidlington, UK), 25 µg/ml ascorbic acid-2-phosphate (Sigma-Aldrich), 1.5 µg/ml fungizone and 50 µg/ml gentamicin). After 24 hours, nonadherent cells were washed off and adherent cells were further expanded. At subconfluency, BMSC were trypsinized, seeded at a density of 2,300 cells/cm² and further expanded. BMSC passages 2-4 were used for the experiment.

Chondrogenic differentiation

Pellet culture

To study cartilage subtype differentiation and stability in vitro, auricular and nasoseptal chondrocytes, as well as articular chondrocytes and BMSC as controls, were chondrogenically differentiated in pellet cultures. Cells were cultured in pellets by centrifuging aliquots of 2 x 10⁵ cells in 0.5 ml medium at 200 xg for 8 minutes in a polypropylene tube. Chondrogenic differentiation was induced by “chondrogenic differentiation medium” based on previous publications (27; 54; 59), consisting of DMEM containing Glutamax, ITS+1 (B&D Bioscience, Bedford, MA, USA), 40 µg/ml L-proline (Sigma-Aldrich), 1 mM sodium-pyruvate (Gibco), 5 µg/ml fungizone and 50 µg/ml gentamicin, 25 µg/ml ascorbic acid-2-phosphate, 10 ng/mL TGFβ2 (R&D systems, Minneapolis, MN, USA) and 10⁻⁷M dexamethasone (Sigma-Aldrich). The medium was changed two times a week.

Flat constructs

To study in-vivo stability of tissue-engineered cartilage from auricular and nasoseptal chondrocytes larger and flat constructs were used because these resemble the clinical need better than pellets. Auricular and nasoseptal chondrocytes from one donor (age 18) were used for this experiment. Aliquots of 5 x 10⁵ auricular chondrocytes or nasoseptal chondrocytes (suspended in 100 µl “chondrogenic differentiation medium”) were pipetted onto a dry 6.5-mm diameter, 0.4-µm pore size polycarbonate Transwell® filters (Corning B.V. Life Sciences, Schiphol-Rijk, the Netherlands), precoated with collagen II (Sigma-

Aldrich), and centrifuged in a 24-well plate for 5 minutes at 200xg (60). Chondrocytes were cultured on “chondrogenic differentiation medium” with medium refreshment three times a week.

General performance of auricular vs. nasoseptal chondrocytes

To directly compare the performance of auricular and nasoseptal chondrocytes, ear and nasoseptal cartilage from the same donor were used, for a total of three donors (age 26, 28, 47). The performance was evaluated in monolayer expansion by number of population doublings till subconfluency and time to subconfluency. All chondrocytes were expanded and trypsinized when subconfluency was reached. Chondrocytes from passage 2 were chondrogenically differentiated for 35 days in pellet culture. To measure the size of the chondrogenic pellet at day 35, a picture was taken of the intact pellet together with a stage micrometer at 10X magnification. Statistical analysis was performed using Microsoft Office Excel 2007. Values are represented as mean \pm standard deviation (SD). To compare rate of expansion in auricular and nasoseptal chondrocytes from the same three donors, a paired t-test was performed. To compare size of pellets and GAG amount an unpaired t-test was performed. The level of significance was set at a *p* value <0.05.

Cartilage subtype differentiation

Characterization of subtype differentiation was studied on pellet cultures at day 35 from auricular and nasoseptal chondrocytes from all donors. This was compared with pellet cultures from articular chondrocytes and BMSC which are known to produce respectively hyaline and hypertrophic cartilage, as well as with sections of native auricular and nasoseptal cartilage. In the flat constructs the cartilage subtype differentiation was evaluated before and after in-vivo implantation. Characterization of cartilage subtype differentiation was performed through immunohistochemistry of collagen II, collagen X, and elastin.

To study the effect of passage number on subtype differentiation, chondrocytes from 2 auricular, nasoseptal and articular donors were not only differentiated at passage 2 but also at passage 4 and 6.

Stability of tissue-engineered cartilage

To study the ability to mineralize the matrix as a measure of stability, 10 mM β -glycerophosphate (BGP) was added to the medium of pellets after cartilage formation had started. Based on previous studies, BGP was added to the medium of auricular and nasoseptal chondrocytes from day 14 on, and these pellets were harvested at day 35. This experiment was performed with auricular and nasoseptal chondrocytes from 3 donors (auricular donors aged 9, 13 and 26, nasoseptal donors aged 26, 33 and 65). As a positive control for this in-vitro mineralization model, pellets of BMSC of 2 donors (age 30, 42) were used. Because of a later onset of cartilage differentiation in BMSC, BGP was added to medium of these pellets from day 35 on, and pellets were harvested at day 49.

To confirm the stable phenotype of auricular- and nasoseptal-derived tissue-engineered constructs that was seen in the previous in-vitro model, a subsequent

experiment was performed implanting tissue-engineered constructs in-vivo. To directly compare the auricular and nasoseptal chondrocytes, cartilage from one single donor (age 18) was used for this experiment and cells were trypsinized simultaneously. Cells were cultured on a Transwell filter as flat constructs because these resemble the clinical need better than pellets. As in the previous experiment cells were chondrogenically differentiated for 14 days. At this time, 6 flat constructs were harvested for histology and biochemistry and 8 flat constructs were implanted into the back of a nude mouse. Four 6-weeks old, male NMRI nu/nu mice (Taconic, Germantown, NY, USA) were used. Two incisions were made along the central line of the spine of the mouse, one at the shoulders and one at the hips. Per mouse two auricular-chondrocyte constructs and 2 nasoseptal-chondrocyte constructs were implanted. Animals were terminated 6 weeks later to harvest the cartilage constructs for histology. This procedure was carried out with approval from the animal ethical committee (EUR1428).

(Immuno)histochemistry

Constructs were fixed in 4% formalin in phosphate-buffered saline (PBS), set in 2% agar, and embedded in paraffin.

Immunohistochemistry for collagen II, collagen X, elastin and vimentine

Antigen retrieval for collagen II was performed through incubation with 0.1% pronase (Sigma-Aldrich) in PBS for 30 minutes, while antigen retrieval for collagen X required 0.1% pepsin (Sigma-Aldrich) in 0.5M acetic acid (pH=2.0) for 2 hours. Both collagen II and collagen X stainings continued with incubation with 1% hyaluronidase (Sigma-Aldrich) in PBS for 30 minutes. Antigen retrieval for elastin was performed through incubation with 0.25% trypsin (Sigma) in PBS for 20 minutes.

Non-specific binding sites were blocked with 10% goat serum in PBS and sections were stained overnight with primary antibodies against collagen II (II/II6B3, Developmental Studies Hybridoma Bank, 1:100), collagen X (Quartett, Berlin, Germany, 1:10), and elastin (BA4, Sigma-Aldrich, 1:1000). To allow the use of monoclonal mouse antibodies on tissue-engineered cartilage constructs implanted in a nude mouse, we used a method described by Hierck *et al* (61). An alkaline-phosphatase-conjugated secondary antibody was used, followed by incubation with Neu Fuchsin substrate (Chroma, Köngen, Germany) to demonstrate alkaline-phosphatase activity with a red staining.

To study whether the flat constructs harvested after in-vivo implantation were from human origin, a monoclonal mouse anti-human vimentin antibody (AMF-17b, Developmental Studies Hybridoma Bank, 1:40) was used. To exclude any unspecific binding of the vimentin antibody to mouse IgGs, the mouse-on mouse HRP-Polymer Kit (Biocare Medical, Concord, CA, USA) was used, according to manufacturer's instructions with some small modifications. In short, slides were incubated in an aqueous 3% solution of hydrogen peroxidase, to inhibit any endogenous peroxidase and allow for a peroxidase-antiperoxidase staining method. Antigen retrieval for vimentin was performed through incubation in Rodent Decloaker® for 60 minutes at 95°C. Non-specific binding sites were blocked with Rodent Block M® and sections were stained overnight with vimentin (V6630, Sigma-Aldrich, 1:40). The MM-

polymer-HRP® secondary antibody was used, followed by incubation with 3'diaminobenzidine chromogen solution (Sigma-Aldrich) to demonstrate positivity for the antibody with a brown staining. A mouse monoclonal negative control antibody (Dako) was used as an isotype control. Slides were counterstained with hematoxylin.

Von Kossa/Thionin staining

For evaluation of mineralization, slides were immersed in 5% silver nitrate solution (Sigma, St Louis, MO) for 10 minutes, rinsed in ultra pure water and exposed to light for 10 minutes. Excess silver nitrate was removed with 5% sodium-thiosulphate (Sigma, St Louis, MO) and cells were rinsed in distilled water. Sections were counterstained with 0.4% thionin in 0.01 M aqueous sodium acetate, pH 4.5 for 5 min, which aside from staining the nucleus also demonstrates glycosaminoglycan (GAG) content.

Biochemistry

Flat constructs were digested overnight at 56°C in 1mg/ml proteinase K (Sigma-Aldrich) in Tris/EDTA buffer (pH 7.6) containing 185 µg/ml iodoacetamide and 1 µg/ml pepstatin A (Sigma-Aldrich). GAG amount was quantified using dimethylmethylene blue (DMB) assay (62). The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ratio A530:A590 was used to determine the GAG amount, using chondroitin sulfate C (Sigma) as a standard. To determine the amount of DNA, each proteinase K-digested sample (50 µl) was treated with 100 µl heparin (8.3 IU/ml in PBS) and 50 µl ribonuclease A (50 µg/ml in PBS) for 30 min at 37°C. This was followed by adding 50 µl ethidium bromide solution (25 µg/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA, USA) using an extinction filter of 340 nm and an emission filter of 590 nm. For standards, calf thymus DNA (Sigma-Aldrich) was used.

Statistical analysis was conducted with Microsoft Office Excel 2007. Values are represented as mean ± standard deviation (SD). An unpaired t-test was performed to compare GAG amount. The level of significance was set at a *p* value of less than 0.05.

Microarray study

Both auricular and nasoseptal cartilage were harvested from the same donor, two donors in total (age 28, 47). Cells were trypsinized at 80% confluency, as judged by the same observer. To avoid a bias in gene-expression data by differences in culture procedures, all cells from P2, P4 and P6 were plated in 24 wells plate at high cell density (50,000 NC / cm²) in "chondrocyte-expansion medium". After three days the medium was replaced, followed by harvesting of the cells exactly 24 hours later. Therefore, from each donor three samples per cell source were available, offering the possibility to statistically adjust for between-donor differences. Cells from P2, P4 and P6 were also chondrogenically differentiated in pellets to determine the chondrogenic capacity after expansion.

RNA isolation and expression profiling

For total RNA isolation, cells were manually homogenized in RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). RNA was extracted with chloroform and purified from the

supernatant using the RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's guidelines with on-column DNA-digestion. Gene expression profiling was performed using Affymetrix 1.0 Human Exon ST arrays, representing all known genes (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. The Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay (kit 900652; 1.0 µg of total RNA) was used to generate amplified and biotinylated sense-strands DNA targets from the entire expressed genome. The manufacturer's instruction (version 4, P/N 701880 Rev. 4) was followed for the hybridization, washing, and scanning steps. Arrays were hybridized by rotating them at 60 rpm in the Affymetrix GeneChip hybridization oven at 45 °C for 17 hours. After hybridization, the arrays were washed in the Affymetrix GeneChip Fluidics station FS 450. Arrays were scanned using the Affymetrix GeneChip scanner 3000 7G system.

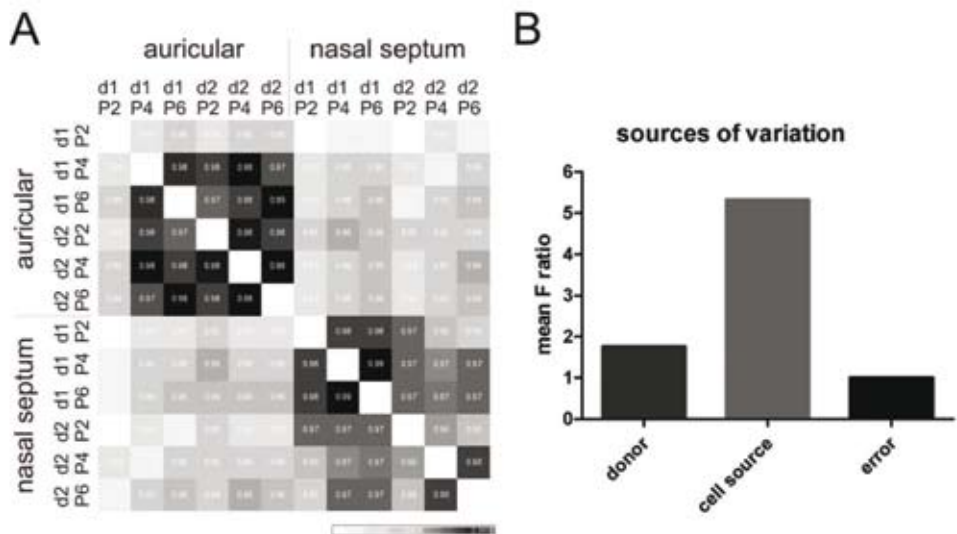


Figure 1. Pearson correlation matrix and sources of variation graph. **A** Pearson correlation coefficients for every combination of arrays. A high correlation (black) was revealed for each of the two tissues. Note that one array on auricular chondrocytes (donor 1, P2) did not show higher correlation to the arrays in the cluster of auricular chondrocytes and was therefore considered an outlier. **B** Sources of variation graph demonstrating that the variation in gene expression in the microarray samples was primarily due to cell source with an average F ratio of 5.32, and not to donor variation with an F ratio of 1.76.

Data extraction and statistical analyses

For quality control, the Affymetrix CEL-files were first imported into Affymetrix Expression Console version 1.1 where control probes were extracted and normalized using the default RMA algorithm. The Area Under the Curve (AUC) of the Receiver Operator Characteristic

was calculated using positive and negative control probes. All arrays had an AUC score above the empirically defined threshold of 0.85 indicating a good separation of positive and negative controls. Pearson correlation coefficients were determined for each and every combination of arrays. For each of the two tissues a cluster of high correlation was revealed. One of the three auricular samples of one donor did not show higher correlation to the arrays in the cluster of its tissue and was therefore considered an outlier and excluded from further analysis (fig. 1). Next, the CEL-files were imported into Partek® Genomic Suite software (version 6.4, Partek Inc., St. Louis, MO, USA) where only core probe sets were extracted and normalized using the RMA algorithm with GC background correction. Core transcript summaries were calculated using the mean intensities of the corresponding probe sets, representing the quantitative expression levels of 17881 genes.

The correspondence of the replicate samples was confirmed using Principle Component Analysis (PCA) and Pearson correlation analysis. P-values and fold differences for the expression differences between auricular and nasoseptal chondrocytes were generated by applying an Anova model. P-values were corrected for multiple testing using the Benjamini–Hochberg method. Genes with p-values smaller than 0.05 and fold differences higher than 2 were functionally annotated and classified by using the functional annotation clustering tool of Database for Annotation, Visualization, and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov/>) (63).

Quantitative real-time RT-PCR

To validate the results from the microarray analysis, the three genes with the highest fold difference between auricular and nasoseptal chondrocytes and the three genes with a fold difference closest to 2 were analyzed by RT-PCR.

Complementary DNA (cDNA) was made using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) and polymerase chain reactions were performed using TaqMan® Universal PCR MasterMix (Applied Biosystems, Capelle a/d IJssel, Netherlands) on an ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA). Taqman gene-expression assays were purchased from Applied Biosystems for STEAP4 (Hs00226415_m1), ALX1 (Hs00232518_m1), IL31RA (Hs00371172_m1), GRK5 (Hs00992173_m1) and TMED3 (Hs00201251_m1). Primer sequences for GAPDH and PRG4 are described elsewhere (64).

Expression was normalized to GAPDH and expressed relatively using the $2^{-\Delta\text{Ct}}$ formula (65). Subsequently, fold differences for each gene were calculated between auricular and nasoseptal chondrocytes.

RESULTS

General performance

To directly compare the performance of auricular and nasoseptal chondrocytes, ear and nasoseptal cartilage from the same donor were used for this experiment, for a total of three donors (age 26, 28, 47). After isolation, the auricular chondrocytes adhering to the flask

varied tremendously in morphology and size while nasoseptal chondrocytes were all round and of similar size. Upon culturing the auricular chondrocytes became more and more uniform in their appearance.

Auricular chondrocytes had gone through 4.37 population doublings (SD \pm 0.30) in two passages, while nasoseptal chondrocytes had gone through 5.45 population doublings (SD \pm 0.20) ($p=0.026$). It took 26.7 days (SD \pm 2.1) for auricular chondrocytes and 19.3 (SD \pm 2.5) days for nasoseptal chondrocytes to perform 2 passages ($p=0.014$).

Chondrocytes isolated from auricular cartilage produced significantly larger ($p=0.023$) cartilage-like constructs (Fig. 2A-F). The average diameter of an auricular-chondrocyte pellet was 2.2 mm (SD \pm 0.71), the average diameter of a pellet of nasoseptal chondrocytes was 1.7 mm (SD \pm 0.13).

Cartilage subtype differentiation

All pellets of expanded auricular and nasoseptal chondrocytes produced cartilage, as was evaluated with thionin staining showing proteoglycans and immunohistochemistry for collagen type II (Fig. 2A, 2D). Pellets of both auricular and nasoseptal chondrocytes expressed collagen X (Fig. 2B, 2E), independent of donor age or passage number, indicating hypertrophic differentiation. In comparison, articular chondrocytes produced abundant collagen type II but no collagen type X (Fig. 2G-H), as was also demonstrated in previous studies. BMSC, known for their potential to generate terminally differentiated chondrogenic pellets and the ability to mineralize, did indeed express both collagen II and collagen X in our experiment (Fig. 2I-J). Pellets of chondrocytes of all sources produced less cartilage-like matrix with increasing passage number (data not shown).

No elastin was detected in pellets from any of the cell sources, including auricular chondrocytes. Even after prolonged culturing (70 days) or when primary auricular chondrocytes were used, no elastin was observed in the matrix. This suggests that either the in-vitro model does not support elastin production of these cells, or that mature elastic chondrocytes are incapable of elastin synthesis.

In comparison, native nasoseptal cartilage stained positively for collagen II, but not for collagen X or elastin. Native auricular cartilage (independent of age) on the other hand stained positively for elastin, collagen type II, as well as collagen X. Therefore the tissue-engineered differed molecularly from the native cartilage the chondrocytes were isolated from, as in vitro nasoseptal chondrocytes produced collagen X and auricular chondrocytes did not produce any elastin.

Stability of tissue-engineered cartilage

Since auricular and nasoseptal chondrocytes formed cartilage positive for collagen X, a marker for hypertrophic cartilage, we studied the ability to mineralize as a measure of stability of the tissue-engineered cartilage formed by auricular and nasoseptal chondrocytes. In this in-vitro model chondrocytes from 1 common donor (age 26) as well as with 2 distinct donors (auricular donors aged 9 and 13, nasoseptal donors aged 33 and 65) were used. In auricular and nasoseptal chondrocyte pellets a collagen-II rich matrix had formed at day 14

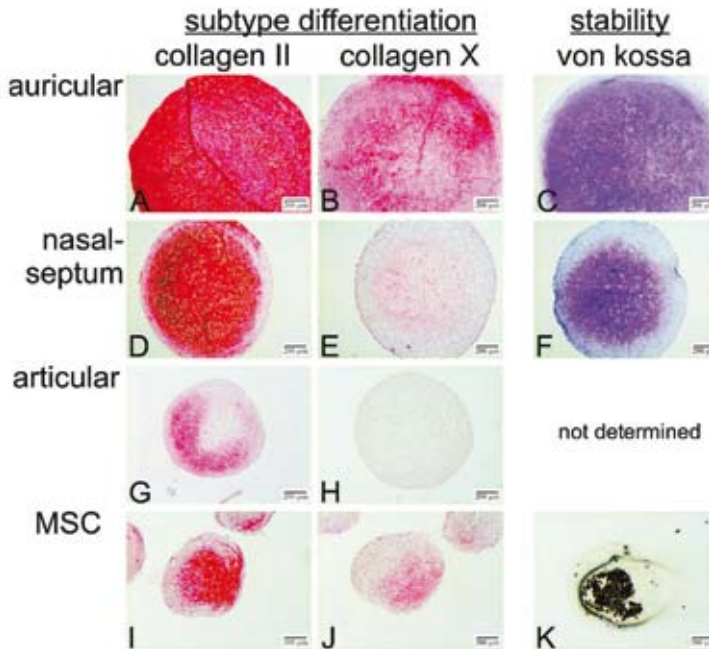


Figure 2. Subtype differentiation and stability of tissue-engineered cartilage in pellet culture. After 35 days of culture on chondrogenic differentiation medium, auricular (A,B) and nasoseptal (D,E) chondrocytes had produced both collagen II and collagen X. Von Kossa/Thionin staining demonstrates a proteoglycan-rich matrix at day 35 without mineralization when BGP was added to the medium for the last 3 weeks (C,F). At day 35 articular chondrocytes had produced collagen II (G), but not collagen X (H) and BMSC had produced collagen II (I), as well as collagen X (J). Von Kossa/Thionin staining demonstrates abundant mineralization in BMSC pellets when BGP was added to the medium from day 35-49 (K). Pellets of auricular and nasoseptal chondrocytes are from the same donor. Note that pellets of auricular chondrocytes (A-C) were larger and contained more cartilage-like matrix than nasoseptal chondrocytes (D-F).

and collagen X was present at this time in the pellets of all except one nasoseptal chondrocyte donor. At the end of the culture period both collagen II and collagen X were observed in all pellets.

Addition of β -glycerophosphate was used to evaluate the capacity to mineralize, resulting in a positive Von-Kossa staining in BMSC pellets (donor age 30, 42) which served as a positive mineralization control (Fig. 2K). No mineralization was observed in any of the pellets of either auricular or nasoseptal chondrocytes (Fig. 2C, 2F).

To compare the performance of auricular and nasoseptal chondrocytes in-vivo in an application set-up, auricular and nasoseptal cells were harvested from the one donor (age 18) seeded in monolayer for expansion and trypsinized simultaneously. Nasoseptal chondrocytes had gone through 5.4 population doublings and auricular chondrocytes through 2.0 population doublings.

After expansion in monolayer, flat constructs were made by seeding the cells in a Transwell culture system and culturing for 14 days on chondrogenic differentiation medium. Both auricular chondrocytes and nasoseptal chondrocytes had produced a cartilage-like matrix consisting of abundant collagen II and scarce collagen X expression. Similar to what was seen in the pellet cultures, cartilage constructs from auricular chondrocytes had produced more collagen II and were larger than constructs from nasoseptal chondrocytes. Moreover, biochemical analysis demonstrated that the matrix of auricular constructs contained significantly ($p < 0.0005$) more GAG than matrix of nasoseptal constructs ($20.8 \pm 5.2 \mu\text{g GAG}/\mu\text{g DNA}$ vs $7.8 \pm 2.4 \mu\text{g GAG}/\mu\text{g DNA}$). Therefore, the difference in size of the cartilage constructs was primarily due to matrix production and not proliferation. In agreement with these findings, flat constructs from auricular chondrocytes were more solid than flat constructs from nasoseptal chondrocytes. However, constructs from both cell sources were still very flexible and fragile after 14 days of in-vitro differentiation.

After six weeks of implantation in a nude mouse all cartilage constructs, except one from nasoseptal chondrocytes, were recognizable and could be harvested. Constructs from auricular chondrocytes were larger and therefore better recognizable in vivo than those from

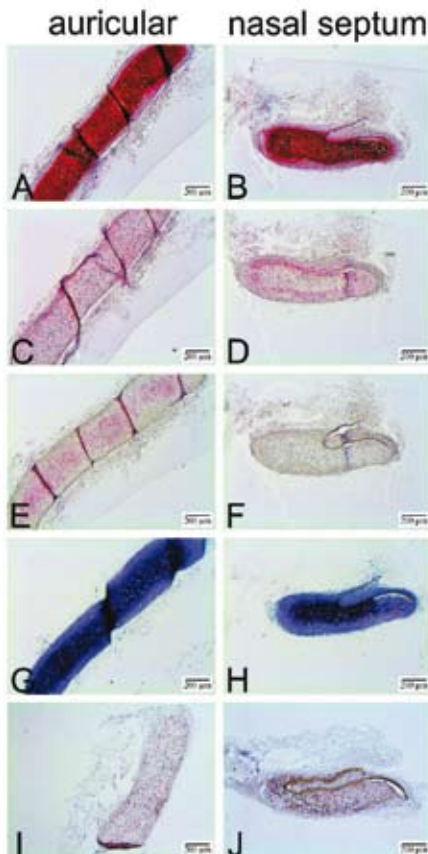


Figure 3. In-vivo stability of tissue-engineered cartilage in flat constructs. Characterization of tissue-engineered constructs of auricular and nasoseptal chondrocytes after 14 days of in-vitro culture, followed by 6 weeks of in-vivo subcutaneous implantation. Constructs of both cell sources had produced abundant collagen II (A-B) and little collagen X (C-D). Constructs of auricular chondrocytes, but not nasoseptal chondrocytes, had produced elastin (E-F). Von Kossa/Thionin staining demonstrated a proteoglycan-rich matrix in constructs of both cell sources, but no mineralizations were observed (G-H). A positive vimentin-staining, sensitive for human vimentin only, demonstrates that the cartilage-like constructs are from human origin, while the surrounding fibrous tissue is from murine origin (I-J). Positivity for the antibody is demonstrated by a red (A-F) or brown (I-J) staining.

nasoseptal chondrocytes. Moreover, these constructs were stiff and strong on handling, while constructs from nasoseptal chondrocytes were still fragile and also macroscopically did not resemble cartilage. Immunohistochemistry demonstrated a rich collagen II and collagen X containing matrix in all harvested constructs of both cell sources (Fig. 3A-D).

Elastin was demonstrated in the matrix of auricular-chondrocyte constructs, but not in nasoseptal chondrocytes (Fig. 3E-F). Von Kossa/Thionin staining demonstrated a proteoglycan-rich matrix in constructs from both cell sources, while no mineralization was present after 6 weeks of subcutaneous implantation (Fig. 3G-H). By means of a vimentin staining, the cartilage-like constructs were demonstrated to be of human origin, while surrounding fibrous tissue was of murine origin (Fig. 3I-J).

Therefore, after in-vivo implantation flat constructs derived from auricular chondrocytes did take on their stable native cartilage subtype, including elastin and collagen X production. Flat constructs derived from nasoseptal chondrocytes on the other hand did not as collagen X was still present in the matrix. However, though collagen X was present in flat constructs from both cell sources indicating hypertrophy, a stable cartilage was produced that did not mineralize.

Microarray data

In the two donors (age 28, 47) used for microarray study auricular chondrocytes had gone through 4.7 population doublings at P2 and nasoseptal chondrocytes had gone through 5.4 population doublings. At P6 cells from both origins had gone through respectively 15.5 and 14.2 population doublings.

Variation in gene expression in the microarray samples was primarily due to cell source with an average F ratio of 5.32, and not to donor variation with an F ratio of 1.76 (fig. 1). Further analysis demonstrated a group of 1090 genes whose expression was significantly different in auricular and nasoseptal chondrocytes (out of 17,881 studied genes in total), of which 487 genes showed a fold difference of 2 or more. No significant difference was observed in the expression of collagen II, FGFR3, BMP2, and ALK1 which were previously reported as markers predicting the capacity of expanded articular chondrocytes to produce cartilage (57).

Of these significantly differentially expressed genes, 58 genes were more than 5 fold higher expressed in auricular chondrocytes compared to nasoseptal chondrocytes (table 1). This list is headed by proteoglycan 4 (88x), a matrix molecule with a lubrication function. Surprisingly, two growth factors with a known anabolic effect on cartilage production were observed in this list of genes expressed higher in auricular chondrocytes than in nasoseptal chondrocytes: BMP5 (37x) and IGF1 (9x).

As expected from the production of elastin in vivo in the previous experiment, 8-times more elastin was expressed in auricular chondrocytes. Fibrillin 2, a glycoprotein essential for the formation of elastic fibers, was also expressed significantly higher (7x) in auricular chondrocytes (table 1). Other components of the elastic fiber, such as fibrillin 1 and 3 or the EMILINs (Elastin Microfibril Interface Located Protein), were not significantly differentially expressed.

Table 1. Genes expressed over 5 fold more in auricular chondrocytes compared to nasoseptal chondrocytes.

Gene symbol	Gene assignment	Benjamini Hochberg	Fold difference
PRG4	proteoglycan 4	1.4E-3	88.0
BMP5	bone morphogenetic protein 5	3.9E-4	37.0
FGL2	fibrinogen-like 2	6.5E-3	36.4
MAB21L2	mab-21-like 2 (C. elegans)	5.3E-4	30.2
DLX5	distal-less homeobox 5	4.9E-4	25.4
MEIS1	Meis homeobox 1	1.8E-4	23.4
SEMA3E	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E	1.9E-3	23.1
FAM107A	family with sequence similarity 107, member A	9.0E-4	22.2
CCKAR	cholecystokinin A receptor	2.3E-2	17.5
OGN	osteoglycin	1.8E-3	16.3
MEIS2	Meis homeobox 2	1.8E-4	15.9
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	4.1E-3	13.1
PI15	peptidase inhibitor 15	2.2E-3	12.9
HMCN1	hemicentin 1	2.6E-3	12.4
EV11	ecotropic viral integration site 1	3.4E-4	12.2
GREM2	gremlin 2, cysteine knot superfamily, homolog (Xenopus laevis)	4.8E-3	12.0
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	7.2E-3	10.9
PCSK1	proprotein convertase subtilisin/kexin type 1	3.1E-3	10.5
CHODL	chondrolectin	3.1E-3	10.2
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	3.5E-3	10.1
MKX	mohawk homeobox	3.9E-4	10.1
IGF1	insulin-like growth factor 1 (somatomedin C)	1.5E-3	9.4
LRRC17	leucine rich repeat containing 17	1.2E-2	8.7
NDP	Norrie disease (pseudoglioma)	1.2E-3	8.4
ELN	elastin	7.2E-3	8.2
FLRT2	fibronectin leucine rich transmembrane protein 2	7.2E-4	8.1
NPTX2	neuronal pentraxin II	1.2E-3	7.9
CGNL1	cingulin-like 1	1.1E-3	7.8
LRFN5	leucine rich repeat and fibronectin type III domain containing 5	1.8E-4	7.7
NTNG1	netrin G1	2.5E-3	7.5
PODXL	podocalyxin-like	2.1E-3	7.3
ELMO1	engulfment and cell motility 1	2.2E-4	7.3
FBN2	fibrillin 2	7.9E-3	7.0
GNG11	guanine nucleotide binding protein (G protein), gamma 11	9.8E-4	6.5
KIAA0746	KIAA0746 protein	6.4E-4	6.5
SFRP1	secreted frizzled-related protein 1	1.1E-2	6.5
SAMD9	sterile alpha motif domain containing 9	7.8E-3	6.5
COLEC12	collectin sub-family member 12	7.1E-4	6.4

CRIP1	cysteine-rich protein 1 (intestinal)	5.5E-3	6.4
NOVA1	neuro-oncological ventral antigen 1	1.2E-3	6.3
CLGN	calmegin	6.4E-3	6.2
PTPRU	protein tyrosine phosphatase, receptor type, U	8.4E-4	6.2
THBD	thrombomodulin	1.5E-2	6.1
EIF1AX	eukaryotic translation initiation factor 1A, X-linked	2.7E-3	6.0
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	1.2E-3	6.0
ETV1	ets variant 1	3.9E-3	6.0
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)	7.5E-3	5.9
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	2.6E-2	5.8
NGEF	neuronal guanine nucleotide exchange factor	1.3E-3	5.8
TIAM1	T-cell lymphoma invasion and metastasis 1	4.7E-3	5.8
MATN2	matrilin 2	1.6E-3	5.8
NPTX1	neuronal pentraxin I	1.6E-3	5.6
CACNB2	calcium channel, voltage-dependent, beta 2 subunit	1.8E-2	5.5
SPRY2	sprouty homolog 2 (Drosophila)	5.3E-3	5.4
GALNT6	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 6	1.0E-3	5.4
DLX6	distal-less homeobox 6	1.6E-2	5.4
G0S2	G0/G1switch 2	3.0E-3	5.3
PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1	3.0E-3	5.1

Table 2. Genes expressed over 5 fold more in nasoseptal chondrocytes compared to auricular chondrocytes.

Gene symbol	Gene assignment	Benjamini Hochberg	Fold difference
STEAP4	STEAP (Six Transmembrane Epithelial Antigen of the Prostate) family member 4	1.5E-3	85.7
ALX1	ALX homeobox 1	1.7E-4	43.6
CPA4	carboxypeptidase A4	3.2E-3	41.4
MMP13	matrix metalloproteinase 13 (collagenase 3)	3.4E-2	39.0
SLPI	secretory leukocyte peptidase inhibitor	2.8E-3	32.3
BEX1	brain expressed, X-linked 1	1.5E-3	29.0
ACTG2	actin, gamma 2, smooth muscle, enteric	8.8E-4	27.0
VCAM1	vascular cell adhesion molecule 1	1.0E-2	24.2
KIAA1199	KIAA1199	4.4E-2	16.8
CH25H	cholesterol 25-hydroxylase	9.7E-4	16.3
CHRD1	chordin-like 1	5.5E-3	14.0
BAMBI	BMP and activin membrane-bound inhibitor homolog	4.7E-3	14.0
KCNK2	potassium channel, subfamily K, member 2	1.7E-4	13.6
DSP	desmoplakin	2.1E-3	13.2
CABLES1	Cdk5 and Abl enzyme substrate 1	2.5E-3	11.5
VAMP8	vesicle-associated membrane protein 8 (endobrevin)	3.6E-3	11.3
PLK2	polo-like kinase 2 (Drosophila)	1.1E-3	10.5
TOX	thymocyte selection-associated high mobility group box	7.2E-4	10.2

COL11A1	collagen, type XI, alpha 1	1.2E-2	9.9
SERPINB7	serpin peptidase inhibitor, clade B (ovalbumin), member 7	1.1E-2	9.0
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.4E-2	8.4
ITM2C	integral membrane protein 2C	3.7E-2	8.3
THBS1	thrombospondin 1	3.3E-2	7.8
ACTA2	actin, alpha 2, smooth muscle, aorta	1.0E-2	7.7
ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltransferase 1	2.0E-2	7.1
KRTAP2-2	keratin associated protein 2-2	1.8E-2	7.1
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	7.9E-4	6.9
P4HA3	prolyl 4-hydroxylase, alpha polypeptide III	7.2E-4	6.8
SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain	2.5E-2	6.7
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	6.1E-3	6.7
PLA1A	phospholipase A1 member A	2.4E-2	6.6
EYA1	eyes absent homolog 1 (Drosophila)	4.8E-3	6.2
SGIP1	SH3-domain GRB2-like (endophilin) interacting protein 1	1.1E-3	5.9
KCNE4	potassium voltage-gated channel, Isk-related family, member 4	1.5E-2	5.8
FZD7	frizzled homolog 7 (Drosophila)	2.3E-3	5.6
DOCK10	dedicator of cytokinesis 10	1.7E-2	5.6
TLR4	toll-like receptor 4	2.2E-3	5.5
EVI2B	ecotropic viral integration site 2B	4.7E-3	5.5
RDH10	retinol dehydrogenase 10 (all-trans)	1.0E-2	5.5
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	3.1E-3	5.4
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	3.4E-2	5.4
PDE5A	phosphodiesterase 5A, cGMP-specific	2.0E-3	5.3
SULF2	sulfatase 2	2.2E-3	5.2
F2RL2	coagulation factor II (thrombin) receptor-like 2	1.8E-2	5.2
RGMB	RGM domain family, member B	2.3E-3	5.2
LEPR	leptin receptor	3.9E-3	5.1
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	9.5E-4	5.1
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	1.8E-3	5.0
PCDH10	protocadherin 10	9.0E-4	5.0

Forty-nine genes were more than 5 fold higher expressed in nasoseptal chondrocytes (table 2). In contrast, no anabolic growth factors were prominent among the genes that were higher expressed in nasoseptal chondrocytes than in auricular chondrocytes. In this list matrix degrading enzymes MMP13 (39x) and ADAMTS5 (5x) were prominent.

The 487 genes that were significantly differentially expressed in auricular and naso-

Table 3. Results of functional annotation clustering.

Annotation cluster 1		Benjamini-
Enrichment score 13.3	count	Hochberg
• developmental process	172	9.9E-16
• multicellular organismal development	136	1.2E-14
• anatomical structure development	126	1.9E-13
• system development	109	7.3E-13
• nervous system development	59	6.5E-9
• multicellular organismal process	168	7.1E-9
• organ development	78	4.9E-8
• cellular developmental process	96	5.3E-7
• cell differentiation	96	5.3E-7
Annotation cluster 2		Benjamini-
Enrichment score 4.83	count	Hochberg
■ glycoprotein	185	3.4E-14
■ signal	144	2.0E-10
Δ signal peptide	135	4.3E-6
Δ glycosylation site:N-linked (GlcNAc...)	158	7.1E-6
• extracellular region	75	1.1E-5
■ secreted	76	1.3E-5
Δ disulfide bond	113	1.7E-2

Table shows the two annotation clusters with the highest enrichment scores. The individual annotation terms are shown (• Gene Ontology term ■ SP-PIR keyword Δ Uniprot sequence feature) that make up the enriched cluster. The number of genes that are involved in the individual term is shown in the next column (count). Finally, the Benjamini-Hochberg stepup (p -value) is shown for each individual term.

septal chondrocytes with a fold difference of ≥ 2 were analyzed with Functional Annotation Clustering in DAVID (Table 3). This analysis identified “development” as the most important process in which these genes are involved (enrichment score: 13.3). Second in line of importance, were processes related to “glycoproteins” (enrichment score: 4.83).

To validate the microarray results, RT-PCR was performed on the three genes with the highest fold difference (STEAP4, PRG4, ALX1), as well as the three genes with a fold difference closest to 2 (TMED3, IL31RA, GRK5). PCR confirmed the trend and order of magnitude in fold difference as observed in the microarray data, indicating the reliability of the data sets (Table 4).

In conclusion, although no difference was observed in mRNA expression of known markers correlated with chondrogenic capacity, auricular chondrocytes do seem to be characterized by a higher expression of anabolic growth factors, while matrix degrading enzymes showed a higher expression in nasoseptal chondrocytes. Moreover, chondrocytes from different specific donor sites seem to preserve specific molecular characteristics even after expansion. These specific molecular signatures are not only indicative of cartilage subtype characteristics (e.g. elastic), but also reflect different developmental processes.

Gene	Microarray	RT-PCR
STEAP4	-85.7	-259.8
PRG4	+88.0	+170.9
ALX1	-43.6	-22.5
TMED3	-2.0	-1.3
IL31RA	+2.0	+9.8
GRK5	-2.0	-1.6

Table 4. RT-PCR validation of microarray. Fold differences in expression of STEAP4, PRG4, ALX1, TMED3, IL31RA and GRK5 are shown for both RT-PCR and microarray analysis. + indicates higher expression in auricular chondrocytes, while – indicates higher expression in nasoseptal chondrocytes.

DISCUSSION

This study demonstrates that both auricular and nasoseptal chondrocytes are promising candidate cell sources to generate cartilage for possible future reconstructions. Both cell sources were capable of producing cartilage. Though both produced collagen X, a marker for hypertrophic differentiation, tissue-engineered cartilage constructs (both pellets and flat constructs) of these cells were stable and did not mineralize. This was demonstrated in an in-vitro model, as well as in-vivo after subcutaneous implantation. A microarray study characterized these chondrocytes more profoundly, and revealed that chondrocytes from ear and nasal septum are different even after expansion and these differences provide possible explanations for the observed higher matrix production by auricular compared to nasoseptal chondrocytes.

This study was the first to examine subtype and stability of tissue-engineered constructs of human auricular and nasoseptal chondrocytes. Human cells were used in our study, while most other publications have studied chondrocytes from different locations in the body in rabbit (51), bovine (52), and porcine samples (66; 67; 68). Another strong point of this study was the elimination of donor differences which usually contributes to a lot of noise in the data sets.

In our study, auricular chondrocytes seemed to produce more cartilage than nasoseptal chondrocytes, confirming a previous study (50; 51). Although we cannot exclude that this was related to an increased proliferation rate of nasoseptal chondrocytes, a study performed by Tay *et al* (69) indicated that the increased proliferation rate of nasoseptal chondrocytes did not influence matrix production. Our microarray study did offer a possible explanation for the observed higher matrix production by auricular chondrocytes. It did not show a different expression of predictive markers of chondrogenic capacity in auricular or nasoseptal chondrocytes (collagen II, BMP2, FGFR3 or ALK1) (57). However, it did demonstrate that two growth factors with a known anabolic effect on cartilage matrix production, BMP5 (70) and IGF1 (71), were significantly higher expressed in auricular chondrocytes. On the other hand, two enzymes involved in cartilage matrix degradation, MMP13 and ADAMTS5, were expressed significantly higher in nasoseptal chondrocytes. This is in line with recent results of Asawa *et al* (50) who found a higher expression of MMP1, 2, 3 and 13 and cathepsin B in nasal septum chondrocytes compared to auricular chondrocytes in 3D conditions. In this study they also demonstrate that the expression of

MMP13 and cathepsin B was already higher in nasal chondrocytes compared to auricular chondrocytes in the monolayer condition. Moreover, in our study the cluster “glycoprotein” was identified by functional annotation clustering as a main target of the genes expressed differently in auricular and nasoseptal chondrocytes.

Cells isolated from auricular and nasoseptal cartilage produce collagen X as well as collagen II, confirming results of Naumann *et al* (72). The subtype of the original donor site does not influence terminal differentiation, as expanded auricular (elastic) and nasoseptal (hyaline) chondrocytes both produced collagen X, while this molecule is only present in native auricular cartilage. In addition, collagen X production was not induced by expansion and dedifferentiation as even primary auricular chondrocytes produced abundant collagen X. The observed difference between chondrocytes from articular joints and the head and neck area, could be caused by the presence of the perichondrium in both nasoseptal and auricular cartilage. The inner layer of the perichondrium is extremely difficult to separate from the underlying cartilage and is therefore likely contaminating cultures. Undifferentiated progenitor cells in this cambium layer are known for their capacity for cartilage regeneration (6; 7; 8; 9). This may explain why culturing chondrocytes with these progenitor cells from the cambium leads to a cartilage matrix which contains collagen X. Since pellets of both auricular and nasoseptal chondrocytes demonstrated collagen X in the matrix, we studied their tendency to mineralize in an in-vitro model. While BMSC mineralized in this in-vitro model, cartilage-like constructs of both auricular and nasoseptal chondrocytes did not and were considered stable. The stability of auricular and nasoseptal chondrocytes was further confirmed by implanting tissue-engineered constructs in vivo.

Although auricular chondrocytes had produced elastin after 6 weeks of in vivo implantation, preceded by 2 weeks of in-vitro differentiation, it was not produced after 10 weeks of in-vitro culture alone, even when primary chondrocytes were used. Other studies have demonstrated as well that elastin was produced by expanded auricular chondrocytes implanted directly in vivo (29; 52; 73), while it was not demonstrated in in-vitro redifferentiated auricular chondrocytes (72). However, de Chalain *et al* (74) demonstrated that elastin production was stimulated in chondrocyte aggregates embedded in hydrogels containing alginate, collagen and κ -elastin. Though this study was performed on primary and not expanded chondrocytes, this indicates that choosing the appropriate in-vitro model and creating a microenvironment that sustains elastin production may be important to tissue-engineer elastic cartilage although the prerequisites for elastin formation are still largely unknown. The presence of elastin in auricular constructs implanted in vivo, but not in nasoseptal constructs, indicates that these expanded cells have retained some characteristics of their native donor site. This was supported by our microarray study. In fact, 1090 genes were expressed on a significant different level in auricular and nasoseptal chondrocytes, of which 487 genes had a fold difference of 2 or more. Fibrillin 2 and elastin were expressed significantly higher in auricular chondrocytes, indicating that a subtype-specific phenotype was still present after expansion. More subtle differences, mostly in the field of developmental processes, were also observed between auricular and nasoseptal chondrocytes. Both auricular and nasoseptal cartilage are formed in the embryo by neural

crest-derived cells, thereby differing from articular cartilage (75). But although the chondrocytes they derive from are from similar origins, auricular chondrocytes produce elastic and nasoseptal chondrocytes hyaline cartilage. Despite this fundamental difference, only little is known about the distinct development processes that lead to these different cartilage subtypes. From embryonic development, it is known that while hyaline cartilage is present in the nose of murine embryos from day 14 on (75), elastic cartilage in the ear does not mature until late fetal and early neonatal periods (76), suggesting that developmental differences in timing and molecular signaling may attribute to cartilage subtype differentiation. More knowledge about these differential developmental processes may reveal clues for engineering specific subtypes of cartilage. For translation to clinical application the culture methods might be optimized, for example by using serum-free medium to expand the cells (77) or a lower oxygen tension (78). This might affect the gene expression profile as well as the amount and type of cartilage produced.

Our data indicate that using expanded chondrocytes from a donor-site that is not directly related to the host tissue, may result in cartilage regeneration of a different molecular character. Whether this may pose a clinical problem is not clear, as it is common practice in head and neck surgery to use auricular, elastic cartilage to repair hyaline cartilage defects of the nose. However, it does emphasize the importance to characterize auricular and nasoseptal chondrocytes and study them in more detail, as these cells seem to have great potential for cartilage regenerative medicine as they produce abundant, stable cartilage and can be harvested without doing harm to the donor-site in contrast to articular chondrocytes.

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3

**FIBROBLAST GROWTH FACTOR RECEPTORS
IN IN-VITRO AND IN-VIVO CHONDROGENESIS:
relating tissue-engineering using adult mesenchymal stem cells
to embryonic development**

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ABSTRACT

Adult bone-marrow derived mesenchymal stem cells (BMSCs) are considered promising candidate cells for therapeutic cartilage and bone regeneration. Because tissue regeneration and embryonic development may involve similar pathways, understanding common pathways may lead to advances in regenerative medicine. In embryonic limb development Fibroblast Growth Factor Receptors (FGFR) play a role in chondrogenic differentiation. The aim of this study was to investigate and compare FGFR expression in in-vivo embryonic limb development and in-vitro chondrogenesis of BMSCs. Our study showed that in in-vitro chondrogenesis of BMSCs three sequential stages can be found, as in embryonic limb development. A mesenchymal condensation (indicated by N-cadherin) is followed by chondrogenic differentiation (indicated by collagen II), and hypertrophy (indicated by collagen X). FGFR1-3 are expressed in a stage-dependent pattern during in-vitro differentiation and in-vivo embryonic limb development. In both models FGFR2 is clearly expressed by cells in the condensation phase. No FGFR expression was observed in differentiating and mature hyaline chondrocytes, while hypertrophic chondrocytes stained strongly for all FGFRs. To evaluate whether stage-specific modulation of chondrogenic differentiation in BMSCs is possible with different subtypes of FGF, FGF2 and FGF9 were added to the chondrogenic medium during different stages in the culture process (early or late). FGF2 and FGF9 differentially affected the amount of cartilage formed by BMSCs depending on the stage in which it was added. These results will help us understand the role of FGF signaling in chondrogenesis and find new tools to monitor and control chondrogenic differentiation.

INTRODUCTION

The potential of cell therapy to stimulate tissue regeneration is currently under investigation. Adult bone-marrow derived mesenchymal stem cells (BMSCs) are a promising candidate cell source for this purpose because of the ease with which they can be isolated and expanded as well as their capacity for multilineage differentiation (79; 80). The possibility of regenerating cartilage with the aid of BMSCs is appealing since cartilage has a very limited spontaneous healing capacity (10; 11; 12). As cartilage consists of only one cell type and does not contain blood vessels or neural innervations, it seems an uncomplicated tissue to engineer. However, to obtain optimal results in reconstruction of cartilage from different anatomical locations, it will be crucial to generate cartilage of a specific subtype. Stable hyaline cartilage should be regenerated for articular surface regeneration, since (tissue-engineered) hypertrophic cartilage may mineralize and perhaps even ossify in vivo via the endochondral route (33, 53). The generation of hypertrophic cartilage, on the other hand, could be advantageous for bone repair (53).

High-cell-density culture systems are helpful in the differentiation of BMSCs towards the chondrogenic lineage. For that reason, pellet culture is an accepted model to induce chondrogenic differentiation. However, BMSCs display signs of chondrocyte hypertrophy, such as production of collagen X and matrix mineralization during an in-vitro pellet culture period of several weeks (33; 53). Therefore, pellet culture of BMSCs has been claimed to be an in-vitro model for endochondral ossification (33). For cartilage regeneration, the consistent terminal hypertrophic differentiation of in-vitro differentiated BMSCs represents a lack of external control over their differentiation (33).

In embryology research, the development of the skeleton is a popular model to study chondrogenesis and endochondral ossification. The first stage of embryonic limb development is the formation of a mesenchymal condensation, characterized by the expression of cell adhesion molecules, such as N-cadherin. As the cells in this condensation differentiate to chondrocytes, expression of the cell adhesion molecules is downregulated, while *collagen II* and *SOX9* expression is upregulated and a cartilage template is formed that consists of a proteoglycan- and collagen II-rich matrix. Next, this cartilage becomes hypertrophic, characterized by a collagen X production, and ultimately it ossifies (5; 39). In embryonic limb development, Fibroblast Growth Factor Receptors (FGFR) 1-3 have been extensively studied since it was discovered that mutations in these receptors cause syndromes with abnormal skeletal development (43). Thus, studies have demonstrated that mRNA levels of *FGFR2* are upregulated in the mesenchymal condensation, while *FGFR3* is expressed during differentiation and *FGFR1* during hypertrophy (43; 82; 83).

We hypothesize that the chondrogenic differentiation of BMSCs in pellet culture takes place in a similar fashion to embryonic endochondral ossification. The high-cell density that is used in this model, suggests that the chondrogenesis in this in-vitro model starts with cell condensation, as in embryonic chondrogenesis. This may then be followed by chondrocyte differentiation and terminal hypertrophic differentiation. Moreover, similar signaling clues

may be involved in in-vitro differentiation and embryonic development. As FGFR activation is associated with endochondral ossification during development it is likely that FGFR activation is related to the consistent terminal hypertrophic differentiation seen in BMSCs in culture. Although studies on mRNA level indicate an important role for FGFRs in embryonic endochondral ossification, data on protein level are lacking. Moreover, FGFR expression has never been studied extensively in in-vitro chondrogenesis of BMSCs.

We therefore analyzed the developmental stages (condensation, differentiation and hypertrophy) of in-vivo embryonic endochondral ossification of the limbs and in-vitro chondrogenic differentiation of bone-marrow derived BMSCs. We studied the expression of FGFR1-3 in these different developmental stages, and compared the expression patterns in both models. Finally, we added FGF2 and FGF9 to modulate FGF signaling during specific stages in chondrogenesis. Our ultimate goal is to learn from embryonic principles to find new tools to monitor and control chondrogenic differentiation in vitro.

MATERIALS AND METHODS

Embryonic chondrogenesis

C57/BL6 female mice were intercrossed with CBA males (Charles River Laboratories, Wilmington, MA, USA). Gestational age was estimated by the vaginal plug method, with the day of plug occurrence designated as day 1 (E1). After gravid female mice had been killed by isoflurane inhalation, embryos were excised following cervical dilatation on E12, E13, E14, E16, E18 and immediately placed into Dulbecco's phosphate-buffered saline (Gibco, Carlsbad, CA, USA). Embryonic age was determined by a combination of external features and somite count.

Cell isolation and expansion

Bone-marrow-derived BMSCs were isolated from femoral shaft biopsies of five patients (4 females, 1 male, age 32-60) undergoing total hip replacement, after informed consent had been obtained in accordance with the local ethical committee (MEC 2004-142). The heparinized aspirate was seeded at a density of $2-5 \times 10^5$ cells/cm² in expansion medium (Dulbecco's Modified Eagle Medium (DMEM)- low glucose (Gibco), supplemented with 10% fetal calf serum (Gibco), 1 ng/ml FGF2 (AbD Serotec, Kidlington, UK), 25 µg/ml ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 1.5 µg/ml fungizone (Gibco) and 50 µg/ml gentamicin (Gibco)). After 24 hours, nonadherent cells were washed off and adherent cells were further expanded. At subconfluency, BMSCs were trypsinized, seeded at a density of 2,300 cells/cm² and further expanded. BMSCs from passage 4 were used for the in-vitro differentiation experiment.

To examine FGFR expression during expansion, BMSCs from passage 4 were expanded in monolayer for another 4 days, followed by immunohistochemistry for FGFR1-3. To evaluate the effect of added FGF2 on FGFR expression, BMSCs from passage 3 were expanded either in medium containing FGF2 or in medium without FGF2, until 4 days into passage 4, when immunohistochemistry for FGFR1-3 was performed.

In-vitro differentiation

After expansion, BMSCs were cultured in pellets, formed by centrifuging aliquots of 2×10^5 cells in 0.5 ml medium at 200g for 8 minutes in a polypropylene tube. Pellets were differentiated in a humidified 37°C/5% CO₂ incubator in DMEM containing Glutamax (Gibco), ITS+1 (B&D Bioscience, Bedford, MA, USA), 40 µg/ml L-proline (Sigma-Aldrich), 1 mM sodium-pyruvate (Gibco), 5 µg/ml fungizone and 50 µg/ml gentamicin, 25 µg/ml ascorbic acid-2-phosphate, 10 ng/mL TGFβ₂ (R&D systems, Minneapolis, MN, USA) and 10⁻⁷M dexamethasone (Sigma-Aldrich).

Examination of FGFR expression during chondrogenesis was carried out with cells from 4 different donors. Pellets were sequentially harvested on different culture days in triplicate, for immunohistochemistry. Pellets from one donor were harvested in duplicate only, because of shortage in cell number. Pellets of donor 1 were used for additional gene-expression analysis.

To study whether FGFR expression in our in-vitro model was regulated by culture conditions independent of chondrogenic differentiation, we used pellets from 1 donor at all experimental time points as a control cell population. These pellets were cultured without dexamethasone and did not undergo chondrogenic differentiation as was demonstrated with a negative collagen II staining after 35 days of culture. The absence of dexamethasone in the culture medium was the only difference with the experimental condition; the rest of the medium (including TGF-β) was the same.

Modulation of FGF signaling

To study whether stage-specific modulation of chondrogenesis is feasible with FGFs, we used BMSCs from two donors: donor 1 that was extensively studied in the previous experiment, as well as a new donor. Pellets were chondrogenically differentiated in the medium mentioned above (including TGF-β₂). 5 nM FGF2 or FGF9 (both Peprotech, Rocky Hill, NJ, USA) was added to this medium either throughout the culture, during early differentiation (day 3-14), or during late differentiation (day 21-35). As a control, pellets were differentiated on chondrogenic medium without FGF. Pellets were harvested in triplicate at day 35 for immunohistochemistry and biochemistry. Three control pellets were also harvested on day 21, to analyze the amount of matrix produced before FGF was added during late differentiation.

Immunohistochemistry

Embryonic limb specimens and pellets were fixed in 4% formalin in phosphate-buffered saline (PBS). Both pellets and limb specimens were embedded in paraffin, after pellets were set in 2% agar. BMSCs in monolayer were fixed for 15 minutes in 4% formalin. To reduce variability in histochemical results, all pellets were fixed overnight and stained in the same procedure.

To allow the use of monoclonal mouse antibodies on murine specimens, an earlier described method (61) was used to link the antibody with anti-mouse antiserum before immunohistochemistry. Antigen retrieval for collagen II was performed through incubation

with 0.1% pronase (Sigma-Aldrich) in PBS for 30 minutes, while antigen retrieval for collagen X required 0.1% pepsin (Sigma-Aldrich) in 0.5M acetic acid (pH=2.0) for 2 hours. Both staining continued with incubation with 1% hyaluronidase (Sigma-Aldrich) in PBS. A heat-induced epitope retrieval was performed for N-cadherin with Target Retrieval Solution (Dako, Glostrup, Denmark), according to the manufacturer's guideline. No enzymatic steps were performed for FGFR1-3.

Non-specific binding sites were blocked with 10% goat serum in PBS and sections were incubated overnight with primary antibodies. Monoclonal antibodies directed against both murine and human antigens were available for N-cadherin (M3613, Dako), collagen II (II/II6B3, Developmental Studies Hybridoma Bank, Oiwa City, IA) and collagen X (2031501005, Quartett, Berlin, Germany). For FGFR1-3 only polyclonal antibodies were available which were directed to both murine and human tissues (sc-121, sc-122, sc-123, Santa Cruz Biotechnology, Santa Cruz, CA, USA). To study the reliability of the use of these polyclonal antibodies we immunohistochemically stained pellets (of human BMSCs) with both these polyclonal antibodies and with monoclonal antibodies against FGFR1 (ab829, Abcam, Cambridge, UK) and FGFR2 (MAB6843, R&D Systems). The staining produced by these polyclonal antibodies showed the same specificity as the staining produced by these monoclonal antibodies, suggesting that the use of polyclonal antibodies (necessary for directly comparing the human and murine model) did not affect the observed FGFR expression pattern.

An alkaline-phosphatase-conjugated secondary antibody was used, followed by incubation with Neu Fuchsin substrate (Chroma, Köngen, Germany) to demonstrate alkaline-phosphatase activity with a red stain.

In each staining procedure, a mouse monoclonal negative control antibody (Dako) was used as an isotype control for the murine antibodies (N-cadherin, collagen II, collagen X and the monoclonal FGFR1-2 antibodies). Rabbit immunoglobulin fraction (normal) (Dako) was used as a negative control for the polyclonal rabbit antibodies (polyclonal FGFR1-3 antibodies). Negative controls were subject to the same antigen retrieval methods as the corresponding antibody. A staining was considered successful and used for analysis only if the control was negative. Slides were counterstained with hematoxylin.

To count the percentage of positive cells for FGFR1-3, the largest section of each pellet (section from the middle of the pellet) was selected. On this section the centre (approximately 50% of the total diameter) and periphery of the pellet were analyzed separately. A cell was considered positive when the cytoplasm stained red. To express a difference in staining intensity the percentage of positive cells was multiplied by a score of 1-3, which represented an increasing intensity of the staining. This resulted in a maximum score of 300 per pellet, meaning that all cells were strongly stained.

N-cadherin was scored by counting the percentage of positive cells in the centre of the pellet, as condensation takes place there.

Scoring for collagen II and collagen X was done by calculating the percentage of the positive-staining surface in at least three different sections throughout a pellet, using a grid of 0.1 x 0.1 mm at 10x magnification. To express a difference in staining intensity this

percentage was multiplied by a score of 1-3, representing increasing intensity. This resulted in a maximum score of 300, representing the whole pellet stained intensively.

All scorings were performed blindly by two independent observers, and per pellet their scores were averaged.

Gene expression analysis

For total RNA isolation, pellets were manually homogenised in RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). RNA was extracted with chloroform and purified from the supernatant using the RNAeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's guidelines with on-column DNA-digestion.

RNA concentration and purity were assessed on a spectrophotometer (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Isogen Life Science B.V., de Meern, The Netherlands). Complementary DNA (cDNA) was made using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) and reverse transcription-polymerase chain reactions (RT-PCR) were performed using TaqMan® Universal PCR MasterMix (Applied Biosystems, Capelle a/d IJssel, Netherlands), as described earlier (84), but on an ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR reactions for *collagen II* and *collagen X* are reported by de Mos et al (85). Oligonucleotide primers for *FGFR* assays were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet SYBRGreen I requirements; gene specificity was ensured by BLASTN search.

Primer sequences for the *FGFRs* were *FGFR1_FwX2* (5'-GTCTGCTGACTCCAGTGCAT-3') and *FGFR1_RvX2* (5'-GGTCTTCGGGAAGCTCATAC-3'); *FGFR2_Fw* (5'-AGCGTTGCCATTCAAGTGACT-3') and *FGFR2_Rv* (5'-CTACGCGCAATGCCTTCA-3'); *FGFR3_FwX* (5'-GCTGCCCGTGAAGTGGAT-3') and *FGFR3-RvX* (5'-AGAGCAGGACCCCAAAGGA-3'). Primers for *FGFR2* and *FGFR3* were designed to detect all currently known isoforms. This was not possible for *FGFR1* in a single assay and our primer set for *FGFR1* detects all currently known splice variants except isoforms 5 and 6. This includes, but not discriminates between, the so-called isoforms IIIb and IIIc.

Amplification efficiencies of all assays were between 90-98% (data not shown). Data were normalized to *GAPDH*, which was stably expressed across all harvest days (data not shown), and presented as relative expression (65).

Biochemistry

Cartilage constructs were digested overnight at 56°C in 1mg/ml proteinase K (Sigma-Aldrich) in Tris/EDTA buffer (pH 7.6) containing 185 µg/ml iodoacetamide and 1 µg/ml pepstatin A (Sigma-Aldrich). Glycosaminoglycan (GAG) content was quantified using dimethylmethylene blue (DMB) assay (62). The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ratio A530:A590 was used to determine the GAG amount, using chondroitin sulfate C (Sigma-Aldrich) as a standard. To determine the amount of DNA, each proteinase K-digested sample was treated with 100 µl heparin (8,3

IU/ml in PBS) and 50 μ l ribonuclease A (50 μ g/ml in PBS) for 30 min at 37°C. This was followed by adding 50 μ l ethidium bromide solution (25 μ g/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA, USA) using an extinction filter of 340 nm and an emission filter of 590 nm. For standards, calf thymus DNA (Sigma-Aldrich) was used.

Statistical analysis

Statistical analysis was conducted using the GraphPad Prism 5 (San Diego, CA, USA) software for Windows. Values are represented as mean \pm SEM. To compare histological scores and gene expression of FGFRs of the different culture days, statistical analysis was performed using one-way ANOVA, followed by a Newman-Keuls test for multiple comparisons. To compare the histological score for the centre of the pellets with that in the periphery on a specific culture day, an unpaired t-test was performed. The same test was used to compare GAG content. The level of significance was set at a *p* value of less than 0.05.

RESULTS

Embryonic chondrogenesis

Condensation (E12)

On embryonic (E) day 12, N-cadherin was observed in the mesenchymal condensation in the centre of the limb bud. Collagen II stained only very weakly in the mesenchymal condensation on E12. No collagen X was seen at this stage (data not shown).

On E12 expression of FGFR1 was lower in the mesenchymal condensation than in the surrounding loose mesenchyme. In contrast, FGFR2 and FGFR3 expression were seen only in the mesenchymal condensation, although the staining for FGFR3 was not very strong (fig. 1A-C).

Differentiation (E13)

At E13, multiple cartilage-like elements had formed, characterized by a collagen II rich matrix. No N-cadherin or collagen X was seen (data not shown).

At this stage, no expression of FGFR1-3 was seen in the differentiating chondrocytes (fig. 1D-F). FGFR1 and 2 were expressed in the periphery of the cartilage templates, outlining the future perichondrium. FGFR2 was also expressed in the mesenchyme located between the future skeletal elements. FGFR3 was not expressed anywhere at this stage.

Hypertrophy (E14-18)

The first signs of hypertrophy were seen at E14, characterized by a collagen X rich matrix (data not shown). These hypertrophic chondrocytes expressed FGFR1-3 at a high level, while the remaining differentiating chondrocytes showed no expression of FGFRs at all (fig.

1G-I). At E18, expression of FGFR1-3 was not only limited to hypertrophic chondrocytes, but was also expressed by some differentiating chondrocytes (fig. 1J-L).

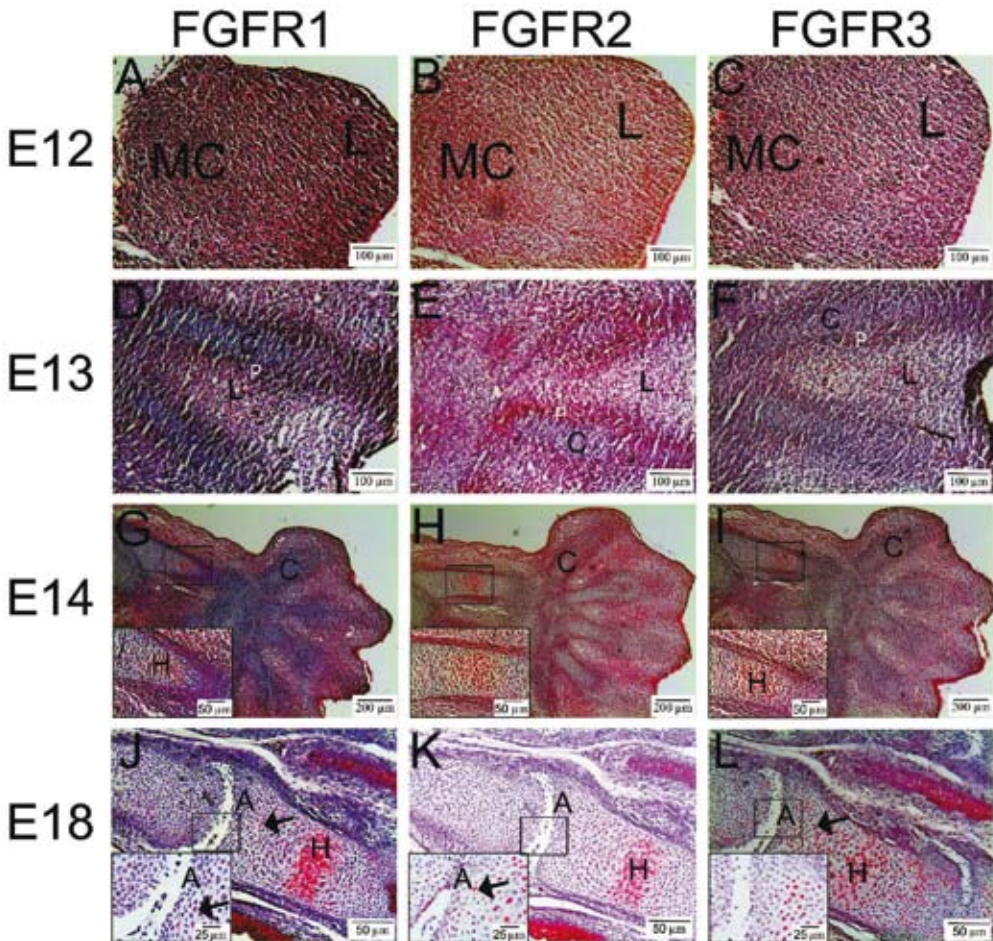


Figure 1. FGFR expression in embryonic chondrogenesis. Red staining demonstrates positivity for the antibody. **A-C** On E12, FGFR1 was expressed throughout the limb bud, but its expression was lower in the mesenchymal condensation (MC) than in the surrounding loose mesenchyme (L). In contrast, FGFR2 and FGFR3 were only expressed in the condensation. **D-F** Autopod skeletal elements of the forelimb are shown. On E13, no expression of FGFR1-3 was seen in the cartilage templates of the digits (C). The future perichondrium (P) expressed FGFR1 and 2, while the loose mesenchyme (L) between the cartilage templates expressed FGFR2. **G-I** On E14, hypertrophic chondrocytes (H) in the central area of the radius expressed all FGFRs, while differentiating chondrocytes did not express any FGFR. **J-L** On E18, expression of FGFR1-3 extended to differentiating chondrocytes (arrow), while no articular chondrocytes (A) expressed any FGFR, as is shown here in the first metacarpal phalangeal joint. Negative controls performed with rabbit immunoglobulin fraction (normal) did not show positive (red) staining.

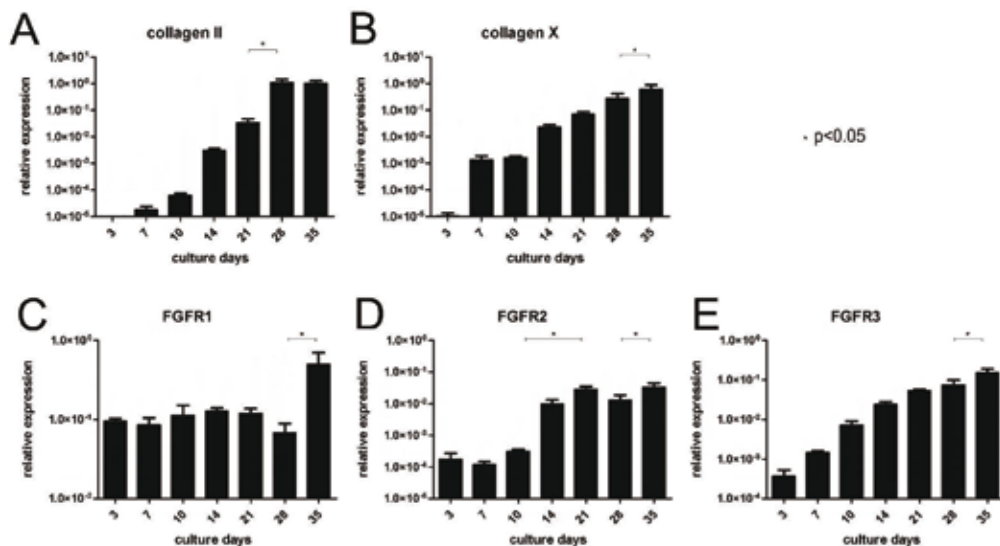


Figure 2. Gene-expression analysis of chondrogenically differentiated BMSCs. Graphs show relative mRNA-expression (determined by RT-PCR) during culture of pellets of donor 1 (homogenous cartilage formation) (A) collagen II, (B) collagen X, (C) FGFR1, (D) FGFR2, and (E) FGFR3. Levels were calculated relative to GAPDH. All data are presented as mean \pm SEM. Data are presented on a logarithmic scale.

Articular cartilage (E16-E18)

To evaluate the expression of FGFR1-3 in permanent cartilage the articular cartilage was studied. Interphalangeal joint cavitation was noted at E16 and E18. The joint surface of the phalanges was covered with a high density layer of differentiated chondrocytes not expressing FGFR1-3. No expression of FGFR1-3 was seen in these articular hyaline chondrocytes at any time point (fig. 1J-L).

In-vitro chondrogenesis

Differentiation stages

After 35 days of culture, pellets of all donors had produced both collagen II and X, suggesting terminal hypertrophic cartilage differentiation. A large, swollen morphology of the BMSCs confirmed their hypertrophic differentiation. Only pellets of donor 1 homogeneously differentiated to cartilage. Of the other 3 donors only a limited area within the pellets showed collagen II and X production (20-75% of the area), indicating that only a subpopulation of the cells in these pellets chondrogenically differentiated during culture. Therefore, gene-expression was performed on pellets of donor1, supporting the immunohistochemical results. mRNA levels of *collagen II* and *collagen X* gradually increased over time (fig. 2A-B), confirming the hypertrophic differentiation of BMSCs.

N-cadherin was clearly expressed in the centre of the pellets of all donors on day 7-10,

indicating condensation stage. N-cadherin expression decreased before collagen II was produced, and increased again mildly during hypertrophy (fig. 3A-D). In donor 1, collagen II was observed on day 21 in the periphery of the pellet, whereas collagen X was still absent (fig. 3F,I). In pellets of two other donors a similar interval was seen between collagen II and X production, while one donor demonstrated the appearance of collagen X together with collagen II at day 21.

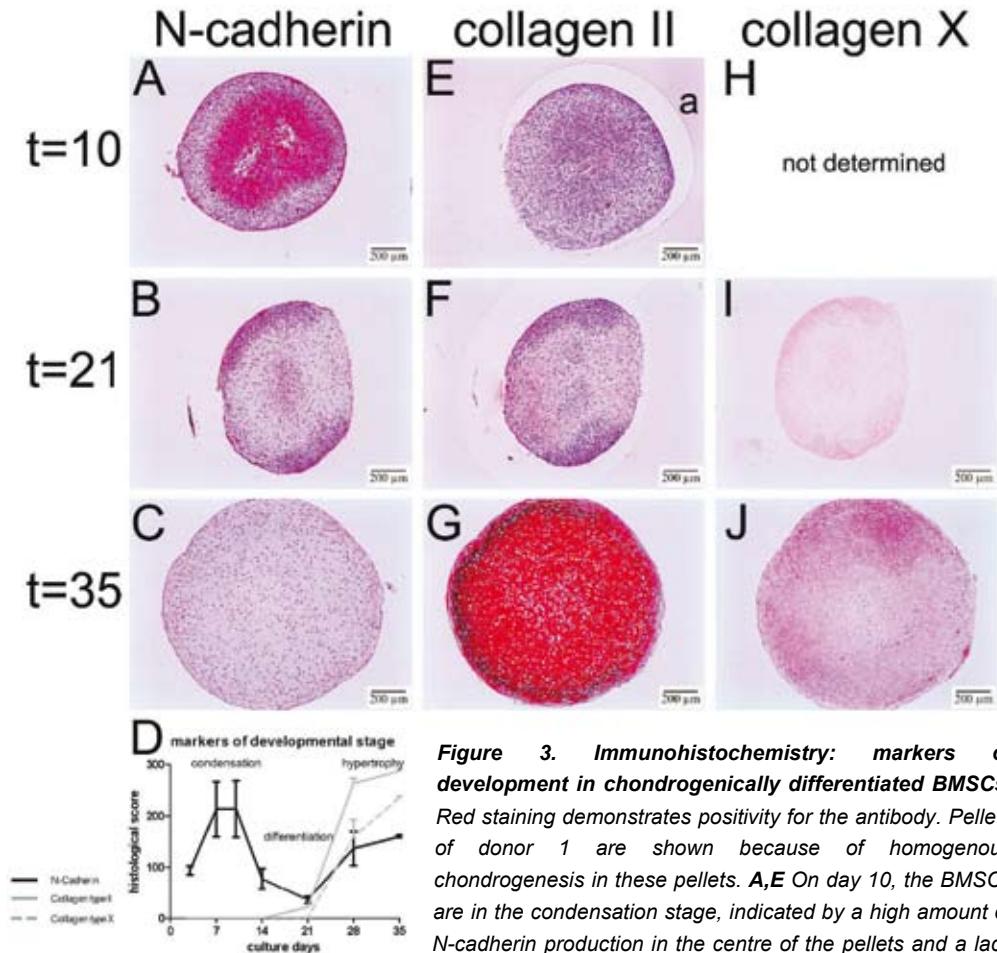


Figure 3. Immunohistochemistry: markers of development in chondrogenically differentiated BMSCs. Red staining demonstrates positivity for the antibody. Pellets of donor 1 are shown because of homogenous chondrogenesis in these pellets. **A,E** On day 10, the BMSCs are in the condensation stage, indicated by a high amount of N-cadherin production in the centre of the pellets and a lack of collagen production. **B,F,I** On day 21, some BMSCs in the periphery of the pellets are chondrogenically differentiated, indicated by collagen II, while they are not yet fully hypertrophic, indicated by a lack of collagen X production. **C,G,J** On day 35 BMSCs in the pellets are hypertrophic differentiated, characterized by a swollen morphology, and collagen II and collagen X production. **D** Histological score for N-cadherin, collagen II, and collagen X during culture of the pellets, demonstrating stages of condensation, differentiation and hypertrophy during chondrogenesis. Note: when agar (a) is still present on the slide during the immunohistochemical staining procedure, it stains red in the Neu Fuchsin substrate. Negative controls performed with mouse monoclonal negative control antibody did not show positive (red) staining.

A,E On day 10, the BMSCs are in the condensation stage, indicated by a high amount of N-cadherin production in the centre of the pellets and a lack of collagen production. **B,F,I** On day 21, some BMSCs in the periphery of the pellets are chondrogenically differentiated, indicated by collagen II, while they are not yet fully hypertrophic, indicated by a lack of collagen X production. **C,G,J** On day 35 BMSCs in the pellets are hypertrophic differentiated, characterized by a swollen morphology, and collagen II and collagen X production. **D** Histological score for N-cadherin, collagen II, and collagen X during culture of the pellets, demonstrating stages of condensation, differentiation and hypertrophy during chondrogenesis. Note: when agar (a) is still present on the slide during the immunohistochemical staining procedure, it stains red in the Neu Fuchsin substrate. Negative controls performed with mouse monoclonal negative control antibody did not show positive (red) staining.



Figure 4. FGFR expression in BMSCs during expansion. Red staining demonstrates positivity for the antibody. Cells are not counterstained. During expansion FGFR1 (A) is highly expressed, while FGFR2 (B) is expressed weakly, and FGFR3 (C) not at all. Negative controls performed with rabbit immunoglobulin fraction (normal) did not show positive (red) staining.

FGFR expression

During expansion, before differentiation in pellet cultures, all BMSCs stained brightly positive for FGFR1. No positive staining was seen for FGFR3 and only a very weak staining for FGFR2 (fig. 4). This expression pattern during expansion was not influenced by adding FGFR2 to the culture medium (data not shown).

As BMSCs of donor 1 formed complete cartilaginous pellets, FGFR expression was scored semi-quantitatively in these pellets. The response of the BMSCs from the other donors were comparable to those of donor 1 within the cartilaginous regions.

Condensation

FGFR1 was clearly expressed in pellets of donor 1 at day 3, but was followed by a significant decrease during the condensation stage, day 7-10 (fig. 5A,D). In contrast, FGFR2 showed a significant peak in expression in the centre of the pellets at day 10 (fig. 5E,H). The area of positive staining for FGFR2 overlapped the staining for N-cadherin, the marker for mesenchymal condensation (fig. 3A, 5E). Staining for FGFR3 was also somewhat positive in this condensing centre of the pellet (fig. 5I), but the difference in scores was not statistically significant (fig. 5L).

The N-cadherin positive centre stained clearly positive for FGFR2 in pellets of all other donors as well, and vaguely positive for FGFR3 in 2 of the 3 remaining donors. FGFR1 staining was almost negative in this area. Thus, during the condensation stage FGFR2 in particular showed a peak in the expression in chondrogenically differentiating BMSCs.

Differentiation

Increased positive staining for FGFR1 was seen in donor 1 on day 21 compared with previous time point, in the area that overlapped with the staining for collagen II (fig. 3F, fig. 5B). No collagen X was seen at that timepoint (fig. 3I). Staining for FGFR2 and FGFR3 were not abundant and similar to previous days (fig. 5F,J). In the pellets of the other donors where a small interval between appearance of collagen II and X was also seen, none of the FGFRs were evidently positive in the area where collagen II was present.

Hypertrophy

FGFR1 and FGFR2 stained brightly positive throughout pellets of donor 1 days 28-35 (fig.

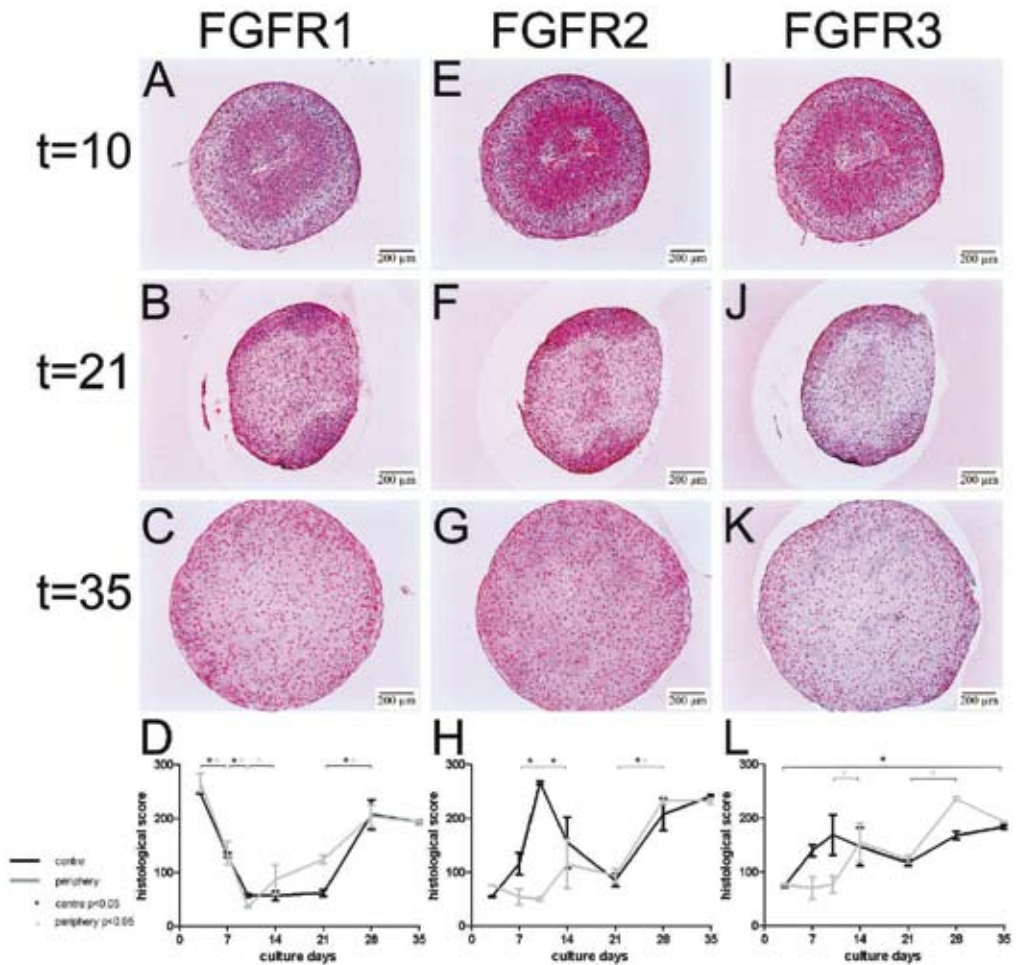


Figure 5. Immunohistochemistry: FGFR1-3 expression in chondrogenically differentiated BMSCs. Red staining demonstrates positivity for the antibody. Pellets of donor 1 are shown. **A,E,I** During condensation, little FGFR1 is expressed, while FGFR2 and FGFR3 are clearly expressed in the centre of the pellets. **B,F,J** BMSCs in the periphery of the pellet, within the cartilage-like matrix, express FGFR1. FGFR2 and FGFR3 are only minimally expressed. **C,G,K** During hypertrophic differentiation, all FGFRs are highly expressed. A magnification is added as inset to highlight the cellular staining, because cells in these pellets have become dispersed due to the production of abundant matrix at this time point. **D,H,L** Histological score of FGFR1- 3, discriminating centre or periphery of the pellets.

5C-D,G-H). Staining for FGFR3 was less intense but, in general, expression of FGFR3 increased throughout the culture period (fig. 5K-L).

In pellets of donors with less homogeneous chondrogenic differentiation, a positive staining for FGFR1-3 overlapped the area that stained positive for collagen II and X (fig. 6). Cells not producing collagen II or X were almost negative for FGFR1-3. Therefore, all FGFRs were specifically expressed by hypertrophic BMSCs.

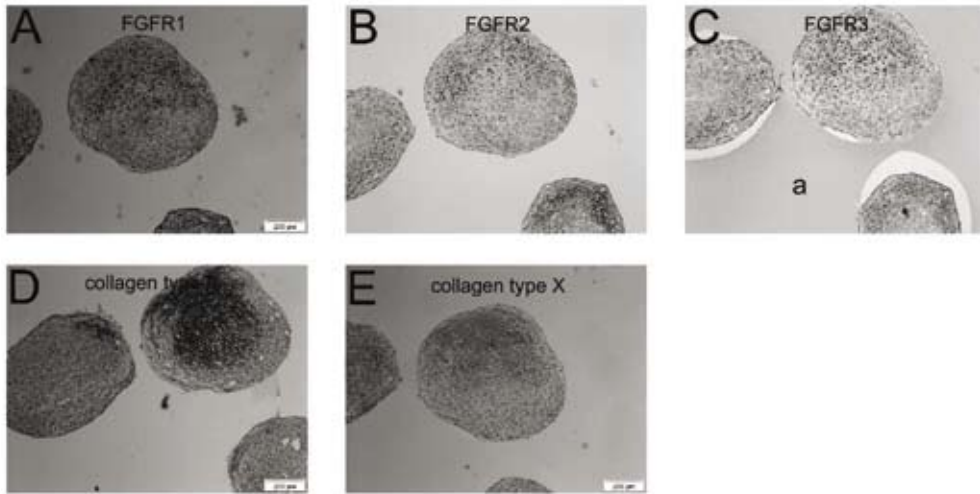


Figure 6. Immunohistochemistry of FGFRs in hypertrophic cartilage of pellets with partial cartilaginous morphology. A typical example of pellets from donor 2 with limited cartilage formation is presented to demonstrate spatial differences in immunohistochemical staining. FGFR1 (A), FGFR2 (B), and FGFR3 (C) expression overlaps with the staining for collagen II (D) and collagen X (E). When agar (a) is still present on the slide during the staining procedure, it stains red in the Neu Fuchsin substrate.

Gene expression

On transcriptional level, *FGFR1* was constantly expressed abundantly during the first 28 days of culture, and increased from day 28 till day 35. The reduced expression during condensation (day 7-10) and the increased expression from day 21 seen on histology, was therefore not observed on a gene-expression level (fig. 2C). *FGFR2* mRNA levels were only minimal at the start of the culture period and significantly increased over time (fig. 2D). Thus, the peak during condensation followed by a decrease during differentiation, seen on protein level, was not seen on mRNA level. This was also the case for mRNA levels of *FGFR3* (fig. 2E).

Control cell population

Pellets cultured on medium without dexamethasone, were used as a control cell population to assure the observed FGFR expression pattern is related to chondrogenic differentiation. No collagen II was produced in these pellets after 35 days of culture. FGFR1 was expressed at day 3, as was seen during expansion in monolayer culture and in the chondrogenically differentiating pellets at day 3. At all subsequent time-points FGFR1 was hardly expressed. FGFR2 and FGFR3 were scarcely expressed at all time points in these pellets.

Modulation of FGF signaling

Because FGFR1 and FGFR2 in particular seemed to have pronounced stage-specific expression patterns in in-vitro chondrogenic differentiation of BMSCs, we studied the possibility to modulate chondrogenesis by stimulation with FGF2, which has a higher binding

affinity for FGFR1 (86), and FGF9, which has a higher binding affinity for FGFR2 (87). FGF was added either throughout the culture (day 0-35), early in the differentiation process (day 3-14), or during a later stage of differentiation (day 21-35).

FGF addition throughout culture

Pellets treated with FGF2 or FGF9 contained significantly less GAG at day 35 than control pellets cultured in chondrogenic medium without addition of FGF (fig. 7A). No significant difference was seen in amount of DNA (data not shown). This was confirmed by histology. Control pellets from both donors demonstrated a strong positive staining for collagen II and X at day 35. Addition of FGF2 throughout the culture period resulted in a weaker staining that was limited to a smaller area. Only a very weak staining was seen in pellets treated with FGF9 (data not shown).

FGF addition during early differentiation

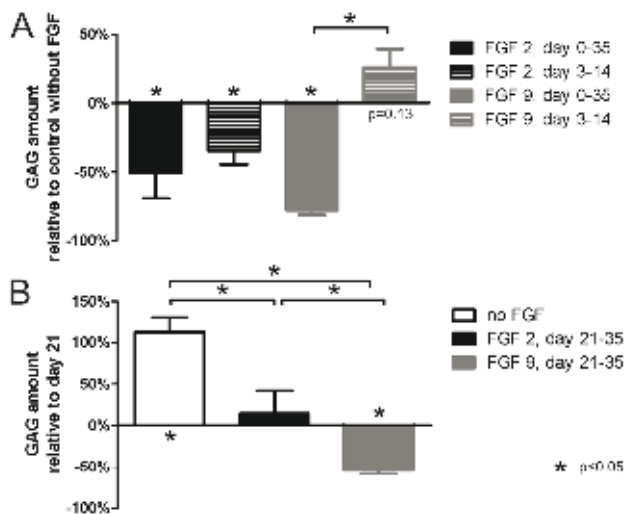
Pellets treated with FGF2 from day 3-14 contained a lower amount of GAG at day 35 than control pellets (fig. 7A). Conversely, a trend towards a higher GAG amount was seen when FGF9 was added from day 3-14 ($p=0.13$). The amount of DNA did not significantly differ (data not shown). While immunohistochemical staining for collagen II and X was observed in all pellets, no clear difference in between the conditions could be observed (data not shown).

FGF addition during late differentiation

In the control condition new GAG was deposited from day 21-35 (fig. 7B). Addition of FGF2 from day 21-35 prevented the accumulation of new GAG (fig. 7B). Addition of FGF9 resulted in a two-fold reduction of the matrix (fig. 7B), indicating that the addition of FGF9 during late differentiation stimulated matrix resorption over matrix production. These data were confirmed by histology.

Figure 7. Modulation of chondrogenic differentiation of BMSCs with FGF.

A Glycosaminoglycan (GAG) content of pellets at day 35, relative to control pellets at day 35. **B** GAG content at day 35, relative to day 21, demonstrating the effect of FGF, when added from day 21 on, on further matrix production.



At day 21, before addition of FGF, collagen II and collagen X were already clearly expressed. An increased staining was seen from day 21 to day 35 in control pellets. When FGF2 was added from day 21-35, the stained area for collagen II and X was somewhat smaller than in the control pellets at day 35. A very weak staining was seen in pellets treated with FGF9.

DISCUSSION

This study demonstrates that FGFR1-3 are expressed in a stage-dependent pattern both in chondrogenic differentiation of adult BMSCs *in vitro* and in embryonic limb development.

To our knowledge, this study is the first to directly relate embryonic chondrogenesis to *in-vitro* differentiation of BMSCs in pellet culture. In both models three stages can be discriminated, condensation, differentiation, and hypertrophy, respectively characterized in this paper by N-cadherin, collagen II, and collagen X expression. However, this study demonstrates that there is a difference in these models when the relative length of these stages is considered. For, example while in embryonic development a differentiation stage is clearly discernable, in *in-vitro* chondrogenic pellet cultures of BMSCs almost no interval exists between production of collagen II and X. We have related the expression of different FGFR subtypes to the differentiation stage in both models. In embryonic endochondral ossification, the mesenchymal condensation expressed FGFR2 and FGFR3. No FGFRs were expressed in differentiating and mature hyaline chondrocytes, while all FGFRs were expressed during hypertrophy. In comparison, in *in-vitro* differentiating BMSCs FGFR2, and to a lower extent FGFR3, were clearly expressed during condensation day 7-10. During differentiation expression of FGFR2 and FGFR3 was low, but FGFR1 was already expressed by cells producing collagen II before collagen X was observed. When collagen X was expressed, during hypertrophy of BMSCs, all FGFRs were expressed. This relation between chondrogenic differentiation stages and FGFR expression was further strengthened by low expression of all FGFRs in pellets that did not undergo chondrogenic differentiation due to withdrawal of dexamethasone.

When trying to relate the *in-vitro* differentiation of BMSCs to embryonic chondrogenesis, it is important to take into account the fundamental differences in these models. Most importantly, chondrogenically differentiating cells in the embryonic limb bud get signals from other cell types surrounding these cells, such as in the future perichondrium and the loose mesenchyme. The signals to these cells are hugely complex and not only involve growth factors such as TGF- β and FGF, but include pathways such as BMP, Ihh, PTHrP, Wnt/ β -catenin and many more (4). In comparison, in pellet cultures of BMSCs only one cell population is present, and the exogenous signals provided to these cells are simplified compared to the signaling in embryonic development. However, studying embryonic chondrogenesis may shed light into the possibilities to enhance the exogenous signal to better control chondrogenic differentiation. In this paper we have focused on FGF

signaling and the possibility to modulate chondrogenesis stage-specifically rather than adding a growth factor throughout the culture period.

FGFR expression in embryonic limb development has been studied before but only at mRNA level (43; 82; 83; 88; 89). In differentiating BMSCs FGFR expression has not been studied extensively. There is only one recently published study that demonstrated expression of all FGFRs in terminally differentiated BMSCs (90), confirming the data in the presented study. Our study added information on FGFR expression during the entire culture time.

A potential drawback of our study is the fact that we compared murine embryonic development to the differentiation of human BMSCs. We used primary antibodies cross-reacting with antigens of both species to minimize this problem. We did not perform RT-PCR during embryonic chondrogenesis and it was, therefore, not possible to directly relate the murine and the human model at mRNA level. Previous studies showed similar FGFR expression patterns in murine and human development at mRNA level (43; 82; 83; 88; 89). Moreover, the BMSCs we used in our experiment were derived from patients suffering hip osteoarthritis. Whether this may have an influence on the chondrogenic differentiation of these cells is unclear, as data regarding this subject are contradictory (91; 92; 93).

Our study on human BMSCs demonstrated a difference in FGFR expression between mRNA level and protein level. This could be due to post-transcriptional regulation. This may also explain why our results at protein level regarding FGFR expression in murine embryonic endochondral ossification differ from previous results at mRNA level. Another explanation for these differences could be the fact that many different isoforms of FGFRs exist. For immunohistochemistry we used antibodies directed to all isoforms. Although we used a single PCR assay to detect all currently known isoforms of *FGFR2* and *FGFR3*, it was not possible to design a single set of primers capable of detecting all isoforms of *FGFR1*. Our PCR primer set for *FGFR1* was developed to detect five of the seven currently known splice variants, including the so-called isoforms IIIb and IIIc, known to play important roles in limb development (94). In addition, to minimize the limitations of immunohistochemistry, we had a consistent, methodical protocol to process and stain the specimens. We, therefore, feel confident that the differences in FGFR expression patterns observed between mRNA level and protein level in the in-vitro model are not subject to the experimental procedures. As a consequence, since our data on protein level on FGFR expression in embryonic endochondral ossification differ from results from previous studies on mRNA level, further experiments dealing with FGF signaling in the developing skeleton should preferentially be based on functional protein expression of the receptors.

We demonstrated that FGFR1 was expressed at the protein level in expanding BMSCs and in early outgrowth of the limb bud, while it was downregulated during condensation. FGF2 has a strong binding affinity for this subtype FGF receptor. When it was added from days 3-14, when FGFR1 was still expressed, it inhibited cartilage differentiation. Therefore, it seems likely that FGFR1 plays a role in mesenchymal stem cell proliferation and retaining pluripotency, and not in (early) chondrogenic differentiation. This is supported by the fact that conditional inactivation of FGFR1 identified it as a positive regulator of limb bud

outgrowth (95). FGFR2 expression showed a peak during condensation, both in embryology and adult BMSC differentiation. Moreover, in BMSC pellets its staining overlapped with the staining for N-cadherin. FGF9, which has a high binding affinity for FGFR2, resulted in a somewhat increased matrix production when added during early differentiation. This suggests that FGFR2 plays a significant role in early chondrogenesis. The importance of FGFR2 during early chondrogenesis is supported by a previous study indicating FGFR2 as a positive regulator in chondrogenesis, by influencing the size of the cartilage templates and the onset of the differentiation (96). The severe syndactyly seen in Apert syndrome, caused by a FGFR2 gain-of-function mutation, supports this view (97).

Since all FGFRs are expressed in hypertrophic chondrocytes in endochondral ossification, while no FGFRs are expressed in hyaline articular chondrocytes, downregulation of FGFRs may well be important for stable chondrocyte differentiation and FGF signaling may prove to be a tool to better control in-vitro chondrogenesis of BMSCs. This is supported by our finding that addition of FGF during hypertrophic differentiation is detrimental for cartilage-matrix production. In our in-vitro study, BMSCs undergoing differentiation displayed only a minimal interval between collagen II and collagen X production, indicating rapid hypertrophic differentiation of these cells. A clear decrease in FGFR3 expression did not take place during differentiation of BMSCs in pellet culture. Therefore, the lack of FGFR3 downregulation could play a role in rapid progression to hypertrophy in pellet culture of BMSCs. Moreover, since FGFR1 was the first receptor upregulated in the cells producing a cartilage-like matrix, it may also play an important role in the early terminal, hypertrophic differentiation of BMSCs in pellet culture. Although all receptors were expressed during hypertrophy, FGF2 and FGF9 had differential effects when added day 21-35. While FGF2 inhibited further matrix deposition, FGF9 increased matrix resorption. This suggests that the FGFRs have specific effects, even during hypertrophy when they are all expressed. Further research is indicated to study the potential to modulate FGF signaling to create stable, hyaline cartilage. In this respect it may be important to realize that during embryonic limb development the cells differentiating towards hyaline, articular cartilage are most likely derived from a distinct progenitor cell population than the cells differentiating towards hypertrophic cartilage in the future bony skeletal elements (98; 99).

We have not only demonstrated that different stages can be discerned in chondrogenic differentiation, but also that the effect of a growth factor depends on the stage in which it is added in the medium. This is in accordance with embryonic development. While FGF9 has an inhibitory effect when added throughout culture, or during hypertrophy, it does not have a negative effect when given during early differentiation and might even be stimulatory when given at the right time interval. Therefore, a more specific stimulation of FGFR2 activation during condensation may be interesting for cartilage tissue engineering. Moreover, since the effects of growth factors and modulation of intracellular signaling are mostly studied throughout culture, more attention to stage-specific effects may be warranted.

In conclusion, we have demonstrated that in-vitro differentiation of BMSCs takes place in stages similar to those in embryonic endochondral ossification. A mesenchymal

condensation (indicated by N-cadherin) is followed by chondrogenic differentiation (indicated by collagen II), and hypertrophy (indicated by collagen X). FGFRs are differentially expressed in these stages, both in chondrogenically differentiating BMSC in vitro and in embryonic endochondral ossification, and first data indicate that modulation with FGF subtypes during different stages affects the amount of cartilage formed by BMSCs. A better knowledge of, and control over, FGF signaling may lead to advances in cartilage tissue engineering and regenerative medicine.

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4

**SMAD SIGNALING DETERMINES
CHONDROGENIC DIFFERENTIATION OF
BONE MARROW DERIVED MESENCHYMAL STEM CELLS:
inhibition of Smad 1/5/8P prevents
terminal differentiation and calcification**

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ABSTRACT

The aim of this study was to investigate the roles of Smad2/3 and Smad1/5/8 phosphorylation in TGF- β induced chondrogenic differentiation of bone-marrow derived mesenchymal stem cells (BMSC) in order to assess whether specific targeting of different Smad signaling pathways offers possibilities to prevent terminal differentiation and mineralization of chondrogenically differentiated BMSCs. Terminally differentiated chondrocytes produced in-vitro by chondrogenic differentiation of BMSCs or studied ex-vivo during murine embryonic limb formation, stained positive for both Smad2/3P and Smad1/5/8P. Hyaline-like cartilage produced in vitro by articular chondrocytes or studied in ex-vivo articular cartilage samples that lacked expression for MMP13 and collagen X only expressed Smad2/3P. When either Smad2/3 or Smad1/5/8 phosphorylation was blocked in BMSC culture by addition of SB-505124 or dorsomorphin throughout culture, no collagen II expression was observed, indicating that both pathways are involved in early chondrogenesis. Distinct functions for these pathways were demonstrated when Smad signaling was blocked after the onset of chondrogenesis. Blocking Smad2/3P after the onset of chondrogenesis resulted in a halt in collagen II production. On the other hand, blocking Smad1/5/8P during this time period resulted in decreased expression of MMP13, collagen X and alkaline phosphatase while allowing collagen II production. Moreover, blocking Smad1/5/8P prevented mineralization. This indicates that while Smad2/3P is important for continuation of collagen II deposition, Smad1/5/8 phosphorylation is associated with terminal differentiation and mineralization.

INTRODUCTION

Development of axial skeletal elements in vertebrates follows a tight schedule of differentiation of mesenchymal stem cells into chondrocytes and finally mineralization and ossification of the matrix. This process starts with cellular condensation, and is followed by differentiation into chondroblasts, chondrocytes, pre-hypertrophic and terminally differentiated chondrocytes (5; 39). Next, the cartilage mineralizes and is replaced by bone.

After traumatic injury or in diseases such as osteoarthritis or rheumatoid arthritis, cartilage can be damaged and the limited reparative capacity of cartilage is a major problem. Therefore, bone-marrow derived mesenchymal stem cells (BMSCs) are widely studied for their potential use in cartilage tissue engineering in order to repair cartilage defects. Chondrogenic differentiation of BMSCs follows a process similar to embryonic limb development. The cells condensate (100), start expressing SOX9 and collagen type II, but eventually the cells become terminally differentiated expressing MMP-13, collagen X and alkaline phosphatase (ALPL) (33; 35; 53). This terminal differentiation of BMSCs may be advantageous for tissue engineering of bone through the endochondral route (53). However, for cartilage reconstructions it is highly unwanted as terminally differentiated cartilage produced by BMSC mineralizes when implanted in vivo (33). So far inhibition of terminal differentiation of BMSCs was only demonstrated through the addition of PTHrP (101; 102). Elucidation of the mechanisms regulating chondrocyte differentiation, and especially terminal differentiation, are crucial to have a better control over the chondrogenic differentiation of BMSCs.

For in-vitro chondrogenic differentiation of bone-marrow derived BMSCs, TGF-beta is commonly used (40; 41). TGF-beta signaling requires binding to complexes of type II and type I serine/threonine kinase receptors, followed by receptor-Smad phosphorylation at their C-terminus. The canonical TGF-beta pathway is the Smad2/3 pathway. However, it has recently been shown that by use of an alternate type I receptor (ALK1 instead of ALK5) Smad1/5/8 is phosphorylated instead of Smad2/3 (103; 104; 105). The Smad1/5/8 route is commonly known as the route activated by Bone Morphogenetic Proteins (BMPs), also a member of the TGF-beta superfamily and a very potent inducer of bone formation (106).

The Smad2/3 route has been found to block terminal differentiation (107; 108), whereas the Smad1/5/8 route is known to stimulate terminal differentiation in murine embryonic endochondral ossification (109). We have recently found that the Smad2/3 route is protective for postnatal articular cartilage and that loss thereof is potentially a hallmark of cartilage degradation (110; 111). In contrast, in osteoarthritis chondrocytes ALK1 expression was related to MMP-13 expression, a marker of terminally differentiated chondrocytes (111).

We postulate that Smad2/3 signaling is important during all stages of chondrogenic differentiation and that Smad1/5/8 signaling is a crucial factor for terminal differentiation. We suggest that blocking Smad1/5/8 signaling is a potential tool to prevent terminal differentiation of BMSCs and thereby generating tissue-engineered cartilage that does not mineralize and more closely resembles the desired stable hyaline cartilage.

Therefore, we set out to investigate the activation of both the Smad2/3 and the Smad1/5/8 pathways in in-vitro chondrogenically differentiated BMSCs, which are known to generate terminally differentiated cartilage. We investigated these pathways in in-vitro cultured articular chondrocytes, which are known to produce stable hyaline-like cartilage (33) and compared Smad signaling in in-vitro cultured cartilage with in-vivo terminally differentiated cartilage in murine embryonic limbs, and in-vivo human healthy articular cartilage. Then, we assessed the outcome of blocking either Smad2/3 or Smad1/5/8 phosphorylation during different stages of in-vitro chondrogenic differentiation of BMSCs. Finally, we tested whether blocking Smad1/5/8 phosphorylation not only prevented hypertrophic differentiation but also resulted in a more stable cartilage construct that does not mineralize.

MATERIALS AND METHODS

Harvesting cartilage samples

To study Smad signaling in healthy hyaline and terminally differentiated cartilage in vivo, cartilage was processed directly for immunohistochemistry. Human healthy articular cartilage was harvested from left-over material of an amputated knee of a 19 year-old patient. Forelimbs of E16 murine embryo's were used to study terminal differentiated cartilage in the central region of the metacarpal bone. C57/BL6 female mice were intercrossed with CBA males (Charles River Laboratories, Wilmington, MA, USA). Gestational age was estimated by the vaginal plug method, with the day of plug occurrence designated as day 1 (E1). After gravid female mice had been killed by isoflurane inhalation, embryos were excised following cervical dilatation on E16 and immediately placed into Dulbecco's phosphate-buffered saline (Gibco, Carlsbad, CA, USA).

Cell isolation and expansion

BMSCs were isolated from a bone-marrow biopsy from the femoral shaft of five patients (age 20-44) undergoing total hip replacement, and articular cartilage was obtained from 2 donors (age 3-8) undergoing triple arthrodesis to treat clubfoot deformity, after informed consent had been obtained in accordance with the local ethical committee of Rotterdam (MEC-2007-032 and MEC 2004-142).

For isolation of BMSCs, the heparinized aspirate from the femoral shaft biopsy was seeded at a density of $2-5 \times 10^5$ cells/cm² in DMEM-LG (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (Gibco), 1 ng/ml FGF2 (AbD Serotec, Kidlington, UK), 25 µg/ml ascorbic acid-2-phosphate (Becton Dickinson), 1.5 µg/ml fungizone (Gibco) and 50 µg/ml gentamicin (Gibco). After 24 hours, nonadherent cells were washed off and adherent cells were further expanded. At subconfluency, BMSCs were trypsinized, seeded at a density of 2,300 cells/cm² and further expanded. BMSCs from passage 2-4 were used.

For isolation of chondrocytes, cartilage slices were rinsed with saline and subsequently digested through incubation for 2 hours at 37°C with 2 mg/ml Pronase E (Sigma-Aldrich, St. Louis, MO, USA), followed by overnight incubation with 1.5 mg/ml Collagenase B

(Boehringer Mannheim, Germany) in DMEM containing Glutamax (Gibco), and 10% fetal calf serum (FCS) (Gibco). Next, cell suspensions were filtered through a 100 µm filter, centrifuged and washed with saline. For expansion, chondrocytes were seeded at a density of 7500 cells/cm² in DMEM containing glutamax, with 10% FCS and 1.5 µg/ml fungizone (Gibco) and 50 µg/ml gentamicin (Gibco). When chondrocytes reached subconfluency they were trypsinized and further expanded. Chondrocytes from passage 4 were used for the experiments.

In-vitro chondrogenic differentiation

Cells were cultured in pellets, formed by centrifuging aliquots of 2×10^5 cells in 0.5 ml medium at 200 xg for 8 minutes in a polypropylene tube. Chondrogenic differentiation was induced by DMEM containing Glutamax, ITS+1 (B&D Bioscience, Bedford, MA, USA), 40 µg/ml L-proline (Sigma-Aldrich), 1 mM sodium-pyruvate (Gibco), 5 µg/ml fungizone and 50 µg/ml gentamicin, 25 µg/ml ascorbic acid-2-phosphate, 10 ng/mL TGFβ₂ (R&D systems, Minneapolis, MN, USA) and 10^{-7} M dexamethasone (Sigma-Aldrich). The medium was changed two times a week. Pellets were cultured for 35 days in total and harvested in triplicates for immunohistochemistry and gene expression.

To study differentiation and Smad expression during the course of chondrogenic differentiation of BMSC, pellets of donor 2 and 5 were harvested days 7, 14, 21, 28 and 35. In addition, pellets of BMSC donors 3 and 4 were harvested days 14, 21 and 35 and articular chondrocytes were harvested days 7, 14, 21, 28 and 35.

Modulation of Smad-signaling

To study the effect of inhibition of Smad2/3 phosphorylation on chondrogenic differentiation of BMSCs, 10µM SB-505124 (Sigma-Aldrich) was added to the medium during chondrogenic differentiation of BMSC from three different donors (donor 1-3). SB-505124 blocks the kinase domains of ALK4, -5 and -7, thereby preventing Smad2/3 phosphorylation. To study the effect of inhibition of Smad1/5/8 phosphorylation, 10µM dorsomorphin (Biomol international, Exeter, United Kingdom) was added to the medium of BMSC from three donors (donors 2-4). Dorsomorphin blocks kinase domains of ALK1, -2, -3 and -6, thereby preventing Smad1/5/8 phosphorylation. Before using these compounds in the chondrogenic differentiation of BMSC their specific inhibitory effect on phosphorylation of either Smad2/3 or Smad1,5,8 was confirmed by Western Blotting cell cultures in monolayer (data not shown).

As we have previously demonstrated the importance to modulate chondrogenesis of BMSC stage-specifically (112), these compounds were not only added to the medium throughout the culture period (day 0-35) but also from day 14-35 and day 21-35. After two or three weeks of chondrogenic differentiation of BMSC, some collagen II production is usually seen but this is donor dependent. Therefore, we used two different time points for the addition of dorsomorphin or SB505124 to study their effect on cartilage production and terminal differentiation without influencing the initial onset of chondrogenic differentiation. The effect of blocking Smad signaling during onset of differentiation only was not

investigated as the aim of our study was to investigate the involvement of different Smad pathways in terminal differentiation.

In the donor with the best chondrogenic capacity, blocking of Smad1/5/8 phosphorylation days 14-35 resulted in inhibition of markers of terminal differentiation (donor 4). To study whether stage-specific blocking of Smad1/5/8 after the onset of chondrogenic differentiation results in more stable cartilage construct that does not mineralize, 10 μ M dorsomorphin was added to the culture medium from day 14 on as in the previous experiment using cells from the donor with the best chondrogenic capacity (donor 4). In this experiment culture continued after 35 days with the addition of β -glycerophosphate to the medium (containing TGF- β +/- dorsomorphin) to allow for mineralization. After a total culture period of 49 days pellets were harvested for histology.

(Immuno)histochemistry

Samples were fixed in 4% formalin in phosphate-buffered saline (PBS) and embedded in paraffin. Pellets were first set in 2% agar before embedding in paraffin. To enable reliable direct comparison of intensity of the staining sections of treated pellets and controls were put on the same slide.

Antigen retrieval for collagen II was performed through incubation with 0.1% pronase (Sigma-Aldrich) in PBS for 30 minutes, while antigen retrieval for collagen X required 0.1% pepsin (Sigma-Aldrich) in 0.5M acetic acid (pH=2.0) for 2 hours. Both stainings continued with incubation with 1% hyaluronidase (Sigma-Aldrich) in PBS. Sections were incubated overnight with primary antibodies for collagen II (II/II6B3, Developmental Studies Hybridoma Bank) or collagen X (2031501005, Quartett, Berlin, Germany) after blocking of non-specific binding sites with 10% goat serum in PBS. An alkaline-phosphatase-conjugated secondary antibody was used for these immunohistochemical stainings, followed by incubation with Neu Fuchsin substrate (Chroma, Köngen, Germany) to demonstrate alkaline-phosphatase activity with a red stain. To allow the use of these monoclonal mouse antibodies on murine specimens, an earlier described method (61) was used to link the antibody with anti-mouse antiserum before immunohistochemistry.

MMP-13 staining was preceded by incubation of the sections in chondroitinase ABC (Sigma-Aldrich) 250 units/ml for 1 hour at 37 °C. For Smad2P, Smad1/5/8P and MMP-13 endogenous peroxide was blocked with 1% hydrogen peroxide in methanol for 30 minutes, followed by antigen retrieval through incubation in citrate buffer (0.1M sodium citrate + 0.1M citric acid) for two hours at room temperature. Sections were incubated overnight with primary antibodies against Smad2P, Smad1/5/8P (both Cell Signaling Technology, Danvers, MA, USA) or MMP-13 (Santa Cruz Biotechnology) after blocking of non-specific binding sites with 5% goat serum. A biotin labeled secondary antibody was used (DAKO, Glostrup, Denmark) for these immunohistochemical stainings, followed by a biotin-streptavidin detection system according to manufacturers protocol (Vector Laboratories, Burlingame, CA, USA). DAB reagent was used to demonstrate bound complexes with a brown stain.

Sections were critically studied for presence or absence of a positive staining. In case of collagen II and collagen X immunohistochemistry a red staining was considered positive,

while in the case of Smad2P, Smad1/5/8P and MMP-13 a brown staining was positive. As the antibody for Smad2P detects Smad3 phosphorylated at equivalent sites according to the manufacturer, we considered positivity for this antibody as positivity for Smad2/3P.

To evaluate mineralization a Von Kossa staining and a Alizarine Red staining were performed. For the Von Kossa staining, slides were immersed in 5% silver nitrate solution (Sigma-Aldrich) for 10 minutes, rinsed in ultra pure water and exposed to light for 10 minutes. Excess silver nitrate was removed with 5% sodium-thiosulphate (Sigma-Aldrich) and cells were rinsed in distilled water. For the Alizarine Red staining, slides were immersed in a saturated Alizarine Red S solution (Sigma-Aldrich) (5 gr/L) for one minute, and rinsed in 70% ethanol.

All (immuno)histochemical stainings were quantified using Bioquant Osteo 7.2 (Bioquant Image Analysis Corporation, Nashville, TN, USA). Quantification was performed on a section through the middle of the pellet. First colour-definitions were set per staining to define a positive staining. In the case of staining for collagen II, collagen X, MMP13, Von Kossa and Alizarine Red, the positive staining was then automatically selected and the size of the positive staining area was defined. Next, the total area of the pellet was manually selected and the size defined. The positive staining fraction over the total pellet area was calculated and used for further analysis.

Positive staining for Smad2/3P or Smad1/5/8P was observed in the cell, indicating active signaling via this pathway. Quantification using Bioquant Osteo was performed on a 200x magnified image in a collagen II rich area of the pellet. Positive cells were automatically selected by the software and the percentage of positive cells was calculated. For the aim of this paper we focused on whether active signaling was present or not, and the intensity of the staining was not further quantified.

We have attempted to perform Western Blots for Smad2/3P and Smad1/5/8P on pellets, but unfortunately these were not successful due to lengthy protein extraction procedure and the fact that the extraction contained large amounts of matrix constituent. Furthermore, baseline Smad-levels were not studied as the aim of this study was to investigate the role of altered active Smad signaling, as indicated by phosphorylated Smads. We did not study whether altered Smad phosphorylation was caused by alteration in basal Smad levels since, although interesting, this will not alter the conclusions of this study.

Gene expression analysis

For total RNA isolation, pellets were manually homogenized in RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). RNA was extracted with chloroform and purified from the supernatant using the RNAeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's guidelines with on-column DNA-digestion.

RNA concentration and purity were assessed on a spectrophotometer (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Isogen Life Science B.V., Belgium). Complementary DNA (cDNA) was made using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) and polymerase chain reactions were performed using TaqMan® Universal PCR MasterMix (Applied Biosystems, Capelle a/d IJssel, Netherlands), as

described earlier (113), but on a ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA). Primers used for real-time RT-PCR reactions for *SOX9*, *CBFA1*, *collagen II*, *collagen X* and *ALPL* were reported previously by Farrell et al (53), and for *MMP13* by de Mos et al (114).

Amplification efficiencies of all assays were between 90-98% (data not shown). After testing *GAPDH*, *18S*, *HPRT*, *ACTB*, *UBC* and *B2M* as housekeeping genes on selected samples, *GAPDH* was chosen to normalize the data to. Relative expression was calculated according to the $2^{-\Delta CT}$ formula (65).

Since in previous experiments we have seen that undifferentiated BMSCs express the genes of interest at a very low or undetectable level (unpublished data) these conditions were not included.

Statistical analysis

Statistical analysis was conducted using SPSS 15.0 and the GraphPad Prism 5 (San Diego, CA, USA) software for Windows. Values are represented as mean \pm SEM. Mixed model analysis and independent sample T-test were performed and the level of significance was set at a *p* value of less than 0.05.

RESULTS

Both Smad2/3 and Smad1/5/8 signaling are active in terminally differentiated cartilage, while only Smad2/3 is active in hyaline cartilage

Pellets of BMSCs of all five donors contained abundant collagen II and collagen X after 35 days of culture (fig. 1A). *Sox-9*, *CBFA1*, *collagen II*, *collagen X* and *ALPL* mRNA increased in time with limited variation between donors (fig. 2) and were highly expressed in all pellets at day 35 confirming the chondrogenic and subsequent terminal differentiation of these *in-vitro* differentiated BMSCs. Moreover, *MMP13* was highly expressed on both protein and mRNA level (fig. 1B,2). Staining for both Smad2/3P and Smad1/5/8P demonstrated positive cells dispersed throughout the pellets at all time-points (data not shown). A strong positive staining for both Smad2/3P and Smad1/5/8P was seen in all terminally differentiated cells embedded in collagen II and collagen X matrix (fig. 1C,D). In-vivo terminally differentiated cartilage in the central region of embryonic (E16) metacarpal bones, rich in collagen X and *MMP13*, stained positive for both Smad2/3P and Smad1/5/8P (fig. 3A-C). Therefore both Smad signaling pathways are active in terminally differentiated cartilage, both in in-vitro differentiated BMSC as well as in-vivo during endochondral ossification.

Pellet cultures of expanded articular chondrocytes showed abundant collagen type II at day 35 (fig. 1E). Chondrogenic differentiation of these cells was confirmed by a high expression of mRNA for *sox-9* and *collagen II*. Articular chondrocytes reached a high expression of these chondrogenic markers earlier in culture than BMSCs, but the level of expression was comparable (fig. 2). Collagen X, *MMP13* and *ALPL* expression was only

minimal on protein and mRNA level (fig. 1E-F, 2) indicating that issue-engineered cartilage from articular chondrocytes resembles hyaline cartilage. Chondrocytes in these pellets stained strongly positive for Smad2/3P while no Smad1/5/8P immunostaining was observed (fig. 1G,H). This was similar to the healthy human hyaline articular cartilage samples (fig. 3E,F).

Thus signaling through Smad2/3 seems related to hyaline-like chondrogenic differentiation and signaling through Smad 1/5/8 is related to terminal differentiation and specifically the upregulation of MMP13 expression.

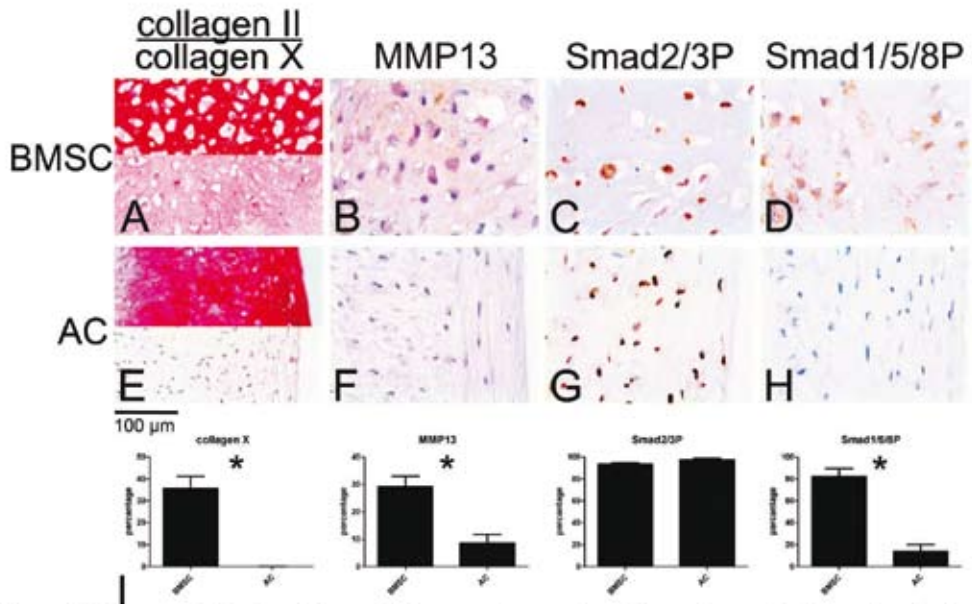


Figure 1. Immunohistochemistry of tissue-engineered cartilage from BMSC and articular chondrocytes. A-H Representative donors are shown of a total of 5 BMSC donors and two articular chondrocyte donors. Samples were harvested in triplicates for each donor after 35 days of chondrogenic differentiation. A red staining demonstrates positivity for the collagen II and collagen X antibody, while a brown staining demonstrates positivity for the MMP13, Smad2/3P and Smad1/5/8P antibody. Sections are counterstained with hematoxylin (purple). A-D Pellets of BMSCs of all five donors contained abundant collagen II and collagen X (A), and both cells and matrix stained positive for MMP13 (B). Cells in this tissue-engineered terminally differentiated cartilage stained positive for Smad2/3P (C) as well as Smad1/5/8P (D). E-H Tissue-engineered cartilage of articular chondrocytes contained collagen II, but no collagen X immunostaining was observed (E). Only minimal expression of MMP13 was observed (F). The cells in this tissue-engineered hyaline-like cartilage were positive for Smad2/3P (G), but not for Smad1/5/8P(H). I Percentage of collagen X and MMP13-positive staining fraction and percentage of Smad2/3P and Smad1/5/8P positive cells in BMSC pellets and articular chondrocytes pellets.

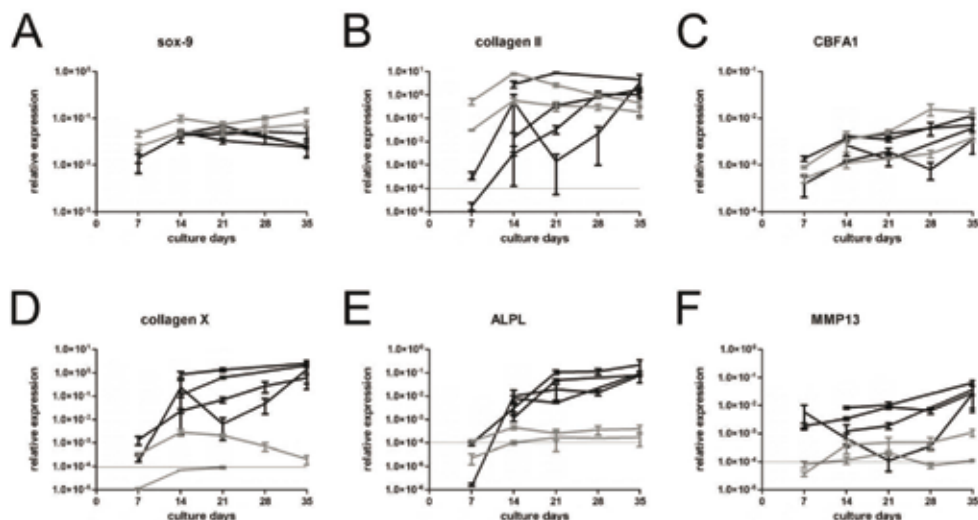


Figure 2. Gene expression during time course of chondrogenic differentiation of BMSC and articular chondrocytes. Gene expression for sox-9 (A), collagen II (B), CBFA1 (C), collagen X (D), ALPL (E) and MMP13 (F) is demonstrated for four donors (donors 2-5) with triplicate samples per donor of bone-marrow derived stromal cells (black lines) or and two donors of articular chondrocytes (gray lines). Detection levels below 1×10^{-4} are considered as no gene expression. Collagen X expression was below detection level in all samples of day 28 and 35 from one articular donor.

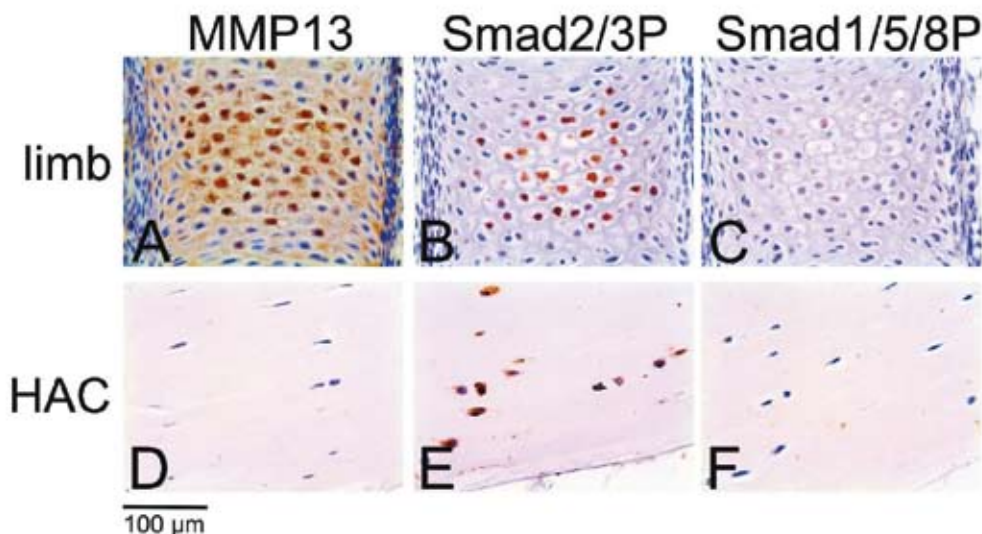


Figure 3. Immunohistochemistry of healthy terminally differentiated and hyaline cartilage in vivo. A brown staining demonstrates positivity for the antibody. A-C Terminally differentiated cartilage in the central region of a murine metacarpal bone (E16) stains positive for MMP13, Smad2/3P and Smad1/5/8P. D-F Healthy human articular cartilage shows no immunostaining for MMP13. The articular chondrocytes stain strongly positive for Smad2P, while no staining for Smad1/5/8 is seen.

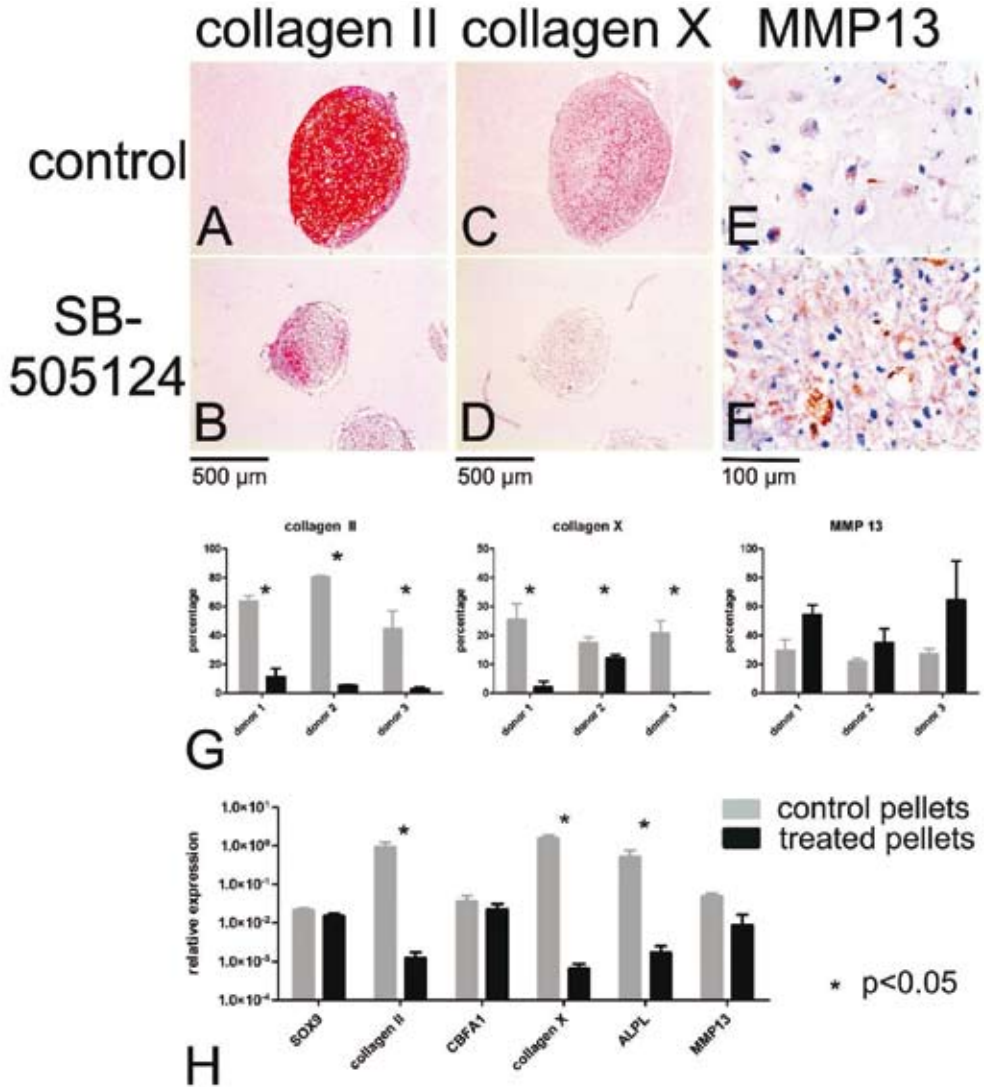


Figure 4. Immunohistochemistry and gene-expression of BMSC pellets at day 35 of chondrogenic differentiation, with or without blocking Smad2/3 phosphorylation day 14-35. A-F Immunohistochemistry is shown of a representative pellet of donor 1 with or without addition of SB-505124. **A-B** Blocking Smad2/3 phosphorylation, through addition of SB-505124, inhibited collagen II production. The amount of positive staining seen in treated pellets after 35 days of culture, was similar to the amount seen before the addition of SB-505124 at 14 days. **C-D** SB-505124 exposed pellets showed less collagen X positivity than control pellets. **E-F** MMP13 was expressed in both control and SB-505124 treated pellets. **G** Quantification of collagen II, collagen X, and MMP13 in donor 1-3 (triplicate samples), with addition of SB-505124 from day 14 on in donor 1 and from day 21 on in donor 2 and 3. Addition of SB-505124 inhibited collagen II and X production, and had no inhibitory effect on MMP13 expression. **H** Gene-expression data is demonstrated of all three donors with triplicate samples per donor. Blocking Smad2/3 phosphorylation by addition of SB-505124 day 14-35 significantly downregulated the gene expression of collagen II, collagen X and ALPL.

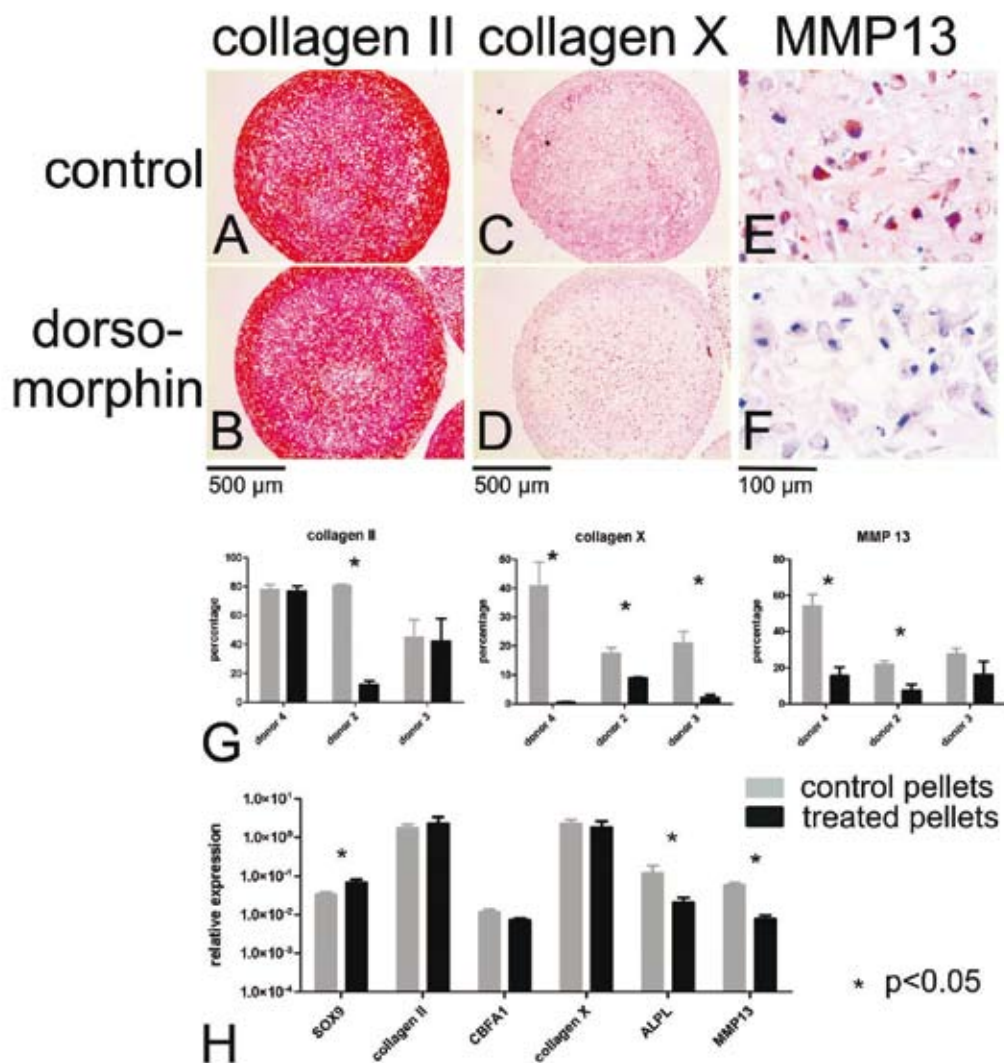


Figure 5. Immunohistochemistry and gene-expression of BMSC pellets at day 35 of chondrogenic differentiation, with or without blocking Smad1/5/8 phosphorylation day 14-35. A-F Immunohistochemistry is shown of representative pellet of donor 4 with or without addition of dorsomorphin. **A-B** Blocking Smad1/5/8 phosphorylation, through addition of dorsomorphin, did not inhibit collagen II production. **C-D** Addition of dorsomorphin resulted in a less intense staining for collagen X. **E-F** Dorsomorphin inhibited the expression of MMP13. **G** Quantification of collagen II, collagen X, and MMP13 in donor 2-4 (triplicate samples), with addition of SB-505124 from day 14 on in donor 4 and from day 21 on in donor 2 and 3. The effect of dorsomorphin on collagen II production depended on the amount of collagen II present from the start of the addition. Dorsomorphin significantly inhibited collagen X production and MMP13 expression. **H** Gene-expression data is demonstrated of all three donors with triplicate samples per donor. Blocking Smad1/5/8 phosphorylation by addition of dorsomorphin day 14-35 significantly upregulated Sox-9 gene expression, while it significantly downregulated the expression of ALPL and MMP13.

Inhibition of Smad2/3 phosphorylation blocked chondrogenic differentiation of BMSCs

To investigate the importance of Smad2/3 signaling for chondrogenic differentiation, SB-505124 was added to BMSCs from 3 donors (donor 1-3) in chondrogenic pellet cultures. Exposure to SB-505124 from the onset of the culture completely blocked chondrogenic differentiation and collagen II production (data not shown).

Although no clear donor-to-donor differences were observed in gene-expression of *collagen II* in the time-course experiment, timing of collagen II deposition in the matrix as seen on immunohistochemistry differed from donor to donor. Only in donor 1 collagen II was already present at day 14. Inhibition of Smad 2/3 phosphorylation with SB-505124 prevented further collagen II production. While collagen II was present throughout the pellets in the controls, very limited collagen II production was seen in SB-505124 treated pellets after 35 days of culture (fig. 4A,B). Donor 2 and 3 did not have a collagen II containing matrix at day 14, and addition of SB-505124 from day 14 on resulted in pellets without collagen II at day 35 (data not shown). When SB-505124 was added to these donors from day 21 on, when the first spots of collagen II depositions were seen in the matrix, further collagen II production was completely blocked as was the case in the first donor (fig. 4C). Collagen X production was not seen in pellets treated with SB-505124, either from day 14 or day 21 on, while no inhibitory effect was seen on MMP13 expression.

These protein data were supported by gene-expression analysis. In all three donors, the addition of SB-505124 from day 14 on significantly inhibited gene-expression of *collagen II*, while expression of *Sox-9* was not significantly influenced (fig. 4G). *Collagen X* expression was inhibited on mRNA level corresponding to protein level in treated pellets of all three donors, and so was gene-expression of *ALPL*. This is most likely due to the inhibited chondrogenic differentiation, hence delaying further terminal differentiation. Staining for *MMP13* was positive in both treated and control pellets (fig. 4E,F), and its gene expression was not significantly inhibited by addition of SB-505124 (fig. 4G). In figure 4 gene-expression is displayed for pellets treated with SB-505124 from day 14 as addition from day 21 on resulted in similar trends.

Summarizing, we conclude that inhibition of Smad2/3 phosphorylation by the addition of SB-505124 prevented further cartilage differentiation and cartilage matrix production after the onset of chondrogenic differentiation, while it had no inhibiting effect on MMP13 expression.

Inhibition of Smad1/5/8 phosphorylation inhibited terminal differentiation and mineralization in differentiated chondrocytes but completely blocked early chondrogenic differentiation

To test the hypothesis that Smad1/5/8 signaling is related to terminal differentiation, dorsomorphin was added to the chondrogenic differentiation medium of BMSCs of three donors (donor 2-4) to prevent phosphorylation of Smad1/5/8. Addition of dorsomorphin throughout the culture period completely prevented chondrogenic differentiation of BMSCs. Thus, Smad1/5/8P is important during the onset of chondrogenesis.



Figure 6. Blocking Smad1/5/8 phosphorylation prevents mineralization. A-B Alizarine Red staining, demonstrating calcium-phosphate depositions. When β -glycerophosphate was added to the medium day 35-49 mineralization was observed in control pellets (A), while no mineralization was demonstrated in pellets in which Smad1/5/8 phosphorylation was blocked through addition of dorsomorphin from day 14 on (B). (C) Quantification of Von Kossa and Alizarine Red staining. Pellets of donor 4 were harvested in triplicate for each condition.

Addition of dorsomorphin from day 14 on had no evident inhibitory effect on further cartilage formation in the donor where cartilage matrix was already deposited at day 14 (donor 4). In these pellets abundant collagen II was seen on immunohistochemistry in treated pellets after 35 days of culture, not differing from the control (fig. 5A,B). In the two other donors with less chondrogenic capacity, no collagen II was present at day 14 and only small spots at day 21. Although, in these cases addition of dorsomorphin inhibited collagen II production compared to the control, the collagen II positive area further increased in time and at day 35 more collagen II was seen than after the addition of SB-505124. Collagen X production and MMP13 expression were inhibited in dorsomorphin-treated pellets compared to the controls. Most importantly, in the donor where an abundant cartilage matrix was deposited in the presence of dorsomorphin after the onset of chondrogenesis, this collagen II rich matrix was lacking collagen X and MMP13 expression, indicating that this cartilage-like matrix was not terminally differentiated.

This effect of dorsomorphin, inhibiting terminal differentiation while allowing collagen II production, was even more evident when studying gene-expression. *Sox9* expression was significantly higher than in the controls (fig. 5G). Though no significant difference was observed in gene-expression of *CBFA1* or *collagen X* at day 35, the intensity of the collagen X staining was clearly diminished in the treated pellets compared to the control (5C,D). Gene-expression of *ALPL* was somewhat reduced when dorsomorphin was added to the medium, though not significantly ($p=0.13$) (fig. 5G). Moreover, both gene-expression and immunohistochemistry for MMP13 was significantly reduced in treated pellets compared to the control (fig. 5E,F,G). Therefore, inhibiting Smad1/5/8 phosphorylation by addition of dorsomorphin after initial chondrogenic differentiation, allowed further cartilage production and inhibited terminal differentiation.

To study whether blocking Smad1/5/8 phosphorylation after initial chondrogenic differentiation not only inhibits terminal differentiation but also prevents mineralization, an additional experiment was performed with cells from the donor with very good chondrogenic

capacity. β -Glycerophosphate was added to the medium from day 35-49 to allow mineralization of the matrix. While mineralization was clearly present in control pellets, blocking Smad1/5/8 phosphorylation completely prevented mineralization as was demonstrated by Von Kossa and Alizarine Red staining (fig. 6).

DISCUSSION

Generation of stable hyaline cartilage from BMSCs is currently still a challenge. Although chondrogenesis and cartilage formation are achieved, it eventually leads to terminal differentiation of chondrocytes instead of the production of stable hyaline cartilage. Furthermore, the tissue-engineered cartilage construct is not stable when it is implanted in vivo but mineralizes (33; 53). The process of in-vitro chondrogenic differentiation therefore seems a recapitulation of endochondral bone formation. A better knowledge of mechanisms determining chondrocyte differentiation and terminal differentiation is therefore crucial to control the chondrogenic differentiation of BMSCs. To our knowledge this study is the first to show the possibility to direct chondrogenic differentiation of BMSCs towards a more stable hyaline-like phenotype by targeting Smad signaling stage-specifically.

Both Smad2/3 and Smad1/5/8 phosphorylation is required for onset of chondrogenic differentiation as blocking of either one from the start of culture completely prevented chondrogenesis of BMSCs. Our results are supported by a study by Roark et al, demonstrating in chick limb mesenchymal cells that both TGF- β and BMP are important for early chondrogenesis (115). However, in the present study we demonstrate that the different Smad signaling pathways have a very distinct function during later stages of chondrogenesis. By affecting Smad signaling in BMSC pellets from day 14-35, it was possible to study the function of both pathways after the onset of chondrogenic differentiation. Blocking Smad2/3 phosphorylation prevented further increase in collagen II expression both on mRNA and histology level, but did not alter MMP13 expression. This confirms our previous findings that expression of ALK5, one of the main activators for Smad2/3 phosphorylation, correlates with collagen II expression in human articular cartilage, but not with MMP13 expression (111). Smad2/3 phosphorylation has been shown to block terminal differentiation (107). Thus, besides impairing cartilage formation directly, blocking Smad2/3 might release the block on terminal differentiation and via this way may allow MMP13 expression. This is also suggested by the enhanced terminal differentiation, in addition to the cartilage loss, seen in Smad3 deficient mice (108), and the fact that reduced TGF-beta signaling via Smad2/3, by proteosomal degradation of Smad2 and 3 through enhanced expression of Smurf2, stimulates endochondral ossification. Smad2/3 signaling is not only important for chondrogenesis, but also for the reparative response of cartilage. We have previously shown a reduced ALK5 expression and reduced responses to TGF-beta in aged mice (116). Moreover, reduced Smad2 phosphorylation was accompanied by an impaired repair response of the cartilage.

In contrast to Smad2/3, blocking Smad1/5/8 phosphorylation from day 14 on did allow further cartilage matrix production. Moreover, it prevented expression of both collagen X and

MMP-13 on histology and inhibited mineralization. *Cbfa1* and *Smad1* and *5* have been suggested crucial for terminal differentiation (117; 118). We found no effect on *cbfa1* mRNA level, but *cbfa1* requires Smad interaction for induction of an osteogenic signal (118; 119). *Cbfa1* is required for MMP13 expression and it can be anticipated that the lack of *Smad1/5/8-Cbfa1* interaction in presence of dorsomorphin is a direct cause for the reduction in MMP13 found in these chondrogenic pellets (120; 121).

Our findings are in line with the results of Valcourt et al (122) showing the involvement of *Smad1,5,8* in BMP2-induced terminal differentiation of chondrocytes and our previous study that demonstrated that ALK1 correlates with MMP13 expression in articular cartilage whereas ALK5 does not (111). *Smad1* and *5* are crucial for terminal differentiation during embryonic development, as was previously demonstrated in *Smad1/5^{CKO}* mutants which had a disorganized growth plate with loss of terminal chondrocytes and very limited collagen X expression (109).

The observed *Smad1/5/8* signaling in BMSCs may be due to signaling of the exogenous TGF-beta to the ALK1 receptor. However, we have seen in a previous study that chondrogenically differentiated BMSCs express BMP2 (data not published). Therefore, it is possible that the observed Smad signaling is not only due to exogenous but also endogenous signaling involving BMP. The precise mechanism of Smad phosphorylation would require further research.

These experiments confirm the importance of using different donors because of the variability in timing of chondrogenic differentiation. Although gene-expression of *collagen II* did not obviously differ from donor to donor, timing of collagen II gene expression and deposition in the matrix did differ, thereby influencing the results of blocking Smad-signaling. The results of inhibition of *Smad1/5/8* phosphorylation by dorsomorphin clearly demonstrate the importance of targeting the chondrogenic processes at the exact right moment. BMSCs of a donor that had an excellent chondrogenic capacity and formed cartilage within 2 weeks performed very well with dorsomorphin. Cartilage was formed, terminal differentiation was inhibited and mineralization was prevented. However, chondrogenesis of BMSCs of another donor that took 3 weeks to form a small amount of cartilage was prevented by adding dorsomorphin from day 14, whereas addition from day 21 did allow further cartilage formation and inhibited terminal differentiation. This variation in cultures highlights the stage specificity of Smad signaling during chondrogenesis. For future studies it may be interesting to study whether a more restricted administration of dorsomorphin upon the onset of terminal differentiation will completely block terminal differentiation or will just merely temporarily inhibit this process, avoiding any adverse effects the continuous administration of dorsomorphin may have. However, the donor-to-donor variation in timing of differentiation will make it difficult to identify during culture the exact timing of the onset of terminal differentiation and the administration of inhibitors.

In conclusion, our data strongly suggest that the role of *Smad2/3* and *Smad1/5/8* phosphorylation in chondrogenesis by human BMSCs is stage dependent. Based on our experiments the timing of *Smad2/3* and *Smad1/5/8* phosphorylation, relative to the expression of known chondrogenic differentiation markers, is schematically demonstrated in

figure 7. Both pathways are crucial for initial chondrogenesis. While Smad2/3 is important for ongoing cartilage matrix production, Smad1/5/8 is crucial for terminal differentiation and mineralization. Most importantly, our data imply that in-vitro cartilage tissue engineering might benefit from blocking the Smad1/5/8 route after chondrogenic differentiation of BMSCs is induced, to prevent terminal differentiation and mineralization while sustaining further cartilage-matrix production.

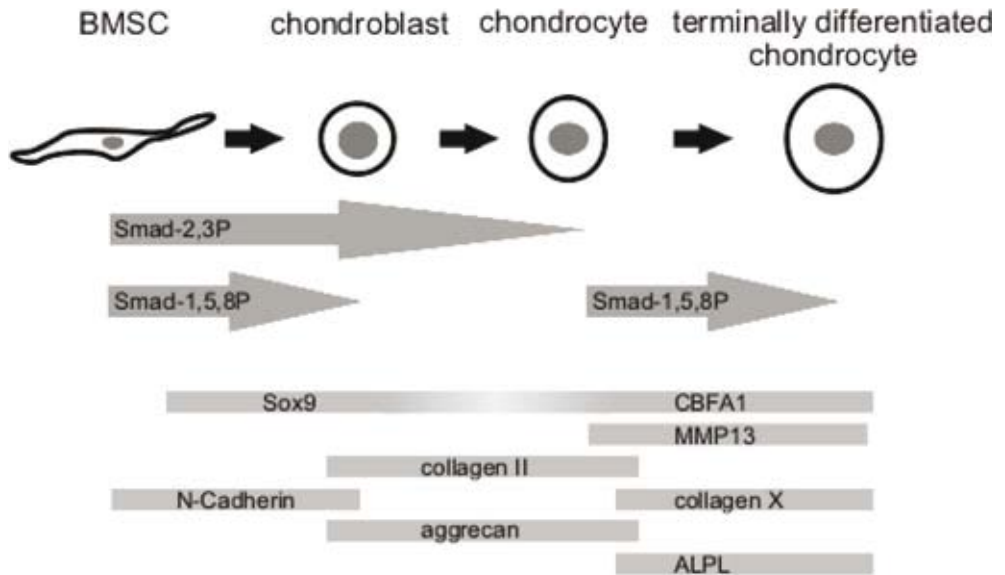


Figure 7. Schematic figure of probable timing of Smad2/3 and Smad1/5/8 signaling during chondrogenic differentiation of BMSCs, relative to some known markers of differentiation.

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5

**EFFECTS OF
TRANSFORMING GROWTH FACTOR-BETA SUBTYPES
ON IN-VITRO CARTILAGE PRODUCTION AND MINERALIZATION
OF HUMAN BONE MARROW STROMAL-DERIVED
MESENCHYMAL STEM CELLS**

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ABSTRACT

Human bone-marrow stromal-derived mesenchymal stem cells (BMSC) will differentiate into chondrocytes in response to defined chondrogenic medium containing Transforming Growth Factor – beta (TGF- β). Results in literature suggest that the three mammalian subtypes of TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3) provoke certain subtype-specific activities. Therefore, the aim of our study was to investigate whether the TGF- β subtypes affect chondrogenic differentiation of in-vitro cultured BMSC differently. BMSC pellets were cultured for 5 weeks in chondrogenic media containing either 2.5, 10 or 25 ng/ml of TGF- β 1, TGF- β 2 or TGF- β 3. All TGF- β subtypes showed a comparable dose-response curve, with significantly less cartilage when 2.5 ng/ml was used and no differences between 10 and 25 ng/ml. Four donors with variable chondrogenic capacity were used to evaluate the effect of 10 ng/ml of either TGF- β subtype on cartilage formation. No significant TGF- β subtype-dependent differences were observed in total amount of collagen or glycosaminoglycans. Cells from a donor with low chondrogenic capacity performed equally bad with all TGF- β subtypes, while a good donor overall performed well. After addition of β -glycerophosphate during the last 2 weeks of culture, the expression of hypertrophy markers was analyzed and mineralization was demonstrated by alkaline phosphatase activity and Alizarin Red staining. No significant TGF- β subtype-dependent differences were observed in expression collagen X or VEGF secretion. Nevertheless, pellets cultured with TGF- β 1 had significantly less mineralization than pellets cultured with TGF- β 3. In conclusion, this study suggests that TGF- β subtypes do affect terminal differentiation of in-vitro cultured BMSC differently.

INTRODUCTION

Each year, approximately one million patients undergo some type of cartilage reconstruction (123). These cartilage reconstructions can be performed due to congenital abnormalities, posttraumatic defects or deformities after tumor resection. Cartilage has insufficient capacity for regeneration (124) and therefore defects need to be reconstructed with transplants. As the amount of autologous cartilage donor tissue is limited, cartilage has become a main target in tissue engineering research.

Human bone-marrow stromal-derived mesenchymal stem cells (BMSC) obtained from the bone marrow, are promising cells for cartilage tissue engineering because of their high proliferative capacity and their ability to differentiate along chondrogenic lineages (31). In previous studies it was shown that BMSC can differentiate into chondrocytes in response to defined chondrogenic medium containing Transforming Growth Factor – beta (TGF- β) (41; 123; 125). In-vitro chondrogenic differentiation of BMSC, cultured with TGF- β , resembles in-vivo endochondral ossification (35; 54). After an initial mesenchymal condensation, characterized by the expression of cell-adhesion molecules such as N-cadherin (126), the cells differentiate into chondrocytes, producing cartilage specific extracellular matrix components, such as collagen I and collagen II and glycosaminoglycans (GAG) (31). This stage is quickly followed by terminal differentiation, also called hypertrophy, characterized by the production of collagen X (127; 128). When a phosphate donor is added to the chondrogenic medium, hypertrophy is followed by calcification and mineralization of the extracellular matrix, demonstrated by calcium depositions and an increased alkaline phosphatase (ALP) activity (127). In-vitro cultured cartilage will be of limited clinical use for cartilage reconstructions if it mineralizes or is replaced by bone in vivo. Therefore, in cartilage tissue engineering it is not only important to create a large amount of cartilage, but also to create a stable construct that does not mineralize when implanted in the body.

TGF- β is a multifunctional protein that regulates cell proliferation, differentiation and extracellular matrix metabolism. Three mammalian subtypes of TGF- β (TGF- β 1, TGF- β 2, and TGF- β 3) are commonly used for chondrogenic differentiation of BMSC in different laboratories (33; 35; 41; 53; 129). Despite the fact that the three TGF- β subtypes share 71-76 % sequence identity and they use the same receptors (130; 131), analysis of their in-vivo function by gene knockout mice revealed striking differences. TGF- β 1-null mice have an auto-immune-like inflammatory disease (132), while TGF- β 2-knockout mice exhibit perinatal mortality and severe developmental defects (133), and TGF- β 3-deficient mice have a cleft palate and a defective lung development (134). These differences might indicate that certain TGF- β subtype-specific activities exist that may influence the outcome of in-vitro chondrogenesis of BMSC. Therefore, the choice of TGF- β subtype to induce chondrogenesis may attribute to contradictory results in different studies. As far as we know, only one study has directly compared the effect of all three different TGF- β subtypes on in-vitro chondrogenic differentiation of BMSC. Barry *et al* (135) concluded that TGF- β 2 and TGF- β 3 are significantly more effective in promoting chondrogenesis than TGF- β 1, demonstrated by a significantly higher GAG/DNA ratio at day 21 and an earlier and more

extensive deposition of type II collagen. Would that than make TGF- β 2 and TGF- β 3 more suitable for cartilage tissue engineering of BMSC? As mentioned before, it is also important to create a stable transplant that does not mineralize. In literature many studies investigated the effect of TGF- β on hypertrophy and mineralization (31; 33; 35; 53; 41; 129; 136; 137; 138). Even after comparing studies that use identical cell sources, results were inconsistent. To our knowledge the effects of the different TGF- β subtypes on terminal differentiation and mineralization of BMSC have never been directly compared and may actually cause these previous inconsistent results.

Therefore, the aim of our study was to investigate whether TGF- β subtypes affect chondrogenic differentiation of in-vitro cultured BMSC differently, concentrating on the amount of produced cartilage as well as the degree of terminal differentiation and capacity to mineralize.

MATERIALS AND METHODS

Isolation and expansion of BMSC

BMSC were isolated from femoral shaft biopsies of four female donors (age 32 – 85 years) undergoing total hip replacement, after informed consent was obtained and with approval of the local medical ethical committee (MEC 2004-142). Culture protocols were adapted from previously described procedures (139; 140) as used previously in our laboratory (53; 54; 141) In short, the heparinized aspirates were seeded at a density of 2.5×10^5 cells/ cm^2 in Dulbecco's modified Eagle's medium (DMEM) low glucose (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS) (Gibco), 50 $\mu\text{g/ml}$ Gentamycin, 1.5 $\mu\text{g/ml}$ Fungizone (Gibco), 1 ng/ml Fibroblast Growth Factor type 2 (FGF2) (AbD Serotec, Kidlington, UK) and 25 $\mu\text{g/ml}$ L-ascorbic acid 2-phosphate (Sigma – Aldrich) at 37°C under humidified condition and 5% CO_2 . After 24 hours non-adherent cells were washed off and adherent cells were further expanded. At subconfluency, BMSC were rinsed with phosphate-buffered saline (PBS) (100 mM NaCl, 80 mM Na_2HPO_4 , 20 mM $\text{Na}_2\text{H}_2\text{PO}_4$) and detached with 0.05% trypsin (Sigma – Aldrich). The cells were replated at density of 2,300 nucleated cells/ cm^2 and further expanded up to three or four passages in the medium described above. Medium was changed twice a week.

In-vitro chondrogenic differentiation

BMSC were resuspended in a concentration of 0.2×10^6 cells in 0.5 ml chondrogenic culture medium and centrifuged at $1000 \times g$ for 8 minutes in polypropylene tubes to form pellets. Chondrogenic differentiation was induced by culturing for 35 days in chondrogenic medium (31; 53; 54), consisting of DMEM high glucose, containing Glutamax (Gibco), 1:100 Insuline Transferring Selenic acid (ITS+) (B&D Biosciences, Bedford, MA), 40 $\mu\text{g/ml}$ L-proline (Sigma – Aldrich), 1 mM sodium pyruvate (Gibco), 1.5 $\mu\text{g/ml}$ Fungizone, 50 $\mu\text{g/ml}$ Gentamycine, 25 $\mu\text{g/ml}$ L-ascorbic acid 2-phosphate, 100 nM dexamethasone (Sigma – Aldrich), and concentration of TGF- β type 1, type 2 or type 3 (R&D Systems, Minneapolis, MN, USA) depending on the experiment as described below. Medium was changed twice a

week. A dose-response experiment was performed with BMSC from one donor with good chondrogenic capacity, using the three different TGF- β subtypes in concentration of 2.5 ng/ml, 10 ng/ml or 25 ng/ml throughout the culture period. Because all had equal molecular weight (approximately 25 kDa) and the three TGF- β subtypes were able to induce chondrogenesis with comparable dose-response curves, subsequent experiments were obtained with the commonly used concentration of 10 ng/ml TGF- β . BMSC of four donors with different chondrogenic capacity were cultured in this fashion. In the last 2 weeks of culture, 10 ng/ml β -glycerophosphate (BGP) was added to the medium of some pellets to study the capacity to mineralize.

(Immuno)histochemical staining

Pellets were fixated in 4% formalin in PBS, set in 2% agar and overnight dehydrated and embedded in paraffin. Six-micrometer-thick sections were made, deparaffinized in xylene and rehydrated through graded ethanol.

Collagen II and collagen X

To analyze collagen II expression, antigen retrieval was performed though incubation with 0.1% pronase (Sigma – Aldrich) in PBS for 30 minutes at 37°C, while antigen retrieval for collagen X required 0.1% pepsin (Sigma – Aldrich) in 0.5 M Acetic Acid at pH 2.0 for 2 hours at 37°C. Both procedures continued with a 30 minutes incubation of 1% hyaluronidase (Sigma – Aldrich) in PBS. Slides were pre-incubated with 10% normal goat serum (NGS) (Sigma – Aldrich) in PBS containing 1% bovine serum albumin (BSA) (Sigma – Aldrich). Next, the slides were incubated overnight at 4 – 7°C with respectively mouse monoclonal antibody against collagen II (II-II6B3, Developmental Studies Hybridome Bank, Iowa City, IA) or against collagen X (Quartett X53, Berlin, Germany) in PBS containing 1% BSA. The next day, a biotin-labeled secondary antibody was used (LINK) (Biogenex, HK-325-UK, from LINK-LABEL kit ZA-000-UM), in addition of 5% normal human serum (NHS), followed by an alkaline-phosphatase-conjugated streptavidine (LABEL) (Biogenex, HK-321-UK). Incubation with Neu Fuchsin substrate (Chroma, Kongen, Germany) was performed to demonstrate alkaline phosphatase activity with a red stain. Sections were counterstained with Gill's haematoxylin (Sigma – Aldrich).

Alizarin Red and Von Kossa staining

For evaluation of mineralization, slides were stained in Alizarin Red S solution (Sigma – Aldrich). Sections were immersed in acetone and subsequently in acetone/xylene. Thus, calcium depositions were demonstrated with an orange stain. Von Kossa staining was also used to demonstrate mineralization. For this staining slides were immersed in 5% silver nitrate solution (Sigma-Aldrich) for 10 minutes, rinsed in ultra pure water and exposed to light for 10 minutes. Excess silver nitrate was removed with 5% thiosulfate (Sigma-Aldrich) and slides were rinsed in distilled water. Sections were counterstained with 0.4% thionine in 0.01 M of aqueous sodium acetate pH 4.5 for 5 minutes, demonstrating proteoglycan content of the matrix with a purple stain. Calcium depositions were demonstrated with a black stain. Alizarin Red staining and Von Kossa staining showed similar trends and therefore only the Alizarin Red staining is demonstrated in the figures. Quantification of

mineralization was determined by measuring the positive calcium-phosphate fraction over the total pellet area with the program ImageJ, an image processing program developed at the National Institutes of Health (142). An area was appointed as a positive calcium-phosphate fraction where Alizarin Red and Von Kossa staining stained the same area. This resulted in a percentage of mineralized area. Averaged results of three sections per pellet were used for further analysis.

Biochemistry

DNA

Pellets were digested overnight at 56°C in 1 mg/ml proteinase K (Sigma – Aldrich) in TRIS/EDTA buffer (pH 7.6) containing 185 µg/ml iodoacetamide and 1 µg/ml pepstatin A (Sigma – Aldrich). To determine the amount of DNA, samples were treated with 100 µl heparin (8.3 IU/ml in PBS) and 50 µl ribonuclease A (50 µg/ml in PBS) for 30 minutes at 37°C. This was followed by adding 50 µl ethidium bromide solution (25 µg/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA, USA) using extinction filter of 340 nm and an emission filter of 590 nm. For standards, calf thymus DNA (Sigma – Aldrich) was used.

GAG

Proteinase K-digested samples were used to quantify the amount of GAG, by using dimethylmethylene blue (DMB) assay. The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ration A530 : A590 was used to determine the amount of GAG, using chondroitin sulfate C (Sigma – Aldrich) as a standard (62).

Total collagen

Cells were suspended in milli-Q, hydrolyzed at 108°C for 18 to 20 hours in 6 M HCl and dried and redissolved in 100µL water. Hydroxyproline contents were measured by colorimetric method (143) (extinction, 570 nm), with chloramine-T and dimethylaminobenzaldehyde as reagents and hydroxyproline as standard (Merck, Damstadt, Germany).

VEGF secretion

To determine VEGF secretion, 48-hours conditioned culture medium was centrifuged at 1000 x g for 15 minutes immediately after collection and the supernatant was stored at – 80°C until further use. The concentration of VEGF in the medium was measured using a commercially available sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) according to suppliers information.

ALP activity

ALP activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1M diethanolamine buffer supplemented with 1 mM MgCl₂ at pH 9.8) in the digest of medium for 10 min at 37 °C. The reaction was stopped by adding 0.06 M NaOH. Absorption was measured at 405 nm. Results were adjusted for DNA content of the cell lysates.

Statistical analysis

Analysis of (immuno)histochemistry, biochemistry and VEGF expression were performed on three pellets of each condition. Biochemical data of TGF- β 1 treated pellets of the second donor were lost due to a technical error. Mixed model analysis and an independent sample T-test were performed on day 21 and day 35 between culture days and between different TGF- β subtypes. A p -value less than 0.05 was considered to indicate statistically significant differences. Statistical analysis was conducted with Microsoft Office Excel 2000 and SPSS 15.0.

RESULTS

Dose-response curves

To study whether the different TGF- β subtypes can be directly compared using the same concentration, dose-response curves were performed of the three TGF- β subtypes on BMSC from a donor with good chondrogenic capacity. After 35 days of culture, control pellets without TGF- β were small, had no positive collagen II staining and contained only a little amount of GAG production per DNA (GAG/DNA) (data not shown). Chondrogenic differentiation with 2.5 ng/ml of either TGF- β subtype resulted in less cartilage production, than with 10 ng/ml or 25 ng/ml of the same TGF- β subtype, as demonstrated with limited localisation of collagen II staining towards the central regions (fig. 1) and a significant lower amount of GAG/DNA ($p < 0.05$) (fig. 2). In contrast, with either 10 ng/ml or 25 ng/ml of any TGF- β subtype, a rich collagen II containing matrix was found throughout all pellets. No significant difference was seen between those two concentrations in intensity of the collagen II staining (fig. 1) or in GAG/DNA content for any TGF- β subtype (fig. 2).

Amount of cartilage production

Based on the previous experiment, we concluded that TGF- β 1, - β 2 and - β 3 have comparable dose-response curves and can be directly compared using the same concentration. As 10 ng/ml is commonly used in literature to induce chondrogenesis and the higher concentration of 25 ng/ml did not seem to have a superior effect, subsequent experiments were performed with 10 ng/ml of TGF- β . To fully evaluate the capacity of the TGF- β subtypes, we performed the experiments with cells of four selected donors with variable chondrogenic capacity. From previous studies the first donor was known to have a very good capacity for chondrogenic differentiation, while the second was qualified as good, the third as low-moderate and the last donor hardly differentiated chondrogenically at all. Indeed, the first and second donor already had some positive collagen II staining at day 21 in the peripheral regions of the pellets, and at day 35 these pellets had abundant staining throughout the pellet, irrespective of TGF- β subtype. In contrast, the third and fourth donor did not show any collagen II production at day 21 and at day 35 only a very limited area stained positive (data not shown). Furthermore, GAG/DNA was significantly higher in the first two donors, compared to third and fourth donor ($p < 0.05$). Addition of 25 ng/ml of TGF- β did not increase the amount of cartilage production by these donors with low chondrogenic

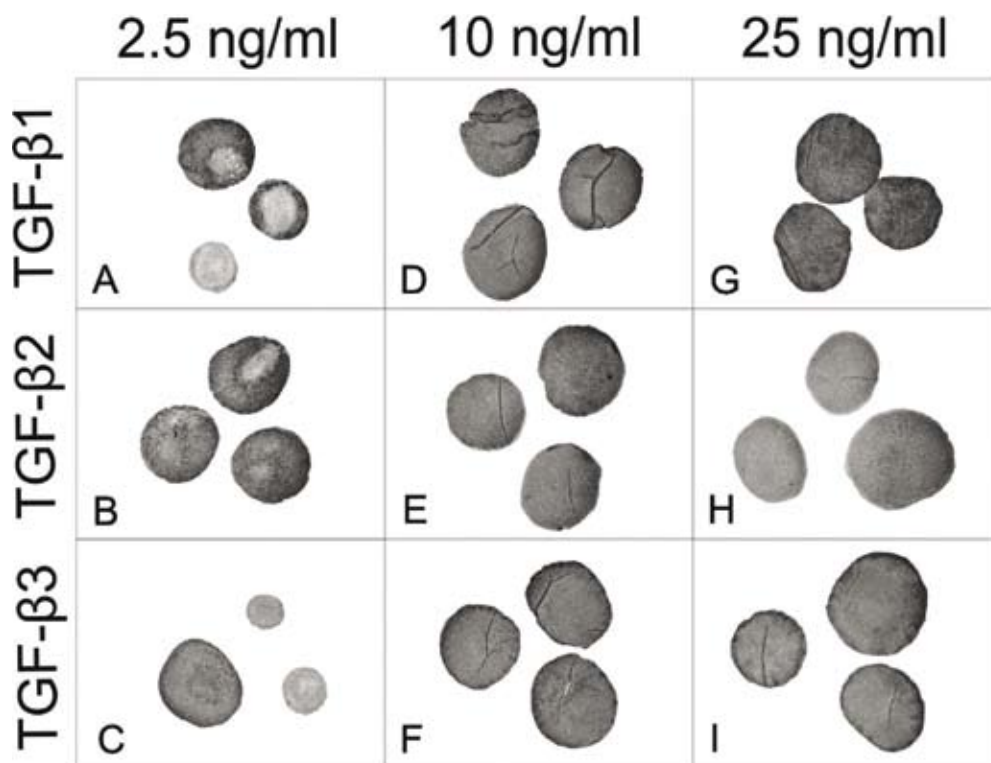


Figure 1. (Immuno)-histochemical staining of collagen II of BMSC pellets from a donor with good chondrogenic capacity. Pellets were cultured for 35 days with 2.5 ng/ml, 10 ng/ml or 25 ng/ml of TGF-β1, -β2 or -β3. Photographs were taken at magnification 40x.

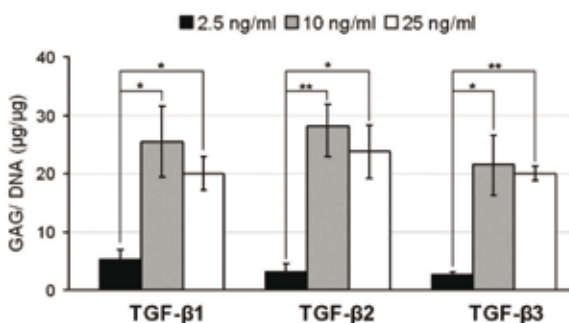


Figure 2. Content of GAG/DNA in BMSC pellets from a donor with good chondrogenic capacity. Pellets were cultured for 35 days with 2.5 ng/ml, 10 ng/ml or 25 ng/ml of TGF-β1, -β2 or -β3. Each bar represents the average and standard error of the mean of three replicates. Asterisk indicates a significant difference with a p-value of * < 0.05 or ** < 0.01.

capacity (data not shown).

At day 21 and day 35, no clear differences in collagen II staining were observed between the TGF- β subtypes (data not shown). After analyzing the amounts of DNA, collagen/DNA and GAG/DNA of each donor separately, no significant differences between the TGF- β subtypes were found. However, in the first, second and fourth donor a trend was seen towards a higher amount of cartilage produced in presence of TGF- β 2 (fig. 3). Still, when pellets of all four donors were analyzed together this trend was not recognizable (data not shown).

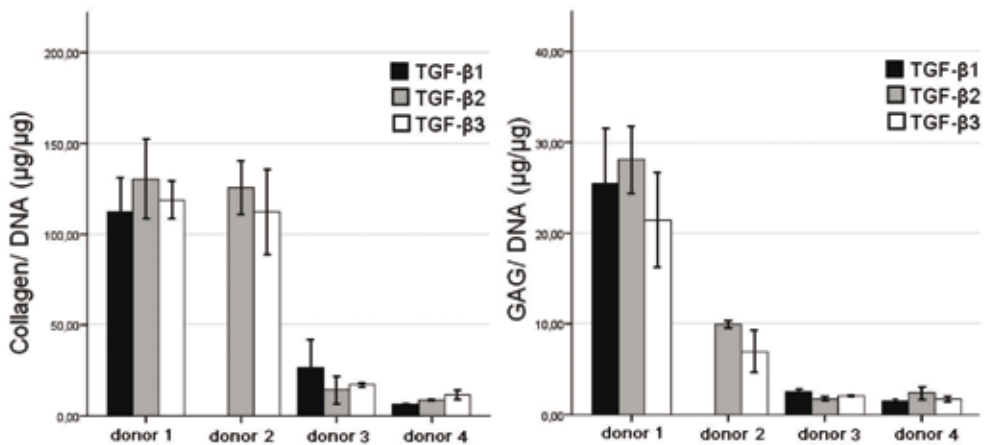


Figure 3. Content of collagen/DNA and total GAG/DNA in BMSC pellets. Pellets were cultured for 35 days with 10 ng/ml of TGF- β 1, - β 2, or - β 3. Each bar represents the average and standard error of the mean of each of the four donors separately, with three replicates per donor. Biochemical data of TGF- β 1 treated pellets of the second donor were lost due to a technical error indicated with an asterisk.

Expression of hypertrophy markers

At day 35 a positive collagen X staining was detected throughout the pellet in all conditions of the first and second donor, demonstrating terminal differentiation of the tissue-engineered cartilage. The third and fourth donor with low chondrogenic capacity did not show any positive collagen X staining. No clear differences were observed in collagen X staining between the TGF- β subtypes in all donors (data not shown). Furthermore, VEGF was secreted in the medium at day 35, although no significant differences were detected between the TGF- β subtypes (data not shown). These data indicate that terminal differentiation was induced in chondrogenically differentiated BMSC pellets, irrespective of the subtype of TGF- β used for culturing.

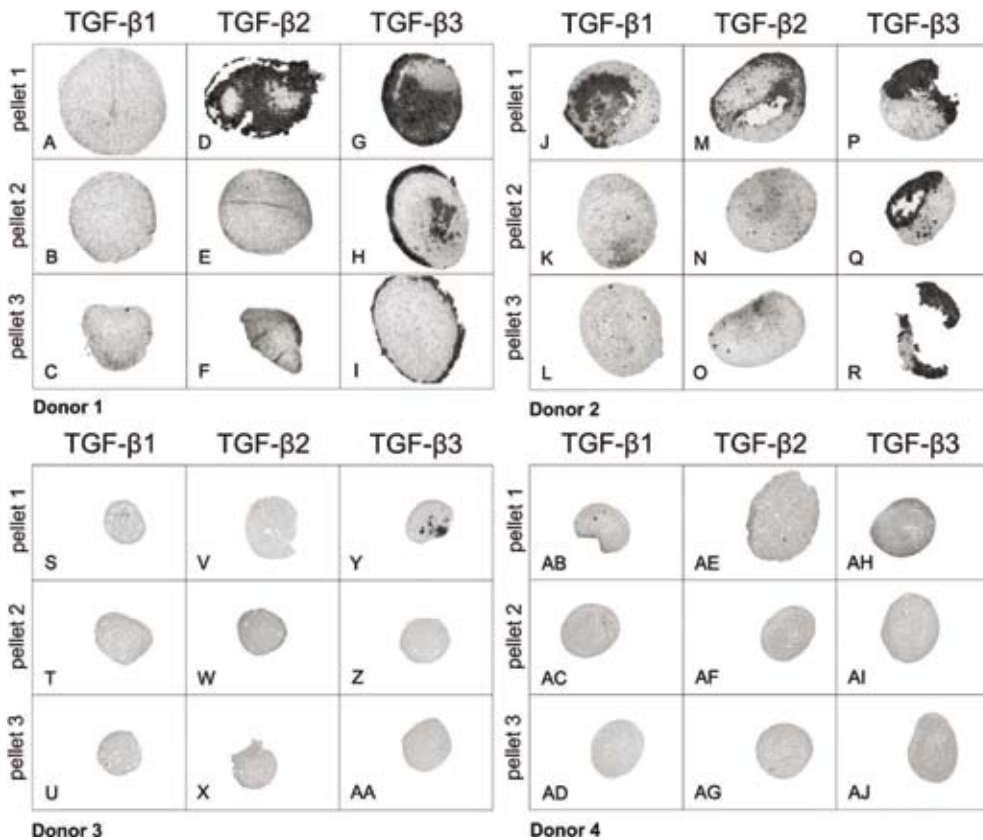


Figure 4. Alizarin Red staining of BMSC pellets. Pellets were cultured for 35 days with 10 ng/ml TGF-β1, -β2 or -β3 with addition of BGP in the last 2 weeks of culture. Positive staining, demonstrating calcium-phosphate depositions, is demonstrated with an orange stain. Results of all individual pellets from 4 donors with varying chondrogenic capacity are shown. Photographs were taken at magnification 40x.

Capacity to mineralize

Alizarin Red and Von Kossa staining showed similar patterns, therefore Alizarin Red staining was used to further evaluate mineralization. As expected no calcium-phosphate depositions were observed in pellets cultured on chondrogenic medium in the absence of BGP as a phosphate donor. If BGP was added to the medium during the last 2 weeks of culture, mineralization was clearly observed at day 35 in good chondrogenic donors indicating that the tissue-engineered cartilage was not only terminally differentiated but also had a tendency towards matrix mineralization. In the first donor with good chondrogenic capacity, mineralization was observed in three out of three pellets cultured with TGF-β3 (fig. 4G-4I). All three pellets cultured with TGF-β2 were also mineralized (fig. 4D-4F), however the

mineralized area was smaller compared to TGF- β 3. In comparison, only one pellet cultured with TGF- β 1 had a very small amount of mineralization, the others were negative (fig. 4A-4C). The second donor with good chondrogenic capacity showed abundant mineralization throughout all three pellets with TGF- β 3 (fig. 4P-4R) and slightly less mineralization with TGF- β 2 and TGF- β 1 (fig. 4J-4O). Pellets of the other donors with less chondrogenic capacity contained significantly less cartilaginous matrix and consequently almost no mineralization in any of the TGF- β subtypes (fig. 4S-4AJ). Addition of a higher concentration of TGF- β (25 ng/ml) did not increase either cartilage matrix production or mineralization (data not shown). Quantification of mineralization was determined as a percentage of the area that stained positive with Alizarin Red, over total pellet area of all four donors. The percentage of mineralization was significantly larger in TGF- β 3 pellets, compared to TGF- β 1 (fig. 5).

To further investigate TGF- β subtype-specific effects on mineralization, ALP activity was measured in chondrogenically differentiated pellets at day 35. Because differences in mineralization were more prominent in a donor with good chondrogenic capacity, pellets of the second donor were used for this additional analysis. In line with the histology, significantly more ALP activity/DNA was present in TGF- β 3 pellets, compared to TGF- β 1 pellets (fig. 6).

Figure 5. Percentage of mineralized area over total pellet area. Quantification was performed on Alizarin Red and Von Kossa staining of BMSC pellets from all four donors cultured for 35 days with 10 ng/ml of TGF- β 1, - β 2, or - β 3 with addition of BGP in the last 2 weeks of culture. Each bar represents the average and standard error of the mean of four donors with three replicates per donor. Asterisk indicates a significant difference with a p -value of $* < 0.05$.

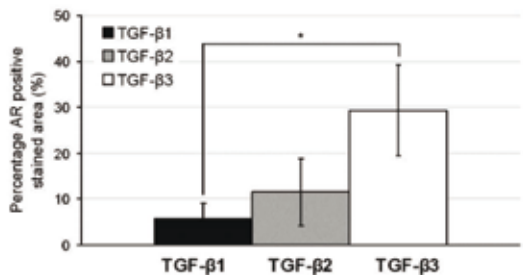
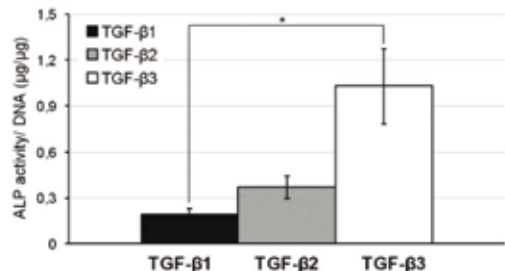


Figure 6. Alkaline Phosphate (ALP) activity in BMSC pellets from a donor with good chondrogenic capacity. Pellets were cultured for 35 days with 10 ng/ml TGF- β 1, - β 2 or - β 3 with addition of BGP in the last 2 weeks of culture. Each bar represents the average and standard error of three replicates. Asterisk indicates a significant difference with a p -value of $* < 0.05$.



DISCUSSION

To induce chondrogenic differentiation of BMSC, all three mammalian TGF- β subtypes are commonly used in different laboratories in varying concentrations (31; 33; 35; 53; 54; 129). In this study we demonstrate that all three subtypes are able to induce chondrogenic differentiation with comparable dose-response curves. A variation in degree of chondrogenic differentiation between samples cultured under same conditions is often described as a general issue in BMSC cultures (41; 53) and was also noticed in all conditions in our study. Although the smaller variation which was seen in pellets that were cultured with 25 ng/ml of TGF- β , 10 ng/ml can be used as an affordable and suitable dosage to induce chondrogenic differentiation in BMSC with all three TGF- β subtypes. Despite the fact that the three TGF- β subtypes share 71-76 % sequence identity and they use the same receptors (130), we demonstrated in this study that TGF- β subtypes do affect chondrogenic differentiation differently in in-vitro pellet cultures of BMSC. Although no TGF- β subtype-dependent differences were found in cartilage production, a striking difference was seen in the capacity of the tissue-engineered cartilage to mineralize. TGF- β 3 treated pellets had significantly more mineralized extracellular matrix than TGF- β 1 treated pellets.

Only one other previous study (135) has directly compared the effect of all three different TGF- β subtypes on in-vitro chondrogenic differentiation of BMSC. However, this study only studied the amount of cartilage production and they performed their experiment on BMSC from one single donor only. They concluded that the use of TGF- β 3 and TGF- β 2 resulted in significantly more cartilage production than TGF- β 1. In a study comparing two TGF- β subtypes, TGF- β 1 and TGF- β 3, no differences were found after comparison of BMSC pellets of three donors (144). Those results support our study in which we did not see significant TGF- β subtype-dependent differences in cartilage matrix production. Nevertheless, pellets treated with TGF- β 2 did produce more cartilage matrix than TGF- β 1 and TGF- β 3, in three out of four donors after 35 days of culture. Because culture methods of these studies were similar, donor-dependent differences might play a role in these inconsistent results. Even when cultured in the same fashion, BMSC from different donors vary in chondrogenic capacity, as was clearly demonstrated in our study. In our experiments an association with donor-age and amount of produced cartilage matrix is obvious; the first and second donor (respectively 35 and 32 years old) performed equally well with all TGF- β subtypes, while the third and fourth donor (respectively 84 and 68 years old) performed equally bad. An explanation for the limited chondrogenic differentiation capacity of older donors might be found in a decreased expression of the TGF- β receptors with age (116; 145). However, a study with 60 donors did not find a correlation between donor age and extent of chondrogenic differentiation (41). This may be explained by the lower age range (14 – 55 years old) in this previous study, compared to our study (32 – 84 years old).

The effects of the different TGF- β subtypes on maintenance of the chondrocytes phenotype of chondrogenically differentiated BMSC have never been directly compared. Nevertheless, collagen X production and as a consequence terminal differentiation were previously demonstrated in chondrogenically differentiated BMSC pellet cultures, in the

presence of TGF- β 1 (125; 129), TGF- β 2 (53; 54) and TGF- β 3 (33; 35; 41). This suggests that terminal differentiation of BMSC occurs in the presence of all TGF- β subtypes, which is supported by our results. VEGF, a proangiogenic factor produced by hypertrophic chondrocytes (35; 146) was secreted in our pellet cultures, and no significant differences were observed between the TGF- β subtypes. Thus concentrating on two important markers of terminal differentiation, hypertrophic alterations occurred similarly in the presence of any of the three TGF- β subtypes. Conversely, in literature TGF- β 1 was used in studies demonstrating an inhibitory effect on terminal differentiation of in-vitro cultured epiphyseal chondrocytes (136), rabbit costal chondrocytes (137) and chick embryonic limb mesenchymal cells (138). As for clinical application it is required to tissue engineer a cartilage construct that is stable and does not mineralize in vivo, direct comparison of the effects of TGF- β subtypes on terminal differentiation and the relation to mineralization is important.

Despite the finding that all TGF- β subtypes induce terminal differentiation to a similar degree, TGF- β subtype-dependent differences were observed in the capacity of the tissue-engineered construct to mineralize. After addition of a phosphate donor to the medium, pellets cultured with TGF- β 3 showed significantly more cartilage matrix mineralization than pellets cultured with TGF- β 1. Furthermore, significantly more ALP activity/DNA was seen in TGF- β 3 treated pellets, compared to TGF- β 1. Although in their study, Mueller *et al* concluded that TGF- β 3 and TGF- β 1 were not different, a closer look at the data indicates a higher ALP activity with TGF- β 3 than with TGF- β 1 (144). The finding that TGF- β 1 seems to inhibit extracellular matrix calcification is supported by the earlier mentioned previous studies (136; 137; 138). Besides the conclusion that TGF- β subtype-dependent effects exist on mineralization, our results also indicate that expression of collagen X and VEGF as markers of terminal differentiation do not predict mineralization. This is in contrast with previous statements (136). For future purposes, it is interesting to analyze the expression of a broad spectrum of genes to study which genes are related to terminal differentiation and which are related specifically to mineralization. Moreover, when tissue-engineered cartilage is developed for cartilage reconstruction, the capacity to mineralize should be included in the study. The in-vitro pellet culture system is a suitable model to study terminal differentiation in BMSC (137). In-vitro testing of mineralization capacity can be performed on pellets by histological and biochemical analysis of calcium depositions and ALP activity, after addition of a phosphate donor to the medium.

In our study we demonstrated that effects of TGF- β subtypes on in-vitro chondrogenesis differ. Questions that arise due to our observations regarding the different mechanisms of TGF- β 1 and TGF- β 3 involved in terminal chondrogenic differentiation and matrix mineralization are still unanswered. A recent study suggested that the differences in TGF- β subtype actions might be caused by their distinct interactions with the TGF- β receptor type I (T β RI) and type II (T β RII) (131). They showed that although all three TGF- β subtypes form their receptor complexes (TGF- β subtype·T β RI·T β RII) equally well, the variations in the receptor type I and type II preferences among the TGF- β subtypes, likely modulate the kinetics of the complex assembly and can explain the functional variation between TGF- β

subtypes (131). In future, it might be interesting to study gene expression, as well as focus on the differences in receptor-binding as well as intra-cellular signalling in in-vitro cultured chondrocytes, to get a closer look at which factors are responsibly for controlling terminal differentiation and which factors are related to mineralization.

We believe this study contributes to the field of cartilage tissue engineering, because TGF- β subtypes differ in their effect on in-vitro chondrogenesis of BMSC cultures. Summarizing, tissue-engineered cartilage would be a promising solution for the limited availability of autologous cartilage for reconstructions of defects. Therefore, we need a large and stable cartilage construct without signs of mineralization. Our experiments show that although no significant differences were found between TGF- β 1, - β 2 and - β 3 cultured pellets, in the quantity of cartilage production or in the expression of hypertrophic markers, the presence of mineralization was significantly higher when TGF- β 3 was used, compared to TGF- β 1. TGF- β 1 is therefore preferred for use in cartilage tissue engineering. Since TGF- β 3, and to a lesser extent TGF- β 2 show signs of matrix-mineralization, those subtypes might be better to tissue engineer bone through endochondral ossification.



6

**CAN ONE GENERATE STABLE HYALINE CARTILAGE
FROM ADULT MESENCHYMAL STEM CELLS?
- a developmental approach**

J Tissue Eng Regen Med, accepted for publication

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ABSTRACT

Chondrogenically differentiating bone-marrow derived mesenchymal stem cells (BMSC) display signs of chondrocyte hypertrophy, such as production of collagen type X, MMP13, alkaline phosphatase (ALPL). For cartilage reconstructions this is undesirable as terminally differentiated cartilage produced by BMSC mineralizes when implanted in vivo. Terminal differentiation is not restricted to BMSC, but is also encountered in chondrogenic differentiation of adipose-derived MSC as well as embryonic stem cells which by definition should be able to generate all types of tissues including stable cartilage. Therefore we propose that the currently used culture conditions may drive the cells towards terminal differentiation. In this manuscript we aim to review the literature supplemented with our own data to answer the question: Is it possible to generate stable hyaline cartilage from adult mesenchymal stem cells? We demonstrate that recently published methods to inhibit terminal differentiation (through PTHrP, MMP13 or blocking phosphorylation of Smad1/5/8) result in cartilage formation with reduction of hypertrophic markers, though this does not reach the low level of stable chondrocytes. A set of hypertrophy markers should be included in future studies to characterize the phenotype more precisely. Finally, we used what is currently known in developmental biology about the differential development of hyaline and terminally differentiated cartilage to provide thought and insights to change current culture models to create hyaline cartilage. Inhibiting terminal differentiation may not result in stable hyaline cartilage, if the right balance of signals has not been created from the start of culture on.

INTRODUCTION

Cartilage tissue has a limited capacity for regeneration. As cartilage lesions can lead to progressive clinical problems, the potential of cell therapy to stimulate cartilage regeneration is currently being investigated (11; 147; 148). Bone-marrow derived mesenchymal stem cells (BMSC) are a promising candidate cell source for this purpose because of the ease with which they can be isolated and expanded and because of their capacities for chondrogenic differentiation (40; 125). However, chondrogenically differentiating BMSCs display signs of chondrocyte hypertrophy, such as production of collagen X, MMP13, alkaline phosphatase (ALPL) (33; 35; 36; 53; 135). This terminal differentiation of BMSC may be advantageous for tissue engineering of bone through the endochondral route (53; 149). However, for cartilage reconstructions it is undesirable as terminally differentiated cartilage produced by BMSC mineralizes and becomes bone when implanted in vivo (33; 150).

Terminal differentiation is not restricted to BMSC. Mesenchymal stem cells isolated from adipose tissue also demonstrated collagen X production and ALPL activity upon chondrogenic differentiation and mineralized after in-vivo implantation (151). This indicates that a predisposition to endochondral ossification of BMSC is not primarily due to their origin from the long bones. Moreover, embryonic stem cells (ESC) which by definition are able to generate all different types of tissue in the body including stable hyaline cartilage, produce hypertrophic cartilage and not stable cartilage after in-vitro chondrogenic differentiation (152). The chondrogenic differentiation protocol for ESCs are based on already established protocols for adult stem cells and chondrocytes (153). This suggests that the current culture conditions drive the cells towards a more terminal differentiation. Recently published methods to inhibit terminal differentiation (through PTHrP (101; 102; 154), MMP13 (155) or blocking phosphorylation of Smad1/5/8 (156) result in cartilage formation with reduction of hypertrophic markers. However, when compared to stable chondrocytes this is only a limited improvement. Therefore we aimed to review the literature supplemented with own data to answer the question: Is it possible to generate stable hyaline cartilage from adult mesenchymal stem cells such as BMSCs? We used what is currently know in developmental biology about the differential development of hyaline and terminally differentiated cartilage to provide thought and insights to change current culture models to create hyaline cartilage.

MATERIALS AND METHODS

Cell isolation and expansion

BMSC were isolated from a femoral shaft biopsy of 4 donors (age 30-44) undergoing total hip replacement, after informed consent had been obtained in accordance with the local ethical committee (MEC-2004-142). BMSC were expanded in DMEM-LG (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS), 1 ng/ml FGF2 (AbD Serotec, Kidlington, UK), 25 µg/ml ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA),

1.5 µg/ml fungizone (Gibco) and 50 µg/ml gentamicin (Gibco). BMSC from passage (P) 2-4 were used for the experiments.

Articular cartilage was obtained as leftover material from 2 donors (age 3-8) undergoing triple arthrodesis (MEC-2007-032). These children were treated for clubfoot deformity and therefore this cartilage was considered normal, as was confirmed by histology. Auricular cartilage was obtained from a patient undergoing rhinoplasty using ear cartilage (age 26) (MEC-2005-359) and a patient (age 12) undergoing protruding ear reconstruction (MEC-2006-186). After resection of the perichondrium cartilage slices were digested through incubation with 2 mg/ml Pronase E (Sigma-Aldrich) and 1.5 mg/ml Collagenase B (Boehringer Mannheim, Germany). Chondrocytes were expanded in DMEM containing Glutamax with 10% FCS and 1.5 µg/ml fungizone and 50 µg/ml gentamicin. Chondrocytes from P2 were used for the experiments.

Chondrogenic differentiation

Cells were differentiated in pellet cultures of 2×10^5 cells in DMEM containing Glutamax, ITS+1 (B&D Bioscience, Bedford, MA, USA), 40 µg/ml L-proline (Sigma-Aldrich), 1 mM sodium-pyruvate (Gibco), 5 µg/ml fungizone and 50 µg/ml gentamicin, 25 µg/ml ascorbic acid-2-phosphate, 10 ng/mL TGFβ2 (R&D systems, Minneapolis, MN, USA) and 10^{-7} M dexamethasone (Sigma-Aldrich). 10µM Dorsomorphin (Biomol international, Exeter, United Kingdom) or 10 ng/ml PTHrP (Peprotech, Rocky Hill, NJ, USA) was added to the medium of BMSC from three donors from day 14 on to inhibit terminal differentiation. To test the ability of the tissue-engineered constructs to mineralize, β-glycerophosphate was added additionally to the medium day 35-49 (156). Pellets were harvested in triplicate for (immune)histochemistry and gene-expression analysis.

(Immuno)histochemistry

Samples were fixed in 4% formalin and embedded in paraffin. Antigen retrieval for collagen II was performed through incubation with 0.1% pronase (Sigma-Aldrich) in PBS for 30 minutes, while antigen retrieval for collagen X required 0.1% pepsin (Sigma-Aldrich) in 0.5M acetic acid (pH=2.0) for 2 hours. Both stainings continued with incubation with 1% hyaluronidase (Sigma-Aldrich) in PBS. Sections were incubated overnight with primary antibodies for collagen II (II/II6B3, Developmental Studies Hybridoma Bank) or collagen X (2031501005, Quartett, Berlin, Germany) after blocking of non-specific binding sites with 10% goat serum in PBS. An alkaline-phosphatase-conjugated secondary antibody was used for these stainings, followed by incubation with Neu Fuchsin substrate (Chroma, Köngen, Germany) to demonstrate alkaline-phosphatase activity with a red stain.

To evaluate mineralization an Alizarin Red staining was performed. Slides were immersed in a saturated Alizarin Red S solution (Sigma-Aldrich) (5 gr/L) for one minute, and rinsed in 70% ethanol.

Gene-expression analysis

Pellets were manually homogenized in RNA-Bee™ (TEL-TEST, Friendswood, TX, USA)

and RNA was extracted with chloroform and purified from the supernatant using the RNeasy Micro Kit (Qiagen, Hilden, Germany).

RNA concentration and purity were assessed on a spectrophotometer (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Isogen Life Science B.V., Belgium). Complementary DNA (cDNA) was made using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) and polymerase chain reactions were performed using TaqMan® Universal PCR MasterMix (Applied Biosystems, Capelle a/d IJssel, Netherlands), as described earlier (53; 113), but on a ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA). Primers used for real-time RT-PCR reactions for *GAPDH*, *SOX9*, *CBFA1*, *collagen II*, *collagen X* and *ALPL* were reported previously by Farrell et al (53), for *SSP1*, *BGLAP* and *IBSP* by Jansen et al (157), for *VEGF* by Verseijden et al (158), and for *MMP13* by de Mos et al (159).

After testing *GAPDH*, *18S*, *HPRT*, *ACTB*, *UBC* and *B2M* as housekeeping genes on selected samples, *GAPDH* was chosen to normalize the data to. Relative expression was calculated according to the $2^{-\Delta CT}$ formula (160).

Statistical analysis

Statistical analysis was conducted using SPSS 15.0 and the GraphPad Prism 5 (San Diego, CA, USA) software for Windows. Values are represented as mean \pm SEM. Mixed model analysis and independent sample T-tests were performed and the level of significance was set at a *p* value of less than 0.05.

Literature review

A literature search was performed in order to do a narrative review in Pubmed from 1948 until march 2010 using the following key words: Mesenchymal Stem Cell, Embryonic Stem Cell, chondrogenesis, cartilage, hypertrophy, hypertrophic differentiation, terminal differentiation, collagen X, MMP13, mineralization, endochondral ossification, development, hyaline, growth plate or synovial joint. We excluded papers that were not written in English.

RESULTS AND DISCUSSION

Characterization of terminal differentiation

While BMSC display signs of terminal differentiation, i.e. production of collagen X (33; 35; 53) and mineralization *in vivo* (33), expanded articular chondrocytes produce stable hyaline cartilage that is negative for collagen X and does not mineralize (33; 34). This confirms a vast amount of research reports that lead to the general acceptance of collagen X as a key marker for hypertrophy. However, we have previously demonstrated that expanded auricular chondrocytes cultured under the same conditions produce a cartilage matrix rich in collagen X that does not mineralize when implanted *in vivo* for 6 weeks (161). This may suggest that terminal differentiation is not easily characterized by the expression of one marker, but a set of markers is required. We studied gene expression of a set of hypertrophic markers by BMSC, auricular chondrocytes and articular chondrocytes that were chondrogenically

differentiated for 35 days in pellet cultures. On histology all three cell types produced cartilaginous matrix and *collagen II* and *SOX9* were expressed in all three cell types indicating chondrogenic differentiation. *Collagen X* expression was significantly higher in BMSCs than in auricular chondrocytes, and was hardly expressed by articular chondrocytes (fig. 1). Expression of two other hypertrophy markers, *MMP13* and *SSP1* (osteopontin), were

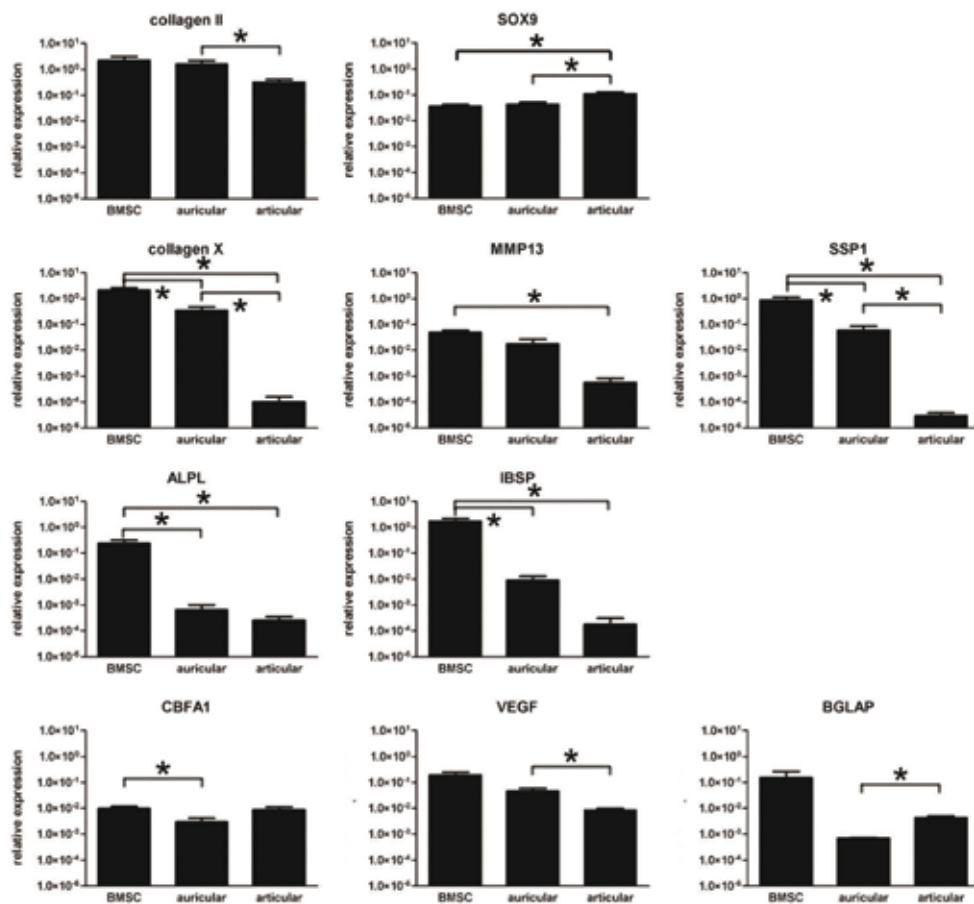


Figure 1. Gene expression of chondrogenic and hypertrophic markers in BMSC, auricular chondrocytes and articular chondrocytes. Cells were chondrogenically differentiated for 35 days. Data are shown of 12 samples from 3 BMSC donors and of 6 samples from 2 donors of articular or auricular chondrocytes. Collagen II and SOX9 were expressed in all three cell types indicating chondrogenic differentiation. Expression of Collagen X, MMP13 and SSP1 was highest in BMSC and lowest in articular chondrocytes with auricular chondrocytes in between. ALPL and IBSP were expressed high in BMSCs compared to both articular and auricular chondrocytes, suggesting a correlation between these genes and matrix mineralization. No clear distinct expression pattern was seen in the other markers associated with terminal differentiation: CBFA1, VEGF, and BGLAP.

also highest in BMSC and lowest in articular chondrocytes with auricular chondrocytes in between. *ALPL* and *IBSP* (bone sialoprotein II) were expressed high in BMSCs compared to both articular and auricular chondrocytes suggesting a correlation between these genes and matrix mineralization. Finally, no clear distinct expression pattern was seen in the other markers associated with terminal differentiation: *CBFA1*, *VEGF*, and *BGLAP* (osteocalcin). The high expression of these genes may indicate that even in articular chondrocytes terminal differentiation is initiated to some extent in vitro. In future studies these gene-expression data should be confirmed on a protein level, to determine whether these proteins are in fact translated. However, these gene-expression data do inform us on the phenotype of these cells in different stages of chondrogenesis and hypertrophy.

In summary, these data indicate that gene expression of *collagen X*, *MMP13* and *SSP1* are markers for increasing hypertrophic phenotype, while *ALPL* and *IBSP* gene expression correlate well to the ability to mineralize the matrix (fig. 2). Therefore, a set of hypertrophy markers should be used in future studies to characterize the progress of terminal differentiation more profoundly.

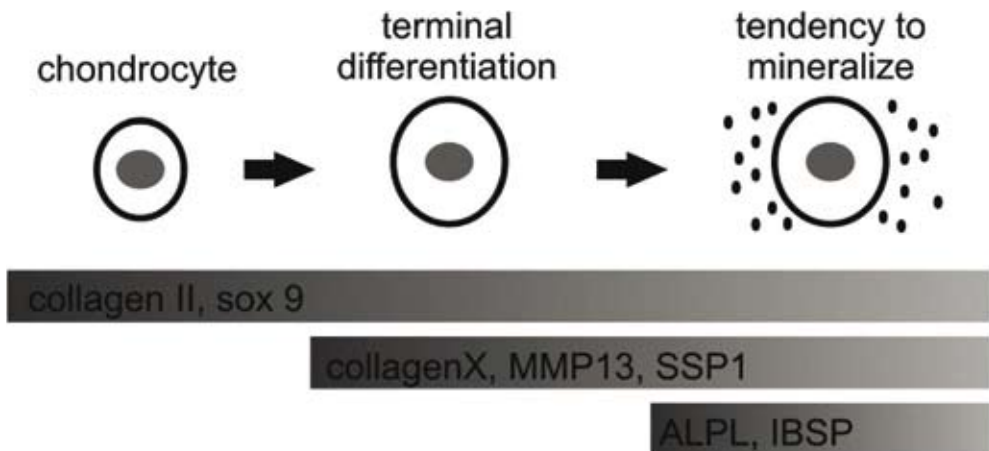


Figure 2. Schematic figure of the gradual progression of chondrogenic differentiation and terminal differentiation towards cartilage that can mineralize. Gene expression of *collagen X*, *MMP13* and *SSP1* are markers for increasing hypertrophic phenotype, while *ALPL* and *IBSP* gene expression correlate well to the ability to mineralize the matrix. *CBFA1*, *VEGF* and *BGLAP* are non-specific markers.

Molecular inhibition of terminal differentiation and mineralization in BMSCs

Recently, a small number of papers have been published on methods to inhibit terminal differentiation of BMSC.

PTHrP

In the growth plate, the negative feedback loop of parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (Ihh) determines the moment when the chondrocytes leave the proliferative zone and enter the transition to hypertrophic zone (162). PTHrP is produced by perichondral cells and chondrocytes at the end of the growing bone, diffuses away and binds to PTH/PTHrP receptors on chondrocytes in the growth plate delaying their conversion into prehypertrophic and then hypertrophic chondrocytes. Chondrocytes sufficiently far away from the source of PTHrP stop proliferating and only then synthesize Ihh. This Ihh then stimulates the synthesis of PTHrP by perichondral cells thereby creating a feedback loop. The function of PTHrP in delaying mineralization was demonstrated using PTHrP knock-out mice (163). The use of PTHrP addition to BMSCs in culture is therefore very logical. PTHrP added throughout the culture period of BMSCs resulted in a significant 5-fold reduction of *collagen X* expression and a two-fold reduction in ALPL activity (101). In another study no significant reduction of *collagen X* mRNA expression was seen when PTHrP was given merely from day 14 on, while a reduction in immunostaining for collagen X, collagen I and CBFA1 was observed (102; 154). The data of these studies were very promising but since a wide range of hypertrophy markers was not taken into account in these studies and the effect on mineralization was not evaluated, we repeated this experiment in our lab. Addition of PTHrP to chondrogenic differentiation medium from day 14 on confirmed an evident reduction of immunostaining for collagen X at day 35 (fig. 3). When β -glycerophosphate was added to the medium from day 35-49 Alizarin Red staining demonstrated a clearly diminished mineralization potential compared to the control without PTHrP. Although gene-expression of *collagen X*, *SSP1* and *IBSP* was significantly reduced in pellets treated with PTHrP (fig. 3), the absolute level of expression was still high compared to the cartilage constructs produced by auricular or articular chondrocytes (fig. 1). Moreover, no significant reduction was observed in gene expression of the other hypertrophic markers (fig. 3), including *MMP13* and *ALPL* that were related to terminal differentiation and mineralization in the previous experiment. Therefore, it seems unlikely that adding PTHrP during chondrogenic differentiation on its own is sufficient to produce a cartilage construct that is stable and does not mineralize in vitro. However, in combination with other factors it may direct BMSC towards a more stable hyaline phenotype. For further studies on PTHrP and inhibition of terminal differentiation of BMSC, it is advisable to study a large array of hypertrophic markers and to include in-vivo experiments to study stability and mineralization of the cartilage construct when used for cartilage reconstruction.

Blocking Smad1/5/8 phosphorylation

The Smad1/5/8 route is commonly known as the route activated by Bone Morphogenetic Proteins (BMPs), a member of the TGF-beta superfamily and a very potent inducer of bone

formation (106). Next to the canonical TGF- β signaling pathway via Smad2/3, it has recently been shown that an alternative type I receptor (ALK1 instead of ALK5) can be used by TGF- β that signals via Smad1/5/8 (104; 103; 105). Smad1/5/8 phosphorylation is known to stimulate terminal differentiation in murine embryonic endochondral ossification (109) and we have demonstrated previously that this route is activated in in-vitro tissue-engineered cartilage constructs derived from BMSCs (156). In contrast, this pathway is not activated in hyaline cartilage. The Smad1/5/8 pathway may be activated by either the exogenous TGF- β in the chondrogenic differentiation medium or BMP or TGF- β subtypes that are expressed endogenously during culturing of BMSCs.

Blocking Smad1/5/8 phosphorylation by adding dorsomorphin to the differentiation medium from day 14-35 resulted in reduced immunostaining for collagen X and a reduction in gene expression of *MMP13*, *SSP1*, *ALPL* and *IBSP*, markers that we have previously characterized to be important for terminal differentiation and mineralization (fig. 3), while sustaining further collagen II production. Therefore, dorsomorphin seems to inhibit terminal differentiation on a broader scale than PTHrP. Moreover, when β -glycerophosphate was added to the medium mineralization was inhibited. However, reduction of hypertrophic markers did not reach the low level of expression seen in hyaline cartilage produced by articular chondrocytes. It is likely that dorsomorphin inhibits terminal differentiation and mineralization temporarily to some extent, but the cartilage construct may become terminally differentiated and mineralized when implanted in an in-vivo environment where dorsomorphin is not added. Further studies on in-vivo stability of cartilage constructs cultured in the presence of dorsomorphin is therefore warranted.

Matrix Metalloprotease Inhibitors

Endochondral ossification is dependent upon proteolysis of the extracellular matrix and MMP13 activity is required for terminal differentiation of bovine fetal epiphyseal chondrocytes (164). A recent study demonstrated that addition of a pan-MMP13 inhibitor prevented chondrogenic differentiation of bone-marrow and adipose-derived MSC (155). A selective MMP13 inhibitor allowed chondrogenesis and reduced ALPL activity two-fold. However, it did not significantly reduce gene expression of *collagen X* and other markers of hypertrophy were not evaluated.

Other approaches to reduce terminal differentiation and mineralization of BMSC

Cells may be sensitive to subtle differences in surface chemistry. Therefore coating the surface of a scaffold material can have an effect on cell differentiation, as can the creation of functional groups (-CH₃, -NH₂, SH, -OH, and -COOH). Changing the chemical composition of the surface may be an alternative approach to prevent terminal differentiation and create hyaline cartilage.

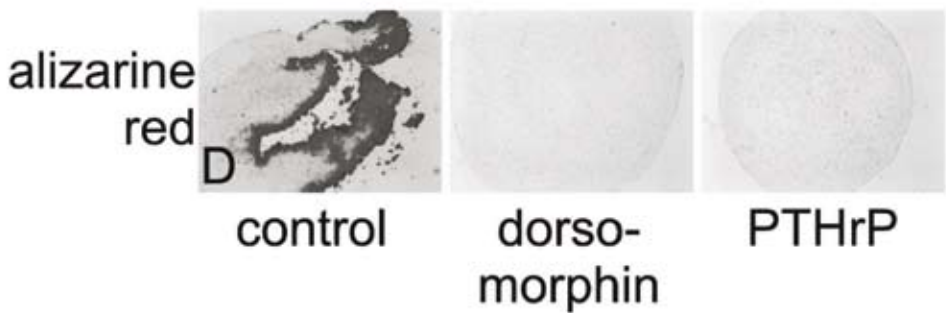
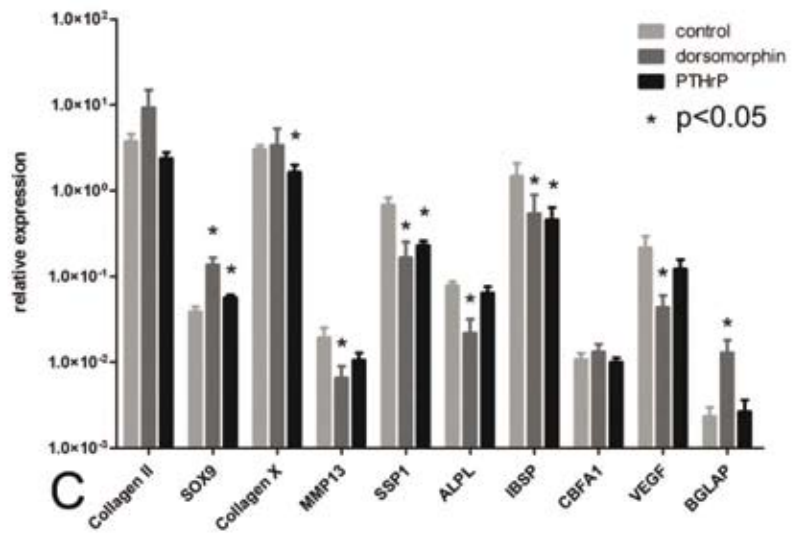
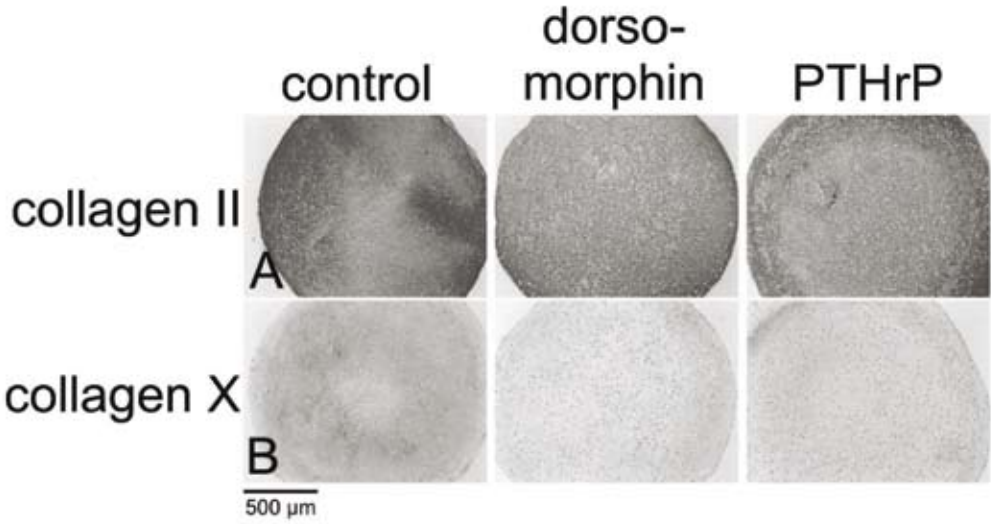
BMSCs cultured on nitrogen-rich plasma polymer coating demonstrated a decreased gene expression for *collagen X*, *ALPL*, *bone sialoprotein*, *osteocalcin*, and *CBFA1 (Runx2)* compared to control BMSCs cultured on commercial polystyrene (165). However, *collagen II* expression did not reach detectable levels and chondrogenic differentiation was thus not

achieved in this experiment with medium containing 10% fetal bovine serum without addition of growth factors. As a consequence it is hard to draw conclusions from this study regarding the potential to inhibit terminal differentiation and create hyaline cartilage.

Coating with cartilaginous extra cellular matrix components creates a microenvironment that better resembles natural hyaline cartilage and may favor hyaline differentiation. Microbeads coated with collagen II or chondroitin sulfate (CS) led to increased glycosaminoglycan and collagen II production compared to pellet culture (166). Collagen X expression was reduced on protein level as well as on mRNA level in microbeads coated with hyaluronic acid or chondroitin sulfate, compared to collagen II-coated microbeads or pellets (166). However, collagen X was still upregulated compared to undifferentiated BMSC and terminal differentiation was not completely prevented. Other hypertrophic markers such as CBFA1 and MMP13 were unfortunately not studied.

An alternative hypothesis why mineralization of chondrogenically differentiated BMSC occurs in vivo is that differentiation of the in-vitro tissue-engineered constructs is not yet fully induced and therefore the cells tend to lose their phenotype after implantation into ectopic sites, such as implantation subcutaneously. Indeed extended in-vitro culture of 12 weeks with TGF- β and IGF1 on PGA scaffolds resulted in stable cartilage that did not mineralize even after 24 weeks of in-vivo implantation, while constructs cultured in vitro for 4 or 8 weeks demonstrated mineralization and vascular invasion after in-vivo implantation (42). Unfortunately, investigation of chondrogenic differentiation was not extended beyond collagen II immunohistochemistry and Safranin O staining and hypertrophic markers were not studied. Hence, it is impossible to say whether extended differentiation results in the production of stable hyaline cartilage. One can only conclude from this study that extended culture using the model performed in this study seems to prevent mineralization upon in vivo implantation. Moreover, this study should be repeated and extended to other models beside PGA and the role of the addition of IGF1 in this study should be further evaluated.

Figure 3 (next page). (Immuno)histochemistry and gene-expression of BMSC pellets with or without addition of dorsomorphin or PTHrP. BMSC were chondrogenically differentiated with or without addition of dorsomorphin or PTHrP day 14-35. **A-B** Immunohistochemistry for collagen II and collagen X at day 35. While collagen X was inhibited in dorsomorphin or PTHrP treated pellets, collagen II production was allowed. **C** Gene expression of chondrogenic and hypertrophic markers in BMSC at day 35. Addition of both dorsomorphin and PTHrP allow expression of chondrogenic markers collagen II and SOX9. Dorsomorphin inhibits expression of hypertrophic markers MMP13, SSP1, ALPL, IBSP and VEGF, while PTHrP inhibits collagen X, SSP1 and IBSP. **D** To test ability to mineralize β -glycerophosphate was added to the chondrogenic medium with or without dorsomorphin or PTHrP day 35-49. Alizarine Red staining demonstrates inhibition of mineralization in dorsomorphin and PTHrP treated pellets. Samples were harvested in triplets from three independent donors. (Immuno)-histochemistry is shown of a representative pellet of one donor, while gene-expression is demonstrated of all three donors with triplicate samples per donor.



Effect of in-vitro culture conditions

The above mentioned approaches to inhibit terminal differentiation in BMSC are not successful in inhibiting the expression of genes such as *collagen X*, *ALPL* and *MMP13* to a level that resembles hyaline cartilage. Assuming that BMSC are theoretically able to generate hyaline cartilage, it is probable that the current in-vitro culture conditions are inducing terminal differentiation in a very early stage. In fact, we have previously demonstrated that, in contrast to embryonic endochondral ossification, the stage of early chondrogenic differentiation and the stage of hypertrophy are not clearly discernable in in-vitro chondrogenic pellet cultures of BMSC where almost no interval exists between production of collagen II and X. Moreover, *FGFR1* which is seen in embryonic endochondral ossification only during hypertrophy was already upregulated in BMSC producing collagen II before collagen X was observed (54).

Expansion

No definitive markers exist to select mesenchymal stem cells from a bone-marrow biopsy, therefore the cells are commonly selected by plastic adhesion. As a consequence a heterogeneous cell population is used for culturing. Osteogenic progenitor cells may well be present in the starting cell population. Medium used for expansion may also select these osteoprogenitor cells, or expansion may induce a more osteogenic phenotype. *FGF2* is often added to the medium during expansion to accelerate population doublings and retain chondrogenic capacity (44; 167). However, this growth factor may select cells that are more driven towards the endochondral route. Nevertheless, hypertrophic cartilage formation by pig BMSCs was also observed when *PDGF* and *EGF* were added to the medium instead of *FGF2* (168). Another factor that may play a role in selecting certain subpopulation is the use of fetal calf or bovine serum for colony formation and proliferation of BMSCs. Serum is a complex natural product that may vary from batch to batch even from a single manufacturer (169). The quality and concentration of both bulk and specific proteins can affect cell behavior and differentiation. A variation in chondrogenic potential due to the used serum is thus expected and indeed observed. However, it is likely that the different laboratories working on chondrogenic differentiation of BMSC use different serum batches and the induction of terminal differentiation of BMSC seems to be universally.

Differentiation model

To induce chondrogenic differentiation of BMSC a 3D culture system is typically used and the cells are cultured in specific differentiation medium. When studying gene expression of hypertrophic markers in time during chondrogenic differentiation in pellet culture it is clear that directly after expansion, before differentiation, *Collagen X*, *MMP13* and *IBSP* are hardly expressed (fig. 4). *ALPL* gene expression on the other hand is high before chondrogenic differentiation, not unsurprisingly as *ALPL* is also a stem cell marker. *ALPL* is quickly downregulated during the first week of differentiation culture. Gene expression of *ALPL*, *collagen X*, *MMP13* and *IBSP* are strongly upregulated during the second week of differen-

tiation, while *CBFA1* and *SSP1* expression increases more or less continuously during culture. This suggests that the current differentiation protocol and not the expansion induces the hypertrophic phenotype of BMSCs. Moreover, expression of these hypertrophic markers very quickly follows, almost overlaps, with the onset of chondrogenesis (expression of *collagen II*). This may even suggest a hypertrophic phenotype is prespecified when chondrogenic differentiation is still in its early stages. This may be an explanation why inhibiting terminal differentiation after the onset of chondrogenesis (i.e. with PTHrP or dorsomorphin) does not result in a truly stable hyaline phenotype although hypertrophic markers are reduced to some extent.

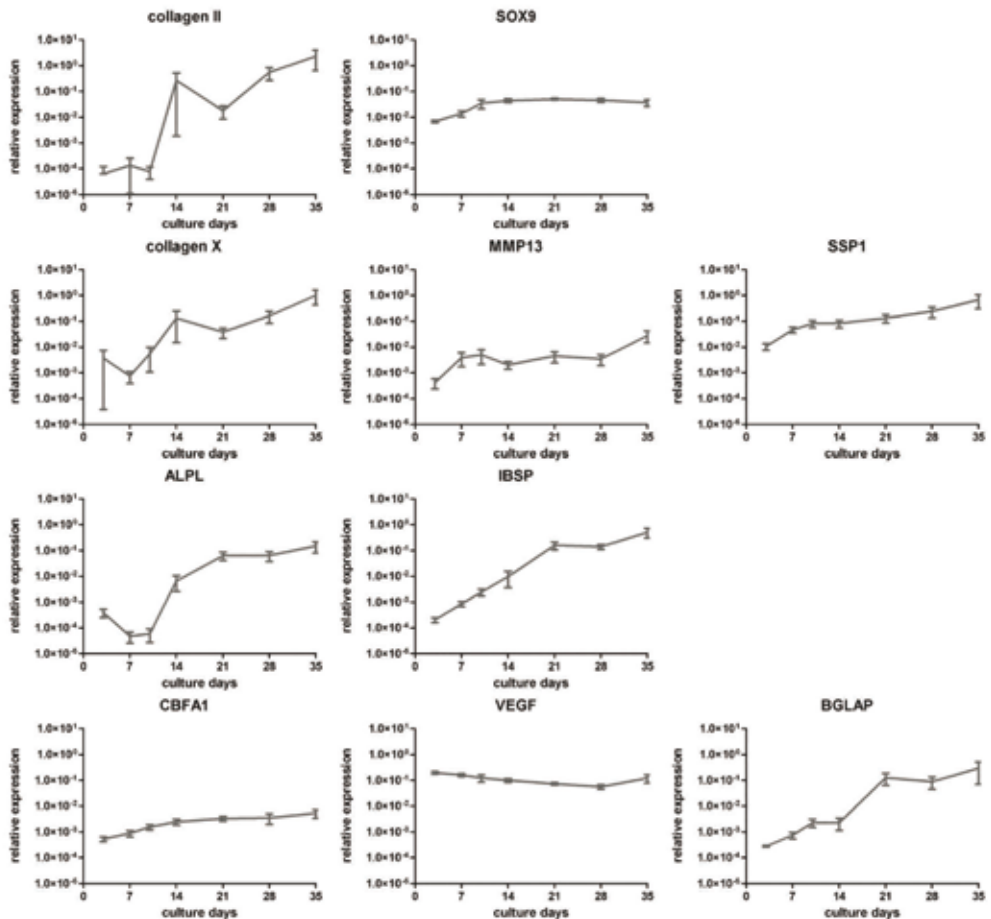


Figure 4. Gene expression of chondrogenic and hypertrophic markers during chondrogenic differentiation of BMSC. BMSC were chondrogenically differentiated for 35 days in pellet cultures. Data are shown of 6 samples from 2 donors. Expression of hypertrophic markers very quickly follows the onset of expression of collagen II.

Pellet culture is a high cell-density model that is often used to study chondrogenic potential of BMSCs (33; 35; 44; 53; 101; 102; 135; 154). Therefore, the terminal differentiation may be associated with the high cell density or the direct cell-cell contact in this culture model. However, hypertrophic differentiation was also seen in polyglycolic acid (PGA) scaffolds (42; 101), fibrin-supported spheroids (168), and alginate beads (36). Direct comparison of pellet and alginate culture indicated expression of *collagen II* as well as *collagen X* at day 14, both somewhat higher in alginate culture (170). Validating these data, we have observed terminal differentiation of BMSC in both pellet and alginate models after 14 and 35 days when using cells from the same donor (unpublished data).

Differentiation medium

From the above it does not seem likely that the choice of 3D model is associated with terminal differentiation. Therefore it may be the choice of differentiation medium that induces hypertrophy. This concept is supported by a study that demonstrated that while in-vitro cultured pig BMSC produced hypertrophic cartilage, in-vivo implantation of BMSC in a full-thickness cartilage defect resulted in a spatially organized repair tissue (168). Hypertrophic cartilage was found in the deep zone in transition to subchondral bone and hyaline cartilage, rich in collagen II and proteoglycan but negative for collagen X, was found in the central region (168). However, in four out of six samples in which BMSC were not transplanted some chondrogenic repair tissue was also observed, mostly limited to the deep zone and thus possibly due to migration of local cells into the osteochondral defect. Moreover, it was not demonstrated whether or not the cartilage matrix lacking collagen X was produced by the implanted BMSC or by other cells that migrate into the defect, for example from the cartilage. However, these data are promising and suggest that the in-vitro microenvironment favors terminal differentiation and that BMSC might be capable of forming of stable cartilage.

Chondrogenic differentiation medium used in the above-mentioned publications evaluating terminal differentiation of human BMSCs contained TGF- β , ITS and dexamethasone (33; 36; 53; 101; 102; 135; 155; 164; 168), occasionally supplemented with IGF (42) or triiodothyronine (35). For the chondrogenic differentiation of AT-MSC BMP6 (151; 155) or BMP7 (102) was additionally used. As these in-vitro chondrogenic differentiation media induce terminal differentiation of BMSCs, one of these factors or a combination thereof may be the trigger. Still, chondrocytes cultured in the same culture medium do not show signs of terminal differentiation as we have demonstrated earlier in this paper. Progenitor cells derived from articular cartilage also form stable hyaline cartilage in a similar culture medium (171), indicating these progenitor cells have more tissue-specific (chondrogenic) properties and are less multi-potent than BMSC. Therefore, directly comparing these different cell types during culture, and studying for example their endogenous expression, may shed light into defining a better culture medium to create stable hyaline cartilage.

Also noteworthy is that the chondrogenic effect of these factors has been studied empirically by adding it throughout the culture period. In a recent paper by Lenas et al, the model of “developmental engineering” was put forward (172). Instead of investigating

empirically by trying various combinations of tools leading to a perpetual cycle of trial-and-error attempts, a rational methodology should be followed based on a fundamental understanding of tissue development. To learn more about the fundamentals of normal differential development of hyaline and hypertrophic cartilage we should therefore review literature dealing with embryonic chondrogenesis.

Induction of terminal differentiation versus hyaline chondrogenic differentiation: What can we learn from embryonic development?

There is a traditional view that the development of articular hyaline cartilage is nothing more than the remnants of the embryonic epiphysis failing to go through endochondral ossification. Based on this view, the cartilage tissue-engineering field has used endochondral ossification during embryonic limb formation as a model for chondrogenesis and methods to inhibit terminal differentiation are often derived from research on the growth plate (5; 39; 173). However, chondrocytes in the growth plate are destined to become terminally differentiated and it therefore may not be the model of choice to study hyaline cartilage formation. Indeed, above-mentioned results in BMSC confirm that these cells are induced towards a terminally differentiated phenotype early on during their *in-vitro* differentiation and that inhibiting terminal differentiation is not sufficient to create true hyaline cartilage.

In fact, in developmental biology the idea that articular cartilage is a remnant of endochondral ossification is increasingly being challenged (174). The hypothesis that articular cartilage is derived from a distinct population of cells becomes more clear (174). This concept is also important for cartilage tissue engineering as it may mean inhibiting terminal differentiation may not result in stable hyaline cartilage. At a certain stage during limb development, a remodeling event takes place at the future joint location (called cavitation); the cells lose their round morphology and become elongated, accompanied by a loss of collagen II and an increase in collagen I expression. This zone is called the interzone and surgical removal of this zone results in fusion of the skeletal elements. The interzone consists of three cell layers; a dense intermediate cell layer and two outer cell layers each facing the epiphyseal end of the joint. The cells in the outer layers become chondrogenically differentiated early in embryogenesis and become incorporated in the epiphyses, while cells in the intermediate zone are responsible for the formation of hyaline articular cartilage (175).

The cells forming the interzone are not simply those cells that per chance occupied the future joint site. Instead peri-joint cells immediately adjacent to the future joint migrate into the site and participate in interzone formation (176). Whether or not the interzone is formed from a distinct subpopulations of cells or not is still under controversy, however it is clear that the development of the interzone and thus the articular cartilage is regulated apart from the transient cartilage template beginning during the prechondrogenic condensation. The formation of the interzone seems to be dependent upon blocking chondrogenic differentiation. One way through which this seems to be regulated is through BMP antagonism and thus blocking the function of chondrogenesis-promoting signals (174; 175). In noggin null mutant mice the mesenchymal condensations become much larger but joint

development is not initiated. Furthermore, at least two human syndromes characterized by multiple synostoses are due to mutations in noggin (174).

A central role in the formation of the interzone is also played by Wnt signaling. Ectopic expression of Wnt is sufficient to induce the formation of a three-layered interzone (177; 178). Again, this effect seems to be based on blocking chondrogenic differentiation as Wnt14 overexpression in micromass culture resulted in severe inhibition of chondrogenesis (177). Besides Wnt14, the interzone also expresses Wnt4, Wnt16 and the canonical Wnt signaling mediator β -catenin (178). In the same way as Wnt14, ectopic expression of an activated form of beta-catenin induced ectopic joint formation while removal of β -catenin promoted chondrocyte differentiation and blocked the activity of Wnt14 in joint formation (178).

A marker often associated with the interzone is growth and differentiation factor 5 (Gdf5), a member of the TGF- β superfamily, as it probably was the first recognized gene marker in the developing joint. In contrast to the above-mentioned proteins it does not induce joint formation, but stimulates chondrogenesis (174; 175). In culture of MSC from adipose tissue, GDF5 was described to increase chondrogenesis as well as collagen X expression (179).

An important concept in “developmental engineering” is that the multistage character of developmental processes allows high observability and controllability (172). In-vitro processes composed of distinct sequential steps that are characterized by markers expressed in a specific stage allow the possibility to intervene with additional factors to direct the cells at the appropriate stages. These data from embryonic development of the interzone suggest that to develop stable cartilage a subtle balance of chondrogenic stimulating as well as inhibiting signals is required, starting from the initial prechondrogenic differentiation, for instance FGF, TGF and Wnt. The timing and interactions of these signals may be the answer to develop stable hyaline cartilage.

Conclusion

We have demonstrated that terminal differentiation is a gradual progression of chondrogenic differentiation towards cartilage that can mineralize, and is thus best characterized with a set of markers indicative of this gradual progression. Although recently published methods to inhibit of terminal differentiation (through PTHrP, MMP13 or blocking phosphorylation of Smad1/5/8) resulted in cartilage formation with reduction of hypertrophic markers, this does not reach the low level of stable chondrocytes. As expression of hypertrophic markers almost overlaps with the onset of chondrogenesis, the hypertrophic phenotype may be prespecified when chondrogenic differentiation is still in its early stages in current culture conditions. These current culture conditions, and the rationale of inhibiting terminal differentiation, are derived from a traditional view that development of hyaline cartilage is nothing more than chondrocytes failing to go through endochondral ossification. However, chondrocytes in the growth plate are destined to become terminally differentiated and it therefore may not be the model of choice to study hyaline cartilage formation. Embryonic development of the interzone suggest that to develop stable cartilage a subtle balance of

Can one generate stable hyaline cartilage from BMSC?

chondrogenically stimulating as well as inhibitory signalling is required, starting from the initial prechondrogenic differentiation. For cartilage tissue engineering this may mean that inhibiting terminal differentiation may not result in stable hyaline cartilage, if the right balance of signals has not been created from the start of culture on.

ACKNOWLEDGEMENTS

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CHONDROGENIC PRIMING OF HUMAN BONE MARROW STROMAL CELLS: a better route to bone repair?

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ABSTRACT

The use of bioengineered cell constructs for the treatment of bone defects has received a lot of attention of late. Often bone marrow stromal cells (BMSC) are used that are in-vitro stimulated towards the osteogenic lineage aiming at intramembranous bone formation. To date, the success of this approach is disappointing. A major concern in these constructs is core degradation and necrosis caused by a lack of vascularization. We hypothesize that stimulation of cells towards the endochondral ossification process would be more successful. In this study we test how in-vitro priming of human BMSC along osteogenic and chondrogenic lineages influences survival and osteogenesis in vivo. Scaffolds that were pre-cultured on chondrogenic culture medium showed collagen II and collagen X production. Moreover, vessel ingrowth was observed. Priming along the osteogenic lineage led to a mineralized matrix of poor quality with few surviving cells and no vascularization.

We further characterised this process in vitro using pellet cultures. In vitro, pellets cultured in chondrogenic medium showed progressive production of collagen II and collagen X. In the culture medium of these chondrogenic cultured pellets VEGF release was observed at days 14, 21 and 35. When pellets were switched to culture medium containing β -glycerophosphate, independent of the presence or absence of TGF- β , mineralization was observed with a concomitant reduction in VEGF and MMP release. By showing that VEGF and MMPs are produced in chondrogenically differentiated BMSC in vitro we demonstrated that these cells produce factors that are known to be important for the induction of vascularization of the matrix. Inducing mineralization in this endochondral process does however severely diminish these capacities. Taken together, these data suggest that optimizing chondrogenic priming of BMSC may further improve vessel invasion in bioengineered constructs, thus leading to an alternative and superior approach to bone repair.

INTRODUCTION

Treatment options for non-unions and large bone defects are plentiful. However, despite the use of external and internal fixation, autologous bone graft transplantation or biophysical stimuli, success rates vary and outcomes are unpredictable (180; 181; 182). Treatment of large bone defects is further complicated by the relatively small amount of available transplantable bone tissue further illustrating the need for alternative treatment options.

One of the strategies focuses on the use of human bone marrow stromal cells (BMSC), which can be harvested with relative ease and can be differentiated towards the chondrogenic and osteogenic lineages *in vitro* (183). Additionally, they can be transplanted autologously, which makes them suitable to use in transplantable bio-engineered cell constructs. However, to date, a vascularized, mechanically competent, osteoconductive construct that could be used to produce bone *in vitro* or cause complete osteogenesis *in vivo* remains to be developed. The issue of core degradation, arising from lack of nutrient delivery and waste removal from the centre of tissue engineered constructs is of major concern in the field of tissue engineering (184). This is caused by insufficient blood supply to the implanted tissue. Firstly, it takes time for blood vessels to invade a tissue and oxygenate it and provide it with nutrients. This is not an instantaneous process and usually occurs too slowly for cells to survive without nutrients. The second problem is that culture of a scaffold *in vitro* for several weeks can lead to formation of an extensive extracellular matrix which can prevent or seriously hamper blood vessel infiltration *in vivo* by sealing the pores of a scaffold.

The approach used for tissue engineering of bone using BMSC is usually similar in different laboratories and involves the induction of direct osteogenic differentiation in a manner akin to intramembranous ossification *in vivo*. As discussed the problem with this approach is the long term survival of these constructs, which is limited due to lack of vascularization. In this study we hypothesized that it might be preferable to induce bone formation *in vivo* via an endochondral ossification approach where hypertrophic chondrocytes, primed *in vitro* and derived from BMSC would be implanted. This priming of chondrogenic three-dimensional cellular constructs can be performed to different stages of the differentiation and maturation process, namely until a cartilaginous extracellular matrix has been formed or when the extracellular matrix is mineralized (185). Often this is seen as an obstacle to cartilage tissue engineering and is seen as something to be avoided (33). However, more recently the benefit of potentially using this phenomenon for bone tissue engineering has been realized (152). Because chondrocytes can survive in hypoxic environments (186) and because they produce anabolic and catabolic factors important for the conversion of an avascular tissue to a vascularized tissue, such constructs might stimulate vessel ingrowth and overcome the problem of core degradation. This approach has the benefits of both *in vitro* and *in vivo* vascularization strategies (187). *In-vivo* prevascularization of a construct in an ectopic site prior to its use has the benefit of creating a material that can be surgically connected to the host vasculature but requires a second surgery to relocate the construct to the implant site (188). *In-vitro* prevascularization avoids

this but still requires some time for anastomosis (189; 190) with the host during which time there will be a hypoxic period. The approach presented here combines the ease of in-vitro priming with the benefit of the ability to withstand the initial hypoxic conditions whilst performing in-vivo vascularization in the target site. Any undifferentiated BMSC at this stage would also stand a better chance of survival than differentiated cells as has also recently been shown (191).

In in-vivo endochondral ossification vascular endothelial growth factor (VEGF) is known to play a critical role in the establishment of vessel ingrowth by effecting angiogenesis, remodeling of extracellular matrix and ossification (146). Additionally, it has been shown that matrix metalloproteinases (MMP) are expressed by chondrocytes and hypertrophic chondrocytes. These molecules play an important role in the conversion of the non-vascularized cartilaginous matrix to a vascularized mineralized tissue in both skeletal development and fracture healing (192; 193; 194; 195). Based on these facts and the lack of success of tissue engineering bone to date, it is more logical to use chondrocytes, which are less oxygen sensitive, to produce bone in vivo via the endochondral ossification route which will then naturally vascularize and mineralize in situ.

In vitro, pellet cultures of chondrogenic differentiated BMSC show a similarity to endochondral ossification; they progressively produce collagen II and collagen X, a protein specifically produced by hypertrophic chondrocytes (196; 197). When a phosphate donor is added to the culture medium mineralization can occur (198). Therefore this culture system offers a simple model for the development of in-vitro tissue engineering approaches for the creation of vascularized bone in vivo. This study aimed to assess the possible advantages of using an endochondral ossification model of bone formation for use in tissue engineering approaches. To that end, BMSC were seeded into highly biocompatible collagen glycosaminoglycan (Collagen GAG) scaffolds (199; 200; 201) and primed in vitro to progress along the osteogenic or chondrogenic routes prior to subcutaneous implantation into nude mice. In vitro, a simple model of endochondral ossification using pellet cultures enabled a further understanding of the process.

MATERIALS AND METHODS

Isolation and expansion of BMSC

BMSC of three donors, all female, 33-60 years of age, were obtained during total hip arthroplasty after informed consent with approval of the local medical ethical committee (METC 2004-142). Bone-marrow aspirates were taken from the greater trochanter. Heparinized aspirates were seeded at a density of 30-90 x 10⁶ nucleated cells per T175 flask. After 24 hours non-adherent cells and cell debris were washed out. BMSC were further expanded in Dulbecco's modified Eagles medium (DMEM), low-glucose with 10% fetal calf serum from a pre-selected batch to maintain the multi potential capacities of the cells, 50µg/ml gentamycin and 1.5µg/ml fungizone (all Invitrogen, Carlsbad, CA, USA), 0.1mM L-ascorbic acid 2-phosphate and 1 ng/ml Fibroblast Growth Factor (Instruchemie

B.V., Delfzijl, the Netherlands). Cells were cultured at 37°C under humidified conditions and 5% carbon dioxide. Medium was changed twice a week. When cultures reached near confluence, they were trypsinized using 0.05 % trypsin and were replated at a density of 2000 cells/cm². Cells from the second to the fourth passage were used for the pellet cultures. BMSC purchased from Cambrex (Now Lonza, Verviers, Belgium) were used in the scaffold study.

BMSC seeding onto collagen-GAG scaffold and subcutaneous implantation

Cells were rinsed with phosphate-buffered saline (PBS, 100mM NaCl, 80mM Na₂HPO₄, 20mM Na₂H₂PO₄) and detached with trypsin-EDTA (Invitrogen). The resulting suspension was centrifuged (650g, 5min at 20°C), re-suspended in 2ml of control medium, and aspirated through a 20-gauge needle to obtain a single-cell suspension of 1.5 x 10⁶ cell/ml. Collagen-GAG scaffolds (8mm² generously donated by MIT, Cambridge, MA), prepared by a freeze-drying method previously described (200), were seeded with 300,000 cells per scaffold as previously described with the exception that a smaller volume of 100µl of cell suspension per side was used (199). Seeding efficiency was estimated to be 50% according to recent experimental data using the same approach (202). These scaffolds are prepared from bovine collagen I and chondroitin sulphate in a freeze-drying process with dehydrothermal crosslinking. After seeding, 3 ml of supplemented DMEM was added to each well. After 2 days medium was replaced with chondrogenic (25µg/ml L-ascorbic acid 2-phosphate, 100 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 1:100 ITS (BD Biosciences, Bedford, MA), 10ng/ml transforming growth factor beta-2 (TGF-β), (R&D Systems, UK,) and 100 nM dexamethasone (Sigma, St Louis, USA)) or osteogenic medium (25µg/ml L-ascorbic acid 2-phosphate, 10mM β-glycerophosphate (Sigma), 10⁻⁸M dexamethasone). Half of the medium was replaced every 3 days. Scaffolds were treated for 21 days and were maintained in a humidified atmosphere of 95% air/ 5% CO₂ at 37°C. At day 21, 5 scaffolds of each condition were harvested for histology and 5 scaffolds were implanted subcutaneously into the backs of nude mice. Two incisions were made along the central line of the spine, one at the shoulders and one at the hips. Two scaffolds were implanted at each point, one to the left and one to the right at random. Animals were allowed to recover and sacrificed 4 weeks later. This procedure was carried out with approval from the local animal ethical committee (EUR334).

Chondrogenic differentiation in pellet cultures

After detachment of the cells with 0.05% trypsin, 0.5 ml medium, containing 200,000 cells were put in polypropylene tubes. Tubes were then centrifuged for 8 minutes at 120g. To induce chondrogenic differentiation pellets were cultured for 21 days in DMEM High glucose medium containing 50µg/ml gentamycin and 1.5µg/ml fungizone (Invitrogen, Carlsbad, CA, USA), L-ascorbic acid 2-phosphate, 100mM sodium pyruvate (Sigma, St Louis, USA), 1:100 ITS (BD Biosciences, Bedford, MA), 10ng/ml transforming growth factor beta-2 (TGF-β, R&D Systems, UK), and 100nM dexamethasone (Sigma, St Louis, USA).

After 21 days of culture, three groups were made that were cultured for another two weeks in different media:

- 1 Chondrogenic differentiation medium: this group was kept on the same medium
- 2 Phosphate-containing chondrogenic medium: in this group 10 mM β -glycerophosphate (BGP) was added to the chondrogenic medium
- 3 Osteogenic medium: this group was switched to medium with 10mM BGP and 10^{-8} M dexamethasone, without TGF- β

In order to induce mineralization of the extracellular matrix, pellets were switched to a phosphate-containing culture medium as in medium 2 above. As it has been shown that the presence of TGF- β in the culture medium might negatively influence hypertrophy (136), the induction of mineralization was also evaluated in pellet cultures switched to a culture medium more resembling an osteogenic differentiation medium (medium 3).

A pure osteogenic condition was not included in the pellet culture experiment based on the lack of sufficient bone formation in the in-vivo condition which we see as a non-viable option for bone repair in these settings.

Table 1. Forward and reverse primers for genes of interest.

Gene symbol	Accession No.		Primer (5'-3')
ALPL	NM_001127501	Fw	GACCCCTTGACCCCCACAAT
	NM_000478	FAM	TGGACTACCTATTGGGTCTCTTCGAGCCA
		Rv	GCTCGTACTGCATGTCCCCT
COL2A	NM_003150	Fw	GGCAATAGCAGGTTACGTACA
	NM_001844	FAM	CCGGTATGTTTCGTGCAGCCATCCT
		Rv	CGATAACAGTCTTGCCCCACTT
COL10A1	NM_000493	Fw	CAAGGCACCATCTCCAGGAA
		FAM	TCCAGCACGCAGAATCCATCTGA
		Rv	AAAGGGTATTTGTGGCAGCATATT
RUNX2	NM_001024630	Fw	GCCTTCAAGGTGGTAGCC
	NM_001015051	FAM	CCACAGTCCCATCTGGTACCTCTCCG
	NM_004348	Rv	CGTTACCCGCCATGACAGTA
SOX9	NM_000346	Fw	CAACGCCGAGCTCAGCA
		FAM	TGGGCAAGCTCTGGAGACTTCTGAACG
		Rv	TCCACGAAGGGCCCGC
GAPDH	NM_002046	Fw	ATGGGGAAAGGTGAAGGTCCG
		FAM	CGCCCAATACGACCAAATCCGTTGAC
		Rv	TAAAAGCAGCCCTGGTGACC

FW, forward; RV, reverse; FAM, FAM-labelled Taqman Probe.

Using averages of triplicate samples for each donor. Due to insufficient RNA extracted from Donor C, PCR analysis could only be performed for RNA from pellets of donors A and B.

Gene expression analysis

At harvesting pellet cultures were suspended in 300 μ l RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). To allow optimal suspension of all cells, pellets were disintegrated using a pestle and were subsequently flushed through a 28 gauge needle (Monoject, Sherwood Davis & Geck, St.Louis, MO, USA). For RNA precipitation a commercially available kit was used (Qiagen, Hilden, Germany). Reverse transcription in cDNA was performed on 200ng of total RNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Primers were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet TaqMan® or SYBR®Green requirements and were designed to bind to separate exons to avoid co-amplification of genomic DNA. BLASTN ensured gene specificity of all primers. The following genes were analyzed: Sox-9 (SOX9), Collagen II (COL 2A1), Collagen X (COL10A1), RUNT-related transcription factor 2 (RUNX2/cbfa-1) and Alkaline Phosphatase (ALPL).

Amplifications were performed as 25 μ l reactions using either TaqMan® Universal PCR MasterMix (ABI, Branchburg, New Jersey, USA) or qPCR™ Mastermix Plus for SYBR®Green I (Eurogentec, Nederland B.V., Maastricht, The Netherlands) according to the manufacturer's guidelines. Real-Time RT-PCR (QPCR) was done using an ABI PRISM® 7000 with SDS software version 1.7. Data were normalized to GAPDH that was stably expressed across sample conditions (not shown). Relative expression was calculated according to the $2^{-\Delta\text{CT}}$ formula

Immunohistochemical and histochemical staining

For immunohistochemistry pellets and scaffolds were fixed overnight in 4% phosphate-buffered formalin and embedded in paraffin. 6 μ m sections were made, deparaffinized in xylene and rehydrated through graded ethanol. Three pellets of each condition were analyzed per donor. Five chondrogenically primed scaffolds and four osteogenically primed scaffolds were analyzed, sectioned at 10 μ m. One osteogenic scaffold could not be located and retrieved. As a control, scaffolds were also cultured in unconditioned medium and analyzed in the same manner (data not shown).

Immunohistochemistry for collagen II and collagen X

To analyze collagen II expression, sections were incubated with 0.1% pronase (Sigma, St Louis, MO) for antigen retrieval and 1% hyaluronidase (Sigma, St Louis, MO). Sections were incubated for 2h at room temperature with mouse monoclonal antibody against collagen II (II-II6B3 antibody, 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD). For collagen X, sections were incubated with 1% pepsin in 0.5 M Acetic Acid at pH 2.0 for 2 hours at 37°C (Sigma, St Louis, MO) and 30 minutes in 1% hyaluronidase (Sigma). The sections were incubated overnight at 7°C with mouse monoclonal antibody against collagen X (1:30, X53, Quartett, Berlin, Germany).

For both staining procedures a biotin labeled secondary antibody was used (Biogenex HK-325, 1:100) followed by alkaline phosphatase conjugated streptavidin (Biogenex HK-321-UK, 1:100). This alkaline phosphatase activity was demonstrated by incubation with a new fuchsine substrate (Chroma, Kongen, Germany). Control for all antibodies was

performed by using an isotype IgG1 monoclonal antibody. Counterstaining was performed with Gill's haematoxylin (Sigma, St Louis, MO).

Quantification was determined by multiplying the intensity of the staining (ranging from 1 to 3, where 1 is light positive staining and 3 intense) by the fraction of stained area over the pellet area, resulting in a maximum score of 300 (3 x 100%). Scores of three sections were averaged. These scorings were performed blind by 2 independent observers.

With regard to our choice of markers, Collagen II is the definitive chondrogenic marker and collagen X is also the most widely accepted marker of chondrocyte hypertrophy/terminal differentiation. For gene expression analyses discussed below we also examined classical markers of chondrogenesis and hypertrophy/osteogenesis, namely the chondrogenesis transcription factor, Sox 9, the osteogenic marker alkaline phosphatase and the early osteogenic/hypertrophic transcription factor *cbfa-1/runx2* (203).

Von Kossa/Thionine staining

For evaluation of mineralization, slides were immersed in 5% silver nitrate solution (Sigma, St Louis, MO) for 10 minutes, rinsed in ultra pure water and exposed to light for 10 minutes. Excess silver nitrate was removed with 5% sodium-thiosulphate (Sigma, St Louis, MO) and cells were rinsed in distilled water. For evaluation of proteoglycan content paraffin sections were counterstained with 0.4% thionine in 0.01 M aqueous sodium acetate, pH 4.5 for 5 min. Thionine stains GAG as can be seen in the chondrogenic conditions (204).

Positive calcium phosphate surface over the pellet surface resulted in the fraction of mineralized area. Averaged results of three sections per pellet were used for further analysis.

VEGF expression and MMP release

Culture medium of day 14, 21 and 35 were collected from each condition. Immediately after collection the medium was centrifuged at 1000g for 10 minutes at 4°C, and stored at -80°C until further use.

VEGF

Expression of VEGF was analyzed on 24-hour conditioned culture medium of day 14, 21, 35. For each condition, medium of three pellets was analyzed in triplicate using a commercially available sandwich ELISA kit (R&D systems, Abingdon, UK) according to the manufacturer's protocol.

MMP release

Release of MMPs was determined by concentrating 100µl of the conditioned culture medium by ultra-filtration (YM-30, Millipore, Billerica, MA, USA), leaving a sample volume of approximately 15µl. Sample loading buffer (Biorad zymogram buffer, BioRad, Hercules, CA, USA) was added to the samples (2:1), and were incubated for 10 minutes at 37°C. Electrophoresis in a gelatin gel (BioRad, Hercules, CA, USA) was performed, at 100V and 25mA, together with a marker (BioRad, Hercules, CA, USA). Thereafter gels were incubated for 2 hours in a renaturing buffer, containing 2.5% Triton X-100 (Sigma, St Louis, MO) in 25 mM Tris-HCl (pH 7.4) followed by incubation for 72 hours in an activation buffer (2mM calcium chloride, 5µm zinc chloride, 25 mM Tris-HCl, pH 7.5, Fluka). Gels were stained

using coomassie brilliant blue and were destained with roti destain (both Carl Roth, Karlsruhe, Germany). Areas of proteolytic activity appeared as translucent halos against a blue background of stained, non-degraded gelatin. Gels were scanned using a Kodak IS scanner.

Statistics

Analyzes of immunohistochemistry and VEGF-expression were performed on three pellets of each of the three donors for all conditions. An ANOVA test and post-hoc Bonferroni test of the means between culture days and between different culture media on day 35 were performed. $p < 0.05$ was considered to indicate statistically significant differences. Statistical analysis was performed on averages of triplicate samples per pellet using Graph Pad Prism 5.00 for Windows (GraphPad Software, San Diego California USA).

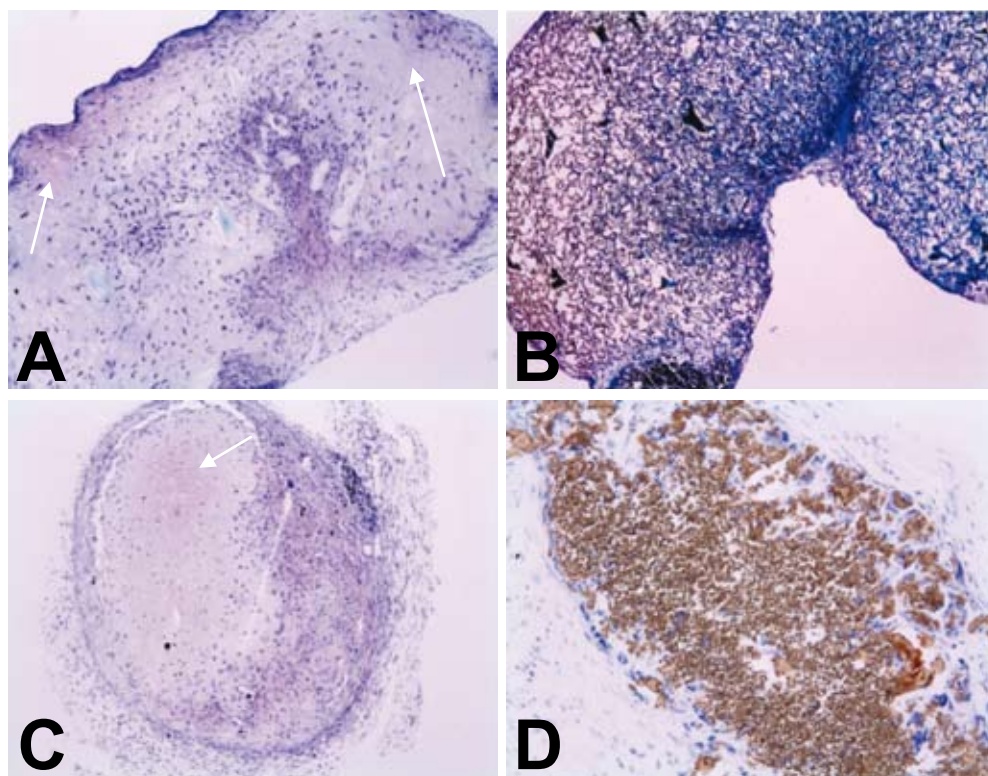


Figure 1. Von Kossa staining with thionine of primed scaffolds. GAG production (thionine staining, white arrows) in chondrogenically primed scaffolds after 3 weeks in vitro (A) which is further developed after 4 weeks in vivo (C) into a cartilage-like matrix with individual cells surrounded by matrix. No mineralization was observed. Following 3 weeks of osteogenic stimulation, mineralized matrix was observed both in vitro (B) and after subsequent implantation in vivo (D) with a similar progressive increase in matrix production. This matrix however did not resemble native bone nor did it contain many viable cells.

RESULTS

Priming of BMSC in vitro and subsequent in-vivo implantation

Following 3 weeks in-vitro priming, chondrogenic scaffolds were positive for GAGs and negative for mineralization (fig. 1A) whereas osteogenically primed scaffolds were mineralized throughout (fig. 1B). Following implantation, the osteogenic scaffolds continued to differentiate along the osteogenic route however producing an inferior matrix with questionable levels of cell survival 4 weeks after implantation as evidenced by reduced cell number within the mineralized portions (fig. 1D). In the chondrogenic condition, despite cell survival and a formation of a cartilage like matrix with some hypertrophic cells visible, no osteogenesis was observed (fig. 1C). No appreciable matrix formation of either kind was observed in control unprimed control conditions (cultured on expansion medium, data not shown) showing the promise and importance of the initial in vitro culture period and the possible benefits of well timed implantation.

Collagen X immunostaining of chondrogenically primed implanted scaffolds showed positive staining for in 5 out of 5 of the retrieved scaffolds (fig. 2A). Osteogenically primed scaffolds showed only very weak positive staining in 1 of 4 scaffolds (fig. 2B).

Importantly, in the chondrogenic scaffolds mature blood vessels were visible in all implanted scaffolds (fig. 2). In 3 out of 5 the scaffolds, blood vessels contained erythrocytes, in the 2 other scaffolds we found what appeared to be blood vessels without red blood cells. This shows not only good cell survival of chondrogenically primed cells but also good host integration and suggests progression along the endochondral ossification route. Conversely, no vascularization was visible in the osteogenically treated scaffolds, possibly due to too much matrix deposition or a lack of release of inductive factors. This lack of vascularization in the osteogenically primed constructs might explain the lack of cells in the scaffolds due to cell death.

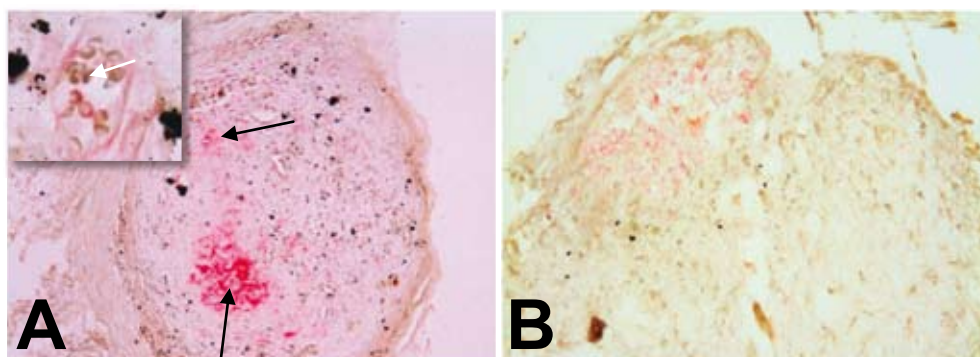


Figure 2. Collagen X staining of implanted scaffolds. Following implantation of primed scaffolds, immunostaining for collagen X showed that all chondrogenically primed scaffolds were positive for collagen X (A) (black arrows) and all osteogenically primed scaffolds were negative (B). More importantly, blood vessels containing erythrocytes were visible (see inset, white arrow indicates erythrocytes). In contrast, no blood vessels were visible in any of the osteogenically primed scaffolds.

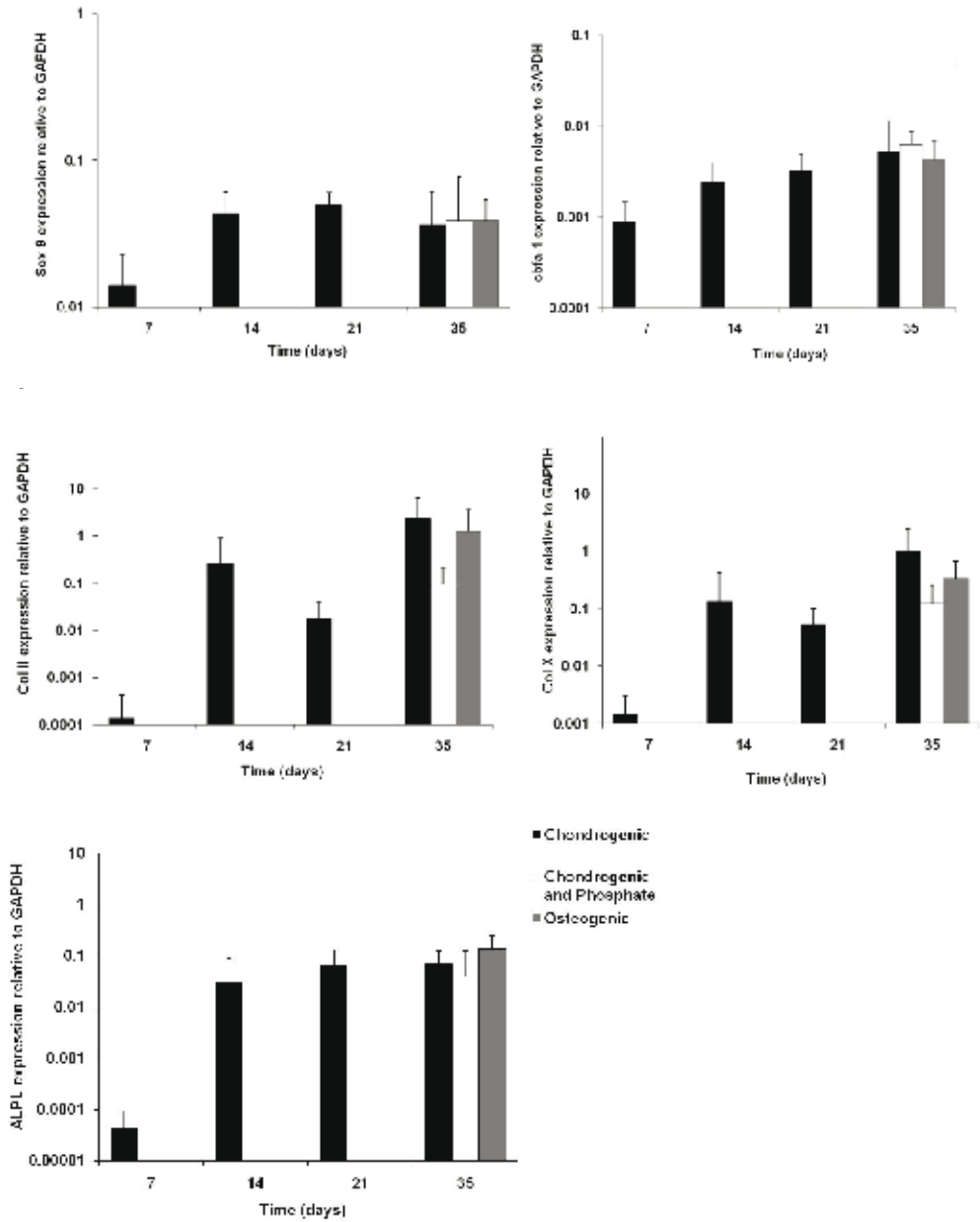


Figure 3. Gene-expression analysis of pellets. Gene-expression analysis of markers of chondrogenesis, hypertrophy and osteogenesis showed a steady increase in time in expression of all markers cultured in chondrogenic medium. Switching to either of the two mineralizing media for 14 days did not lead to significant differences in gene expression of any of the markers. Data represents 3 pellets per donor for Donors A and B only.

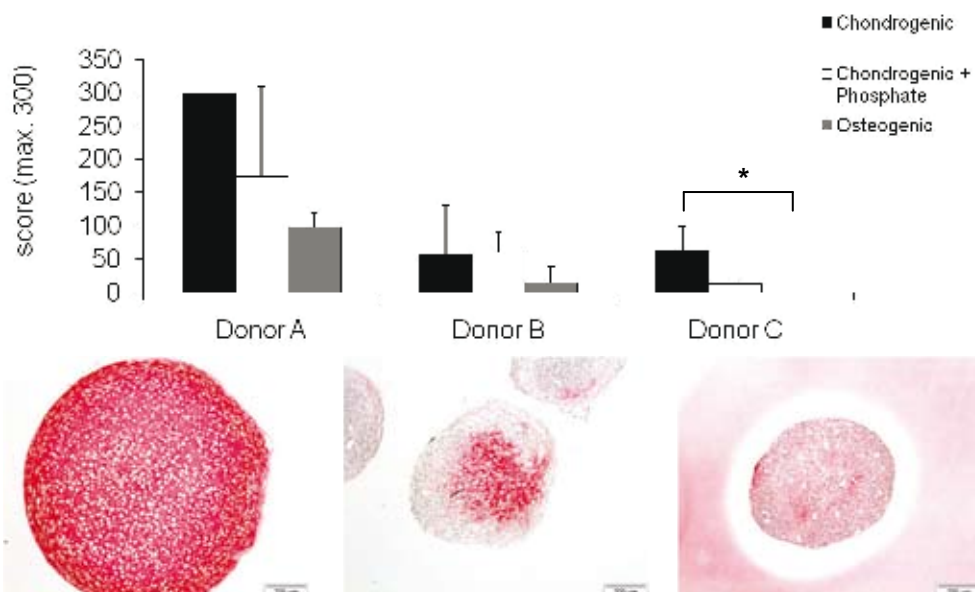


Figure 4. Collagen II immunohistochemistry and scoring of pellets. To some extent, positive collagen II staining (red) was observed in all pellets in a donor-dependent manner, as is often seen. Upon addition of a phosphate donor for 14 days, collagen II expression was vastly reduced in all 3 donors. 3 sections per pellet with 3 pellets per donor were analyzed to score the amount of staining (1 way ANOVA; $*= P<0.05$). Representative images of the chondrogenic condition are displayed for each donor respectively.

In vitro pellet culture system

Gene expression

All genes were observed to increase in expression over time upon addition of chondrogenic factors. At day 35 comparison of expressions in the mineralizing conditions which had been switched 14 days previously was unchanged for Sox 9, *cbfa-1/runx2* and alkaline phosphatase. There was a decrease in expression of collagen II and X upon the addition of phosphate, however this decrease was not observed in the osteogenic condition and in no instances was it significantly different from the chondrogenic condition (fig. 3).

In vitro, collagen II positive staining was found in chondrogenically differentiated pellets of all donors at day 35 (fig. 4A). Although they all showed positive staining, variation in stained area and intensity was high, ranging from a score of 57 to the maximum score of 300. This variation was donor dependent. Collagen II expression was mainly seen in the center of the pellets (fig. 4B).

Collagen X expression was found in all patients at day 35. This expression was again donor dependent and only highly expressed in donor A who also produced more collagen II. Collagen X and II expressions were reduced in both conditions switched to mineralizing media (fig. 5).

Von Kossa staining showed calcium-phosphate depositions in pellets that were switched to phosphate-containing medium after being cultured on chondrogenic differentiation medium for 21 days. Mineralization was found in two out of three donors (A and B) in both the chondrogenic + phosphate switched condition and in the condition switched to osteogenic medium, with no mineralization observed in the chondrogenic condition. The mineralized area was found in the core of the pellet cultures and covered approximately half of the pellet area. Few viable cells were visible in the mineralized portions. In non-mineralized tissue this lack of cells was not seen. No difference was found between the chondrogenic condition containing BGP (culture medium 2) and the osteogenic condition (culture medium 3) (fig. 6).

When pellets were cultured on chondrogenic differentiation medium for 49 days collagen II and X further increased (data not shown). When cultures were switched after 35 days on chondrogenic differentiation medium, the incidence and amount of mineralization was not different to those conditions switched after 21 days. Mineralization again occurred in two out of three donors.

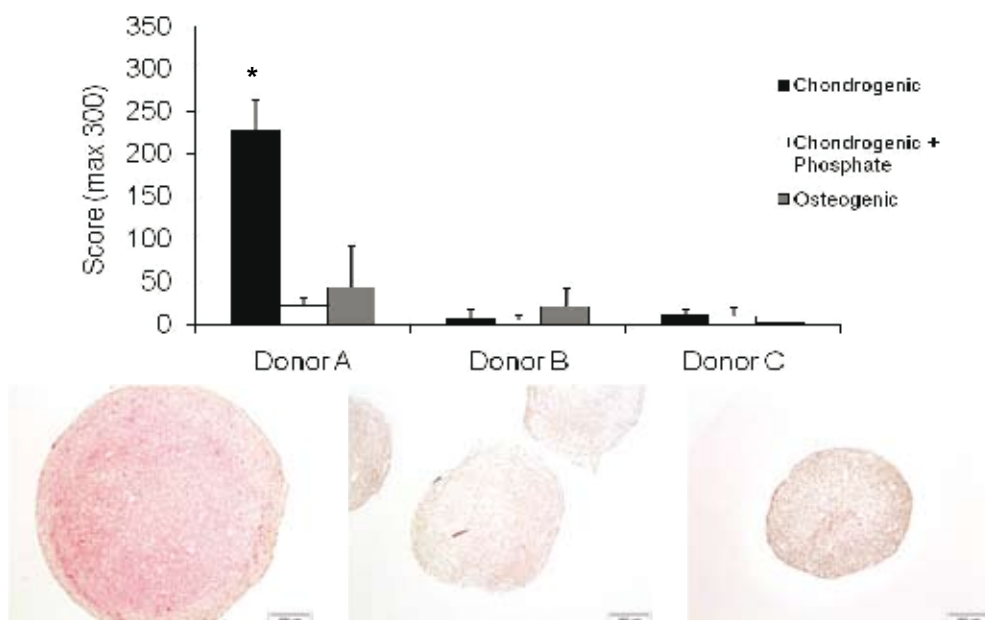


Figure 5. Collagen X immunostaining and scoring of pellets. Staining of pellets for collagen X showed a similar pattern of expression to collagen II with a donor related variation in expression levels with decrease expression in the presence of a phosphate donor. Again sections were scored in triplicate with 3 pellets per donor (1 way ANOVA; *= significant difference from chondrogenic + phosphate and osteogenically switched conditions $P < 0.05$). Representative images show collagen X expression in the chondrogenic condition following 35 days culture.

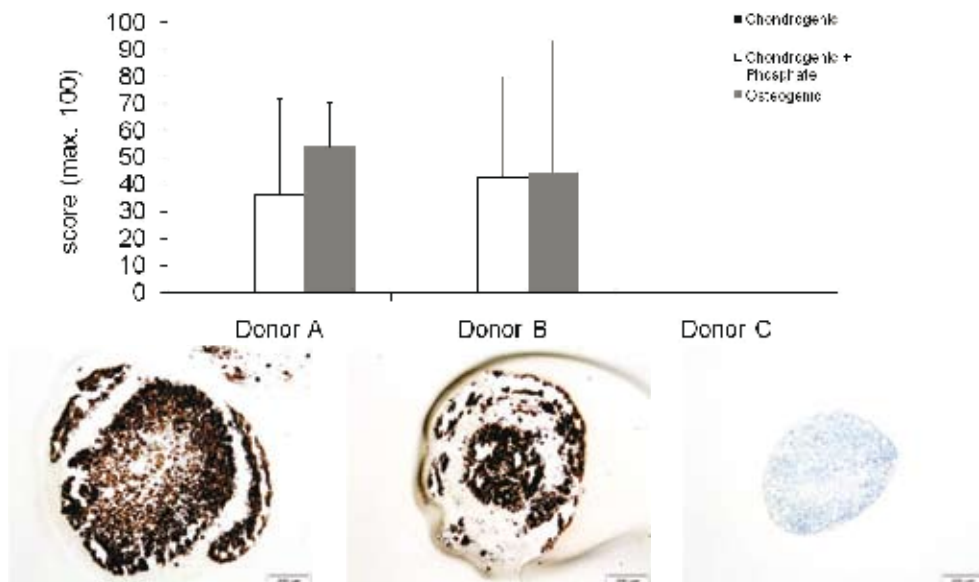


Figure 6. Von Kossa staining of in-vitro cultured pellets. Culture of pellets for 35 days on chondrogenic medium resulted in no mineralization in any pellet from any donor as evidenced by the zero scores for the chondrogenic group. When a source of phosphate was added, mineralization was observed in all pellets in 2 of 3 donors with no difference between the chondrogenic + phosphate and osteogenic conditions. Despite the presence of cells within the pellets, donor C demonstrated no mineralization capability. Representative images of stained pellets show mineralization when phosphate was added to the chondrogenic medium (black stain).

VEGF expression

VEGF was present in medium of pellets on chondrogenic medium on day 14, 21 and 35 except for one donor, which showed VEGF release at day 14 but little thereafter. This donor, Donor C also showed little collagen II and X expression and insufficient RNA was available for gene expression analysis. When pellets were switched to the culture medium containing BGP, independent of the presence of TGF- β 2 or the concentration of dexamethasone, VEGF expression was considerably lower than the chondrogenic condition (fig. 7). Also, the conditions that were switched after 35 days of culture showed no reduced VEGF expression (data not shown). Although some variation between donors was found, the overall effect on VEGF production was the same.

MMP-release

Gelatin zymography demonstrated MMPs present in the culture medium collected at day 35 from all cultured pellets. A steady increase in release from day 14 was also observed in the chondrogenic condition (data not shown). The two bands with high activity showed up around 62 kDa, and around 59 kDa. A third activity band was seen around 50 kDa. Analysis of culture medium collected from pellet cultures that were switched to phosphate containing

culture medium demonstrated far less activity at 62 kDa and no activity at 59 and 48 kDa, both at day 35 and day 49 (not shown) of culture (fig. 7B).

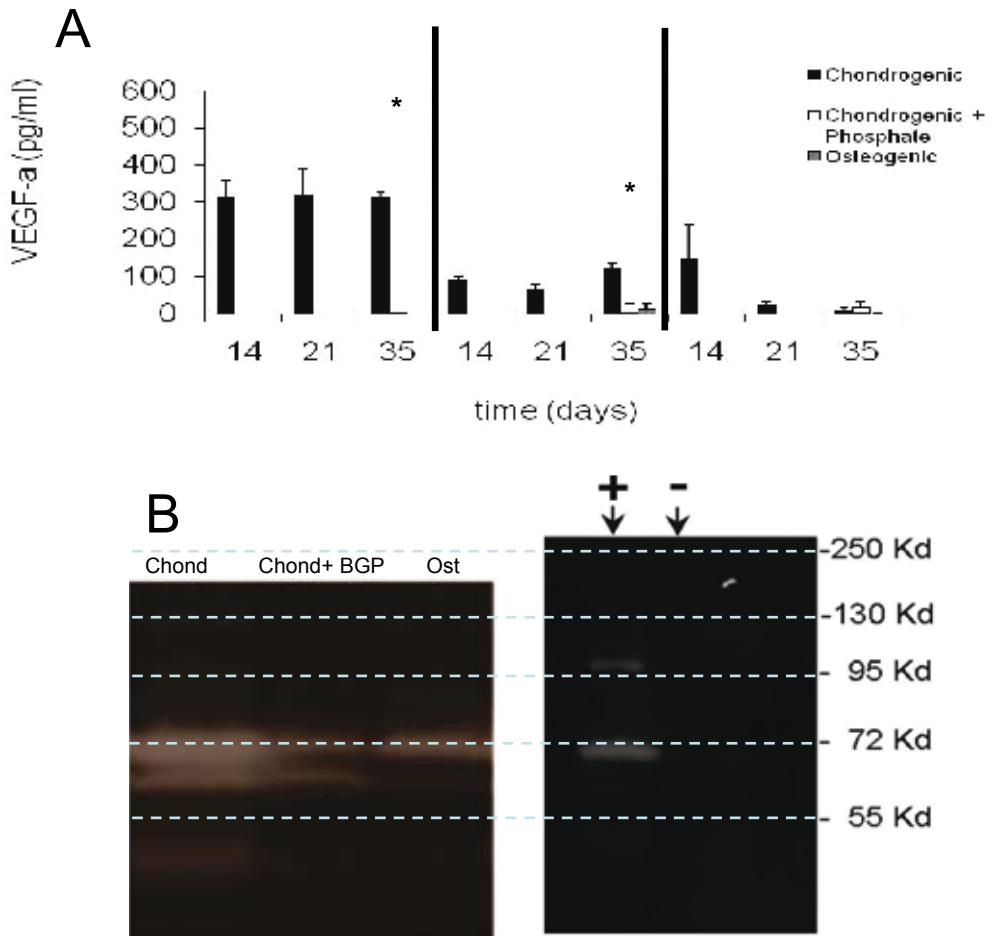


Figure 7. Release of factors related to endochondral ossification. Release of VEGF from pellets was measured over time. As was seen with collagen II and collagen X, release of VEGF from pellets was abrogated following addition of a phosphate donor for 14 days after 21 days in chondrogenic medium (A). It is important to note that VEGF was released continuously until day 35 in the chondrogenic condition with almost no VEGF release 14 days after addition of BGP and pellet mineralization. VEGF levels at days 14 and 21 are for chondrogenic condition only (1 way ANOVA $n=3$ per donor; *= significant difference from chondrogenic + phosphate and osteogenic conditions $P<0.05$). There was also a concomitant reduction in MMP release in these samples at day 35 suggestive of an inhibition of the remodeling processes associated with endochondral ossification (B).

DISCUSSION

In this article we assessed the possibility for in-vitro chondrogenically primed BMSC to offer a better repair option for bone tissue engineering approaches. In vivo we demonstrated improved cell survival following chondrogenic priming of BMSC in vitro, with significant blood vessel invasion after 4 weeks in vivo, although no bone formation was observed, possibly due to insufficient time allowed. We hypothesized that in-vitro priming of BMSC along the chondrogenic lineage would prompt the release of factors leading to tissue vascularization and bone formation in vivo. Here we demonstrate that mineralization of cartilage tissue can be induced in vitro but that this inhibits the angiogenic and remodeling properties of chondrogenically differentiated pellet cultures. Crucially, in a model of endochondral ossification we show that pellet cultures of chondrogenically differentiated BMSC express collagen II and collagen X, produce VEGF and increased amounts of MMPs and that all of these qualities are quickly reduced following mineralization in a model of endochondral ossification as was initially hypothesized. This might suggest that mineralized pellet cultures do not possess the same angiogenic and remodeling properties that can stimulate graft-host integration in vivo and that compared to chondrogenically differentiated pellet cultures the chance to obtain a bony union in bone defects might therefore be reduced.

Progressive production of collagen II and subsequent collagen X, suggests that differentiated BMSC in pellet culture resemble chondrogenic differentiation and hypertrophy, as seen in the growth plate during longitudinal growth. Lack of phosphate in the chondrogenic culture medium does however prevent further resemblance to endochondral ossification because the formation of calcium-phosphate depositions is inhibited. In order to examine if pellet cultures only need the presence of phosphate in the culture medium to mineralize the extracellular matrix or that they must be cultured in a more osteogenic with a lower concentration of dexamethasone and in the absence of TGF β , we evaluated the mineralization of chondrogenic differentiated pellet cultures that were switched after 21 days to these two phosphate containing culture media. 14 days after switching, abundant amounts of mineralization were seen in the core of the pellets at the same area as the cartilaginous tissue production was found, suggesting that this is indeed possible also in vitro given the correct signals and timing. Patterns of VEGF release, collagen expression and MMP release were similar between this condition and a standard osteogenic condition switched from chondrogenic culture at the same timepoint. The spatial production of collagen II, collagen X and mineralization as seen in subsequent sections suggests endochondral ossification had occurred. This is line with published data by Mueller and Tuan, however they observed the requirement for the removal of TGF β for mineralization to occur (35). Gene expression of markers of chondrogenesis and hypertrophy/osteogenesis was also seen to increase over time as has been demonstrated in the past (33; 35; 196). VEGF, which is produced by hypertrophic chondrocytes, is known to have distinctive roles in endochondral ossification. First, it is an important mediator in angiogenesis. Blocking the activity of VEGF inhibits blood vessel formation in the hypertrophic zone (146). Furthermore, it has been shown that VEGF has chemoattractant effects on mesenchymal progenitor cells

and osteoblasts and influences osteoblast proliferation and differentiation (205; 206). Cells of the monocyte-macrophage lineage are also affected; chondroclasts are less present when VEGF activity is blocked (146; 207; 208). Analysis of VEGF on the culture medium of chondrogenically differentiated pellet cultures showed a considerable production of VEGF at 14 days of culture. In two donors the expression remained stable during the five-week culture period. This suggests that these pellet cultures have potential to stimulate incorporation *in vivo* after transplantation. Most importantly, amounts of VEGF release appeared related to collagen II and X expression and were almost completely inhibited by matrix mineralization. This is very much in line with the *in-vivo* findings showing no vascularization of the osteogenically primed scaffolds and some vasculature connected to the host within the chondrogenically primed scaffolds. It will be important to elucidate the timing of these events in the future along with cell-death events to understand the role of cell death in this process. The type of cell death undergone by hypertrophic chondrocytes is a continuing debate (209; 210; 211) and warrants investigation, however it is beyond scope of this article.

To further evaluate the angiogenic and remodeling activities of the cell constructs we performed gelatin zymography to analyze the relative amounts of total MMPs released by the various pellets. In the conditioned medium of chondrogenically differentiated pellet cultures, three bands were found, one around 62 kDa, one around 59 kDa and a third around 50 kDa, the approximate weights of several MMPs including but not exclusively MMP-2, pro-MMP-13 and MMP-13 respectively. What is important to note is that many of these MMPs play an important role in the remodeling of the cartilaginous extracellular matrix during endochondral ossification by breaking down collagen 2 and non-collagenous matrix proteins such as aggrecan to allow vessel in-growth (192; 193; 194; 195; 212; 213). Reduced levels of MMPs in the mineralizing samples are in agreement with the *in vivo* findings where no blood vessel invasion was observed in the osteogenic condition.

It is clear that there was a large inter-donor variability in this study. We show relatively little variation within pellets from the same donors. Often in this research field donors are pre-screened and those that do not “perform” well are not included in experiments. We believe however it is just these donors who will most likely require treatment in the future and it is therefore relevant to examine how they respond to this type of stimulation and to present this data. Donor C produced collagen II and no collagen X and subsequently no VEGF. Having performed all experiments on multiple pellets of each donor we are confident of our results. From this data we conclude that chondrogenic priming of BMSCs will lead to increased VEGF release with a concomitant increase in blood-vessel invasion *in vivo*. Induction of mineralization in pellets or scaffolds prevents this release of VEGF and leads to inferior mineralized matrix production *in vivo* with no blood supply and non-viable cells. The poor performance of Donor C in all respects confirms that this is not simply a standard response of BMSC in pellet culture and that this approach requires further investigation and optimization. This is not the first article to illustrate large variation between donors. Scharstuhl *et al* (214) demonstrated as much as 1000 fold differences in expression of chondrogenic markers between patients and it is important not to overlook this variability

and to use it as a means to develop better treatments and approaches to regenerative medicine.

Also of interest is the better control of this process that could be garnered through further understanding of how it works. Endochondral ossification is tightly controlled by a host of intra and extracellular signaling molecules including but not exclusively, insulin like growth factors (IGF), members of the fibroblast growth factor (FGF) family and the Wnt signaling pathway. It is conceivable that tailored addition of growth factors or agonists at temporally distinct periods in the differentiation process could vastly improve the efficacy of such an approach, particularly for those donors that do not respond well to the standard cocktail of growth factors. This also would apply to attraction of vessels *in vivo*, determining the correct combination to lead to optimum vessel/osteoblast/osteoclast attraction upon implantation.

This study contributes to the view that chondrogenically differentiated pellet cultures of BMSC resembles endochondral ossification, the more so as we demonstrated that mineralization of the extracellular matrix can occur when a phosphate donor is present. More importantly we demonstrate for the first time the critical relationship between VEGF release, MMP release and matrix mineralization that can have huge effects on the success of implanted tissue engineered constructs. While we are aware of the limitations of using an immunocompromised animal in this model we felt it was important to assess the potential of human cells and this was the best model to do so. We demonstrate that induction of mineralization prior to transplantation might have severe effects on the angiogenic and remodeling properties, suggesting that transplantation of chondrogenically primed tissue at the correct stage of differentiation, and prior to mineralization, might prove much more successful than fully differentiated tissue. Finally we show that chondrogenically primed BMSC are capable of expressing the hypertrophic marker collagen X, attracting the host vasculature and surviving long term *in vivo*. While this pattern was observed in all samples, it should be noted that this was performed on commercially provided cells from a single donor. Our data suggest that determination of the optimal time of implantation of these primed cells, combined with a longer *in vivo* period, will lead to successful bone formation *in vivo* via the more common route of endochondral ossification.

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SUMMARY

Cartilage has a very poor capacity for regeneration in vivo. In otorhinolaryngology cartilage defects are usually reconstructed with autologous cartilage from for instance the external ear or the ribs. Cartilage tissue engineering may be a promising alternative to supply tissue for cartilage reconstructions in otorhinolaryngology as well as in plastic surgery and orthopaedics. Different cell sources could be used to tissue engineer cartilage. In this thesis we studied expanded chondrocytes and adult bone-marrow derived mesenchymal stem cells (BMSC). The aim of this thesis was to find new tools by which cartilage tissue engineering can be better controlled, focusing on the generation of cartilage that does not mineralize when implanted in vivo. We hypothesize that new insights to control cartilage tissue engineering can be gained by studying in-vivo chondrogenesis during embryonic development.

In **chapter 2** we evaluated the potential of culture-expanded auricular and nasoseptal chondrocytes as cell sources for generation of stable cartilage. Auricular chondrocytes produced larger cartilage-like constructs than nasoseptal chondrocytes. The matrix formed by auricular and nasoseptal chondrocytes contained collagen X, a marker for hypertrophic differentiation. However, the cartilage constructs did not mineralize in an in-vitro model, nor after in-vivo subcutaneous implantation for 6 weeks. Next, a DNA-microarray study on expanded auricular and nasoseptal was performed to analyze the differences in gene-expression profile of expanded chondrocytes from these specific locations and revealed 1090 differentially expressed genes. No difference was observed in the expression of known markers of chondrogenic capacity (e.g. collagen II, FGFR3, BMP2 and ALK1). The most striking differences were that the auricular chondrocytes had a higher expression of anabolic growth factors BMP5 and IGF1, while matrix-degrading enzymes MMP13 and ADAMTS5 were higher expressed in nasoseptal chondrocytes. This might offer a possible explanation for the observed higher matrix production by auricular chondrocytes. Moreover, chondrocytes from different donor sites seemed to express specific molecular characteristics even after expansion. These specific molecular characteristics were not restricted to known characterization of donor-site subtype (e.g. elastic), but were also related to developmental processes. Therefore, our data indicate that using expanded chondrocytes from a donor-site that is not directly related to the host tissue, may result in cartilage with a different molecular character.

In the next chapters we focused on the potential of BMSC for cartilage tissue engineering. It is known that chondrogenically differentiated BMSC display signs of chondrocyte hypertrophy and mineralize in vivo (33). It has been said that this terminal differentiation and mineralization of BMSC resembles in-vivo endochondral ossification of the limbs. Therefore, in **chapter 3** we compared embryonic chondrogenesis to in-vitro chondrogenic differentiation of BMSC. In both models three sequential stages were discriminated, condensation, differentiation, and hypertrophy, respectively characterized by N-cadherin, collagen II, and collagen X expression. However, a difference in these models is seen when the relative length of these stages is considered. For example while in embryonic development a differentiation stage is clearly discernable, in in-vitro chondrogenic pellet

cultures of BMSC almost no interval exists between production of collagen II and X. Next, we related the expression of different Fibroblast Growth Factor Receptors (FGFR) subtypes to the differentiation stage in in-vitro differentiation and in-vivo embryonic limb development. In both models FGFR2 is clearly expressed by cells in the condensation phase. No FGFR expression was observed in differentiating and mature hyaline chondrocytes, while hypertrophic chondrocytes stained strongly for all FGFR. To evaluate whether stage-specific modulation of chondrogenic differentiation in BMSC is possible with different subtypes of FGF, FGF2 and FGF9 were added to the chondrogenic medium during different stages in the culture process (early or late). In general, addition of FGF to the medium had a negative effect on chondrogenesis. However we did show that the effect of an FGF ligand is dependent upon the FGFR subtype to which it preferentially binds, thereby offering clues to subtype specific functions of FGFR. Moreover, we demonstrated that the effect of a growth factor depends on the stage in which it is added in the medium. This is in accordance with embryonic development. While FGF9 has an inhibitory effect when added throughout culture, or during hypertrophy, it does not have a negative effect when given during early differentiation and shows a trend towards stimulatory when given at the right time interval. Therefore, a more specific stimulation of FGFR2 activation during condensation may be interesting for cartilage tissue engineering. Since the effects of growth factors and modulation of intracellular signaling are mostly studied throughout culture, these results indicate that more attention to stage-specific effects may be warranted.

The concept of stage-specific modulation of chondrogenesis was continued in **chapter 4**. To induce chondrogenic differentiation of BMSC TGF- β is commonly used (31) and intracellular signaling of members of the TGF- β superfamily involves phosphorylation of Smads (104). Therefore we investigated the roles of Smad2/3 and Smad1/5/8 phosphorylation in chondrogenic and hypertrophic differentiation and assessed whether specific targeting of the Smad signaling pathways offers possibilities to prevent terminal differentiation and mineralization of chondrogenically differentiated BMSC. First, we evaluated whether Smad2/3 and Smad1/5/8 pathways are phosphorylated in hypertrophic and hyaline cartilage. Terminally differentiated chondrocytes in cartilage produced in vitro by chondrogenic differentiation of BMSC or studied ex vivo during murine embryonic limb formation, stained positive for both Smad2/3P and Smad1/5/8P. On the other hand, hyaline-like cartilage lacking expression for MMP13 and collagen X only expressed Smad2/3P. When either Smad2/3 or Smad1/5/8 phosphorylation was blocked by addition of SB-505124 or dorsomorphin throughout culture, no collagen II expression was observed, indicating that both pathways are involved in early chondrogenesis. Distinct functions for these pathways were demonstrated when Smad signaling was blocked after the onset of chondrogenesis. Blocking Smad2/3P after the onset of chondrogenesis resulted in a halt in collagen II production. On the other hand, blocking Smad1/5/8P during this time period resulted in decreased expression of MMP13, collagen X and alkaline phosphatase while allowing collagen II production. Moreover, blocking Smad1/5/8P prevented mineralization. This indicates that while Smad2/3P is important for continuation of collagen II deposition, Smad1/5/8 phosphorylation is associated with terminal differentiation and mineralization.

Three mammalian subtypes of TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3) are commonly used to induce chondrogenic differentiation of BMSC in different laboratories (31; 35; 41; 129). In our laboratory we are accustomed to use TGF- β 2. In literature, results with one TGF- β subtype are often extrapolated to the other subtypes. As results in literature suggest that these subtypes actually may provoke certain subtype-specific activities, the choice of TGF- β subtype to induce chondrogenesis may attribute to contradictory results in different studies regarding chondrogenic differentiation of BMSC. Therefore, we compared the three TGF- β subtypes in **chapter 5** for their capacity to induce chondrogenesis in BMSC, concentrating on the amount of produced cartilage as well as the degree of terminal differentiation and capacity to mineralize. Although no significant differences were found between TGF- β 1, - β 2 and - β 3 cultured pellets in the quantity of cartilage production or in the expression of hypertrophic markers, the presence of mineralization was significantly higher when TGF- β 3 was used, compared to TGF- β 1. For future studies TGF- β 1 is therefore preferred for use in cartilage tissue engineering. Since TGF- β 3, and to a lesser extent TGF- β 2 show signs of matrix-mineralization, those subtypes might be better to tissue engineer bone through endochondral ossification.

Although the studies in chapter 4 demonstrated that blocking phosphorylation of Smad1/5/8 resulted in cartilage formation with reduction of hypertrophic markers, this did not reach the low level of stable chondrocytes. This was also the case for other recently published methods to inhibit terminal differentiation, through PTHrP (101; 102; 154) or blocking MMP13 (155). Therefore in **chapter 6** we aimed to review the literature supplemented with own data to answer the question: Is it possible to generate stable hyaline cartilage from adult mesenchymal stem cells? First, we characterized terminal differentiation more precisely as a gradual progression of chondrogenic differentiation towards cartilage that can mineralize. As expression of hypertrophic markers almost overlaps with the onset of chondrogenesis, the hypertrophic phenotype may be predetermined when chondrogenic differentiation is still in its early stages in current culture conditions. These current culture conditions, and the rationale of inhibiting terminal differentiation, are derived from a traditional view that development of hyaline cartilage is nothing more than chondrocytes failing to go through endochondral ossification. However, chondrocytes in the growth plate are destined to become terminally differentiated and it therefore may not be the model of choice to study hyaline cartilage formation. Embryonic development of the interzone suggest that to develop stable cartilage a subtle balance of chondrogenic stimulating as well as inhibiting signal is required, starting from the initial prechondrogenic differentiation. For cartilage tissue engineering this may mean that inhibiting terminal differentiation may not result in stable hyaline cartilage, if the right balance of signals has not been created from the start of culture on.

While the terminal differentiation and mineralization seen in chondrogenically differentiated BMSC is negative for cartilage tissue engineering, it may offer a new route to engineer bone. Often BMSC are in vitro stimulated toward the osteogenic lineage, thereby aiming at intramembranous bone formation (183). However, results of this approach have been disappointing due to core degradation and necrosis of the construct as a result of lack

of vascularization (184). Therefore, in **chapter 7** we studied the possibility of tissue engineering bone through endochondral ossification as an alternative approach. Scaffolds seeded with BMSC were cultured in vitro on either osteogenic or chondrogenic medium before they were implanted in a nude mouse, and survival and osteogenesis in vivo were evaluated. Scaffolds that were pre-cultured on chondrogenic culture medium showed collagen type II and collagen type X production after implantation. Moreover, vessel in growth was observed. In contrast, scaffolds that were osteogenically pre-cultured demonstrated a mineralized matrix of poor quality, with few surviving cells and no vascularization. This process was further characterized in vitro using pellet cultures. Pellets cultured in chondrogenic medium showed progressive production of collagen type II and collagen type X, so a hypertrophic cartilage construct was formed. Moreover, in the culture medium VEGF release and MMP activity were observed, two factors that are known to be important for the induction of vascularization of the matrix. We therefore conclude that chondrogenically differentiated BMSC produce hypertrophic cartilage that supplies vascularization stimulating factors, which may be essential to prevent core degradation and necrosis of the tissue-engineered construct. These data suggest that optimizing the hypertrophic differentiation of BMSCs may further improve vessel invasion in bioengineered constructs and longer implantation may lead to viable bone turnover of the hypertrophic cartilage construct.

Concluding, in this thesis we have demonstrated that expanded auricular and nasoseptal chondrocytes produce cartilage that does not mineralize, and therefore offer great promise for clinical application in cartilage tissue engineering. In contrast, chondrogenically differentiated BMSC become terminally differentiated and mineralize. While this offers a new route to tissue engineer bone, it is disadvantageous for cartilage tissue engineering as stable cartilage is essential for clinical cartilage reconstructions. The chondrogenic differentiation of BMSC is a multi-stage process (condensation, differentiation and hypertrophy) that resembles endochondral ossification during embryology. Therefore, a stage-specific modulation of chondrogenic differentiation seems a promising way to control cartilage formation by BMSC. However, current concepts to control chondrogenic differentiation of BMSC have arisen from developmental research of the growth plate. Since chondrocytes in the growth plate are destined to become terminally differentiated, it may not be the model of choice to study hyaline cartilage formation. Embryonic development of the interzone suggest that to develop stable cartilage a subtle balance of chondrogenic stimulating as well as inhibiting signals is required, starting from the initial prechondrogenic differentiation. For cartilage tissue engineering this may mean that inhibiting terminal differentiation may not result in stable hyaline cartilage, if the right balance of signals has not been created from the start of culture on.



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DISCUSSION AND FUTURE PERSPECTIVES FOR CLINICAL APPLICATION

Embryology as a model for tissue-engineering

Formation of cartilage is only seen during embryonic development (215). The rationale of comparing tissue engineering and embryonic development is that these processes may involve similar pathways, and that understanding these common pathways may lead to advances in tissue engineering. Therefore we compared differentiation of BMSC with embryonic endochondral ossification of the limbs in chapter 3 and 4. When trying to relate the in-vitro differentiation of BMSCs to embryonic chondrogenesis, it is important to take into account the fundamental differences in these models. Most importantly, chondrogenically differentiating cells in the embryonic limb bud get signals from other cell types surrounding these cells, such as in the future perichondrium and the loose mesenchyme (4), whereas BMSC get signals mainly from components added in the medium. The signals to these cells are hugely complex and not only involve growth factors such as TGF- β and FGF, but include pathways such as BMP, Ihh, PTHrP, Wnt/ β -catenin (4). In this thesis we have compared these models to obtain more knowledge about the FGF and TGF- β pathways to enhance the tissue engineering process to better control chondrogenic differentiation.

Stage-specific modulation of chondrogenesis

The effects of growth factors and modulation of intracellular signaling are mostly studied throughout culture. We have demonstrated in chapter 3 and 4 that more attention to stage-specific effects may lead to a better control over chondrogenesis. In a recent paper by Lenas et al (216), the model of “developmental engineering” was put forward. Instead of investigating empirically by trying various combinations of tools leading to a perpetual cycle of trial-and-error attempts, a rational methodology should be followed based on a fundamental understanding of tissue development. An important concept in “developmental engineering” is that the multistage character of developmental processes allows high observability and controllability. In-vitro processes composed of distinct sequential steps that are characterized by markers expressed in a specific stage allow the possibility to intervene with additional factors to direct the cells at the appropriate stages. This thesis has made a first attempt to define chondrogenic differentiation of BMSC as a gradual progression of steps in need of specific stimuli.

Expanded auricular and nasoseptal chondrocytes for cartilage tissue engineering

In this thesis we have demonstrated that both expanded auricular and nasoseptal chondrocytes produce cartilage that is stable and does not mineralize in vivo (chapter 2) in contrast to BMSCs (chapter 3-7). Moreover, while a huge inter-donor variability is seen in chondrogenic capacity of BMSC, in our experience chondrocytes (and especially auricular chondrocytes) seem to be more predictable and have less donor variability. Thus, although BMSC are generally considered a popular cell source for tissue engineering, in the near future chondrocytes seem to be a more likely cell source for successful clinical application of cartilage tissue engineering than BMSCs.

Although tissue-engineered cartilage constructs from auricular and nasoseptal chondrocytes are stable and do not mineralize, both cells sources produce a collagen X rich matrix indicating a progression towards terminal differentiation. This may be due to

progenitor cells in the cambium layer of the perichondrium (6; 8; 28). Still, no mineralization was observed after 6 weeks subcutaneous implantation. Another study on rabbit chondrocytes (20) did demonstrate mineralization after 10 weeks of implantation. These contradictory result may be explained by the different sites of implantation. Tissue-engineered constructs were implanted in the trachea of rabbits in that study, while we implanted the cartilage constructs ectopically in the subcutis of the back of nude mice. Ectopic implantation in the back is often performed in similar studies (33; 34; 55) with small animals, such as nude mice, but may influence the stability of the construct. The subcutaneous fat tissue of the back is completely sterile and not highly vascularized. In contrast, the region of the head and neck is highly vascularized and the tissue aligning the airways has a high immune activity to protect it from the exterior. The combination of these factors in the head and neck region may cause a more active reaction to a tissue-engineered cartilage construct, leading to for instance mineralization or resorption of the construct (21; 23). Therefore, more research is warranted on long-term stability upon implantation of the cartilage construct derived from human chondrocytes in specific orthotopic locations such as the trachea, nose or auricle.

Auricular chondrocytes especially seem attractive for translation to clinical practice, as these chondrocytes seem to produce more cartilage-like matrix (chapter 2). Furthermore, auricular cartilage can be harvested in a minimally invasive procedure. A higher cartilage-matrix production in auricular chondrocytes was also seen in previous studies (50; 51). Nevertheless, our study is the first to directly compare auricular and nasoseptal chondrocytes from the same human donors. What is more, our microarray study did offer a possible explanation for the observed higher matrix production by auricular chondrocytes, as anabolic growth factors BMP5 and IGF1, were significantly higher expressed in auricular chondrocytes while matrix degradation enzymes MMP13 and ADAMTS5 were expressed significantly higher in nasoseptal chondrocytes. These data also highlight the importance in tissue-engineering of not only adding needed growth factors or signaling molecules to the culture medium, but the need to increase knowledge of and to control cellular behavior and production of signaling molecules by the cells themselves. The importance of these endogenously produced factors for cartilage tissue engineering should be further studied.

Even after extended expansion chondrocytes seem to retain some characteristics of their native donor site (chapter 2). This was indicated by elastin production *in vivo* and gene expression of *Fibrillin 2* and *elastin* by auricular chondrocytes as well as more subtle differences in gene expression between auricular and nasoseptal chondrocytes mostly in the field of developmental processes. Therefore, using expanded chondrocytes from a donor-site that is not directly related to the host tissue, may result in cartilage regeneration of a different molecular character. This does not seem to be a vital clinical problem, as it is common practice in head and neck surgery to use hyaline costal cartilage for auricular reconstructions (217) or auricular elastic cartilage to repair hyaline cartilage defects of the nose (14). However, more knowledge about the differential developmental processes of the different cartilage subtypes may reveal clues to tissue engineer cartilage with a more specific character adapted to local circumstances, such as mechanical characteristics or the

presence of elastin, that may improve long-term results. This may prove to be essential especially in paediatric patients, as long-term functionality as well as further development and adaptation of the cartilage construct may be important during growth.

The function of elastin is to maintain shape and flexibility of the cartilage matrix, for instance in the auricle (218). Currently, auricular reconstruction is a multistep procedure using costal hyaline cartilage lacking elastin (217). These procedures result in somewhat unsatisfactory results, but this may be due to the fact that fully matured cartilage is used and formed into shape and may not be caused by the lack of elastin. We were not able to tissue engineer cartilage constructs in vitro from auricular chondrocytes that contained elastin. However, upon 6 weeks of in-vivo implantation elastin was produced and thus the cartilage was transformed in elastic cartilage. Nevertheless, whether presence of elastin is necessary to create a cartilage construct that is suitable for reconstruction of for instance auricular cartilage is not known. Perhaps, before attempting to tissue engineer cartilage of a specific molecular phenotype more knowledge is warranted on the characteristics and mechanical properties, and demands, of the original cartilage in the transplant site. For instance, in case of auricular reconstruction one should know which characteristics of the auricular cartilage are essential to make it both aesthetically acceptable as well as functional in the day-to-day use.

Before translation to the clinic, research in this field should be focused on generating cartilage of a sufficient size and form to be adequate for reconstruction. It is likely that scaffolds will be necessary to achieve this, as is discussed later on in this chapter. Moreover, the cartilage construct should have sufficient mechanical properties. Large animal studies focusing on reconstruction of nasal, auricular or tracheal defects with tissue-engineered cartilage seem feasible on the short term.

In conclusion, especially auricular chondrocytes have great potential for cartilage tissue engineering as these cells produce abundant cartilage matrix, the cartilage construct is stable and does not mineralize, and the cells can be harvested with minimally invasive techniques. Although upon expansion the cells retain characteristics of their donor-site, clinically this may not be relevant and tissue-engineered auricular cartilage constructs may be used without difficulties for cartilage reconstruction elsewhere in the body.

BMSC for cartilage tissue engineering

In chapter 6 we have discussed the significance and problems associated with the terminal differentiation of chondrogenically differentiated BMSCs, making these cells not appropriate for clinical application of cartilage tissue engineering in the near future. In this thesis we have described two methods (addition of dorsomorphin or PTHrP) to inhibit terminal differentiation of BMSCs. Especially dorsomorphin led to a significant reduction of a large variety of hypertrophic markers. In contrast to other studies on inhibition of terminal differentiation of BMSC (101; 102; 154; 155), in this thesis we compared gene expression of BMSCs with stable hyaline cartilage produced by articular chondrocytes. Because of this comparison, we consider the significant reduction of hypertrophic markers as promising but insufficient as it did not reach the low level of stable hyaline cartilage produced by articular

chondrocytes. Nevertheless the inhibiting effect of dorsomorphin on terminal differentiation as well as mineralization, by inhibiting Smad1/5/8 phosphorylation, seems to indicate an important role of this TGF- β signaling pathway. Perhaps by targeting this pathway more upstream, the specific ALK receptors that bind to TGF- β rather than the intra-cellular Smad signaling molecules, the signal can be balanced more subtle and a better control over terminal differentiation of BMSC can be attained. Another approach to inhibit terminal differentiation of BMSCs could be to affect different pathways simultaneously, for instance combining addition of PTHrP and dorsomorphin. Still, one must keep in mind that although a reduction in mineralization was observed in the presence of the inhibitor in vitro, that the inhibition of terminal differentiation may be only temporarily during the presence of the inhibitor. The cartilage construct may become terminally differentiated and mineralized when implanted in vivo without the inhibitor. As a consequence, for future studies on inhibition of terminal differentiation and mineralization of BMSC long-term in-vivo experiments are essential.

We have seen in chapter 5 that although the choice of TGF- β subtype did not have a clear effect on collagen X or VEGF, significantly less matrix mineralization was seen in the presence of TGF- β 1 than TGF- β 3. However, this was tested in our in vitro mineralization model, and should be repeated in in-vivo experiments. Still, this suggests that subtle differences in TGF- β subtype specific signaling pathways may play a role in terminal differentiation and mineralization. Therefore, gaining more insight into these TGF- β subtype-specific signaling pathways may reveal clues to inhibit terminal differentiation and mineralization.

We have demonstrated in chapter 6 that terminal differentiation is a gradual progression of chondrogenic differentiation towards cartilage that can mineralize, and is thus best characterized with a set of markers indicative of this gradual progression. As expression of hypertrophic markers almost overlaps with the onset of chondrogenesis, the hypertrophic phenotype may be predetermined when chondrogenic differentiation is still in its early stages in current culture conditions. These current culture conditions, and the rationale of inhibiting terminal differentiation, are derived from a traditional view that development of hyaline cartilage is nothing more than chondrocytes failing to go through endochondral ossification (5; 39; 173). However, chondrocytes in the growth plate are destined to become terminally differentiated and it therefore may not be the model of choice to study hyaline cartilage formation. Embryonic development of the interzone (174) suggest that to develop stable cartilage a subtle balance of chondrogenic stimulating as well as inhibiting signal is required, starting from the initial prechondrogenic differentiation. For cartilage tissue engineering this may mean that inhibiting terminal differentiation may not result in stable hyaline cartilage, if the right balance of signals has not been created from the start of culture on.

Therefore, clinical application in head and neck surgery of cartilage tissue engineering with BMSC seems to be out of reach in the near future. Instead, it looks as if the research field should go back to the beginning; what determines the hyaline or hypertrophic chondrogenic fate of undifferentiated stem cells during early development. Unless a better

control over chondrogenic differentiation of BMSC is achieved and a long-term stable cartilage construct can be guaranteed, clinical application of tissue-engineered cartilage constructs from BMSC is not recommended in head and neck surgery.

BMSC for bone tissue engineering

This inclination of BMSC to become terminally differentiated can be advantageous for bone tissue-engineering by generating bone through the endochondral route in contrast to direct intramembranous bone formation. Clinically, autologous bone grafts are frequently used to heal large bone defect but this has some major disadvantages (219). First, it requires the generation of a second surgical site with significant donor site morbidity. Secondly, the availability of autologous bone is limited (219). For this reason, a huge interest has emerged to generate autologous bone through tissue engineering. In contrast to bone, cartilage can survive in a defect area without direct connection to a vasculature. Additionally, hypertrophic cartilage produces growth factors to stimulates the in growth of new blood vessels (4). Therefore, taking advantage of the tendency of BMSCs to become terminally differentiated to generate bone via the endochondral route, seems a more promising approach than inducing BMSC directly into the bone lineage. This leads to a potential clinical approach for bone replacement by inducing BMSCs to form a hypertrophic cartilage construct in vitro which upon implantation in vivo is transformed into bone.

We have seen in chapter 7 that the tissue-engineered hypertrophic cartilage indeed excretes VEGF and active MMP, both important to induce vascularization. Moreover, significant vessel formation was observed after 4 weeks in-vivo implantation. However, no mineralization or bone formation was observed, possibly because of insufficient time allowed in vivo to remodel. Therefore, further experiments were warranted to optimize the in-vitro differentiation of BMSC and study the most favorable timing to implant the tissue-engineered constructs to generate bone in vivo through endochondral ossification. Indeed, additional experiments performed by our research group have demonstrated that prolonging the implantation period not only resulted in mineralization of the hypertrophic cartilage construct but that the construct was truly transformed into bone, including bone marrow (220). This endochondral ossification of in-vivo implanted hypertrophic cartilage constructs was also demonstrated in studies from other research groups using BMSC from human (221) or murine (222; 223) origin. The in-vitro terminal differentiation of BMSC could perhaps be further enhanced. For instance, in chapter 7 we have used TGF- β 2 while we have observed in chapter 5 that TGF- β 3 induces cartilage formation that is driven more towards endochondral ossification. A role may also be played in this fashion by FGF9, by stimulating matrix remodeling upon implantation as we have seen in chapter 3. Furthermore, future experiments should be conducted to increase the size of the tissue-engineered constructs as this is necessary for clinical application.

After optimization of the culture protocol, a large animal model could be used to test safety and efficacy of this novel clinical approach to tissue engineer autologous bone. Next, a clinical trial seems feasible on patients that have intra-oral defects that need bone reconstruction to allow dental implant placement. This defect is the perfect candidate to test

the tissue engineered constructs since it is, although challenging, small in size and because a biopsy is part of the routine procedure to generate space to place the implant. This allows histological evaluation of the newly formed tissue.

In conclusion, the hypertrophic differentiation of chondrogenically differentiated BMSC is generally considered as a problem for cartilage tissue engineering in this thesis. However, the hypertrophic cartilage that is the product of the current chondrogenic culture conditions for BMSC may actually be the optimal construct for bone tissue engineering. Although optimization of the current procedure is advisable, the clinical application of bone tissue engineering via endochondral ossification and the chondrogenic differentiation of BMSC seems feasible in the near future.

Important general issues for clinical translation of tissue-engineering

Inter-donor variability

In the experiments throughout this thesis we encountered a problem with inter-donor variability with BMSC, while only little variation was seen within pellets from the same donor. Furthermore, when BMSCs are used from the same donor in a later experiment, even when used in another passage number, the chondrogenic capacity is predictable in our experience. Inter-donor variability is often seen in other research groups as well (41; 224). In fact, often in this research field, donors are pre-screened and those that do not “perform” well are not included in the experiments. However, this poses a huge problem for further advances in tissue engineering and especially in clinical application as results from patient to patient may differ due to the chondrogenic capacity of their BMSCs. Moreover, the chondrogenic capacity cannot be predicted from for instance age, osteoarthritis etiology, BMSC yield or cell size (41). Therefore, further studies are necessary to understand and control inter-donor variability in chondrogenic differentiation potential.

Perhaps the condensation phase may play a role in inter-donor variability as the size of the condensation determines the size of the cartilage template (225). In embryology the mesenchymal condensation results from an active cell movement towards the centre of the outgrowing limb bud and is characterized by expression of adhesion molecules, such as N-cadherin (38; 225). In fact, during limb development functional N-cadherin is required for normal mesenchymal condensation to occur, as well as chondrogenesis, and its expression should be strictly regulated in both a quantitatively and temporally specific manner (38). Although we did not see an evident difference in immunohistochemical staining for N-cadherin during condensation between donors with different chondrogenic differentiation capacity (unpublished data), we have seen that FGFR2 is expressed during condensation and that addition of FGF9 (with a high binding affinity for FGFR2) during early differentiation results in a somewhat increased matrix production. As FGFR2 was indicated as a positive regulator in embryonic chondrogenesis, by influencing the size of the cartilage templates (96) it may indicate that a more precise control over FGF signaling may lead to more control over and predictability of cartilage formation by BMSC of different donors. Moreover, addition of FGF2 during expansion not only increased the proliferation rate but also delayed

loss of chondrogenic potential upon expansion (44). Therefore, the role of intrinsic FGF signaling in inter-donor variability and the interactions between TGF- β and FGF signaling would be interesting to study.

External control and observation of differentiation process

This thesis has made a first attempt to define chondrogenic differentiation of BMSCs as a gradual progression of steps in need of specific stimuli. However, for this approach to tissue-engineering it is essential to know during culturing how far the cells have progressed into their differentiation and thus which stimuli should be added. For the experiments in this thesis we decided beforehand at which time-intervals we would add certain growth factors or inhibitors, but did encounter differing results in outcome due to variability in the progress of differentiation of the BMSC from different donors. Therefore, more research is warranted to study markers, for instance in the medium, that can be used during culturing to define the progress of differentiation. BMSC during differentiation produce all kinds of endogenous signals, some of which are secreted in the medium. More knowledge of stage-specific production of these proteins may lead to a method in which one can predict during culturing from a sample of the medium how far the BMSC have progressed in their differentiation and thus which exogenous factors should be added. This would enhance the possibilities of stage-specific modulation of chondrogenic differentiation of BMSC and enable the concept of “developmental engineering” that was put forward by Lenas *et al* (216) and discussed earlier in this chapter.

Use of scaffold material

Traditionally tissue engineering is performed using bio-degradable scaffolds. However, the well-vascularized subcutaneous transplant site in head and neck surgery in combination of the high immune activity of the airways induces the risk of a strong inflammatory response to the scaffold and resorption of the construct (21). Indeed, in the head and neck region the use of (degradable) scaffolds to create a tissue-engineered cartilage construct has proved unsuccessful (23; 24). Therefore we have performed most of the experiments in this thesis without the use of scaffolds. However, for clinical application this means that one should tissue engineer a cartilage construct in vitro consisting of only cells and their ECM that has sufficient mechanical properties to perform its function from the moment of implantation on. In addition, creating a cartilage construct in a specific predefined shape and form (such as for regeneration of the auricle) could be facilitated by using a scaffold. Therefore, more research is warranted for cartilage tissue engineering in the head and neck region to design a flexible scaffold, that can be shaped into a prespecified form with long-term durability and that does not induce an inflammatory response. One must keep in mind for further studies that the potential benefits and limitations of a scaffold may depend on the site of transplantation.

Recently, clinical application of a tissue-engineered trachea in a 30 year old woman with end-stage bronchomalacia has made headlines (47). For this approach a decellularized trachea was used from a human donor as a scaffold and seeded with BMSC-derived

autologous chondrocytes externally and autologous epithelial cells internally. Research on pigs has demonstrated that this decellularized cartilage matrix is not sufficient on its own to reconstruct the matrix and that autologous cells are necessary for optimal graft survival (226). Although there were no clinical (47), immunological, or histological (226) signs of rejection despite the lack of immune suppression, this tissue-engineering approach relies on a allograft material as a scaffold. Therefore, patients reserving such transplants should be critically monitored and long-term follow-up is required before this approach can be implemented on a larger scale.

Regulatory and ethical issues

Strict regulation of any emerging biotechnology is required to protect patients. Until recently, medical products were regulated in the European Union either as medicinal products (such as drugs), or medical devices (such as pacemakers). Tissue-engineered products fell between these two categories. Therefore a new class of medicinal products has been defined in 2007: Advanced Therapy Medicinal Products (ATMP).

To ensure safety and efficacy the production of tissue engineered products for clinical application requires good manufacturing practice (GMP)-protocols focusing on for instance sterility, identity and purity. Therefore, it is important already in the current not-clinical research setting to start to develop rapid in-process characterization and controls of the product and to aim at reproducibility of the tissue-engineering process. Another requirement for ATMP is absolute traceability, not only of the original cells and the end product but also of all the substances that have come in contact with the cell during the manufacturing procedure.

Characterization of the cells and the end-product is especially important since pluripotent cell theoretically have the risk of ectopic differentiation or tumor production. Although with adult stem cells this appears not to be a problem in animal models, the human situation is more complex and may be different and the long-term effects of tissue-engineered products in humans are not yet known (227). For example, tumor formation may be more likely in patients that become immunodeficient in the long run.

In this thesis cartilage tissue engineering was studied from the perspective of future autologous application of the cells. In this approach the delay from harvesting of the stem cells to the implantation of the tissue may become an issue. As a consequence a broad interest has emerged in tissue engineering of-the-shelf products derived from allogenic stem cells (227). Moreover, the use of characterized allogenic stem cells would facilitate regulatory issues as described above and improve reproducibility and traceability. However, although it has been reported that some populations of stem cells have a low immunogenicity (228), it seems that differentiated cells display all the immunogenic characteristics of the cells in question, thereby inducing a potential immune reaction to a tissue-engineered allogenic off-the-shelf product (227).

An important hurdle in clinical translation of cartilage tissue engineering is the use of animal-derived components. For the experiments in this thesis chondrocytes and BMSC were expanded in fetal calf serum. This is routinely done nowadays, even for clinical

applications. However, fetal calf serum is prone for transmission of viral and prion related material and could also theoretically cause an immune response (229). Therefore, new rules and guidelines ATMPs have stated that xenogeny product free cultures are preferred. A good alternative in clinical application may be the use of autologous serum derived from peripheral blood. Although it has been demonstrated that autologous serum is not inferior to fetal calf serum (229), a minimum concentration of 10% is needed (230; 231). As a consequence, the use of autologous serum seems feasible only in a clinical setting where low numbers of BMSC are needed. When vast numbers of BMSC are needed, such as to tissue engineer a cartilage construct for reconstruction, in current protocols large amounts of culture media are needed and sufficiently large volumes of peripheral blood cannot be obtained from the patient. Furthermore, serum is a complex natural product that may vary from individual to individual thereby introducing an unpredictable variable in the culturing process (169). The same is true for the application of platelet-rich plasma (PRP) to the culture system (232). Therefore further research on defining optimal culture conditions, preferably serum-free, is necessary for clinical application of BMSC.

With respect to the ethics of tissue engineering, it has been suggested that one can roughly distinguish two perspectives (233). On the one hand, this technology could be considered morally good because it mimics nature. On the other hand, tissue engineering could be considered morally dangerous because it defies nature; bodies or organs constructed in the laboratory are seen as unnatural. Nevertheless, tissue engineering with BMSC or chondrocytes does not evoke the same ethical concerns as with embryonic stem cells. Generally there seems to be no discussion in the media about so-called adult stem cells, since the potential benefit appears to be very high and the utilization of the patient's own cells poses no serious ethical conflict (234). Still two issues remain to be discussed. First, animal experimentation seems necessary for future development and clinical translation of tissue engineering to study safety and efficacy of the procedure before clinical trials. Then again, research with (adult) stem cells may lead to organotypic models that may be able to replace animal models in some areas of research. Secondly, the development of tissue engineering techniques requires tremendous funding and potential future clinical applications will most probably be expensive. This may cause a serious public debate, especially when not-lifesaving or esthetical procedures are considered.

There is no doubt that numerous scientific developments have transformed our lives in a way that would have been unthinkable of just a century ago. Whether tissue engineering research will have the same impact as other scientific achievements cannot be foreseen, but the promise seems so great that it seems wise to consider seriously how best to proceed research in a manner that is perceptive to any ethical objections.



10

NEDERLANDSE SAMENVATTING

Kraakbeen heeft een unieke weefselopbouw in die zin dat het geen zenuwen, bloed- of lymfevaten bevat en slechts uit één celsoort bestaat: de kraakbeencel oftewel chondrocyt. Kraakbeencellen maken in totaal slechts 1-5% van het kraakbeen uit, omdat ze zijn ingebed in een grote hoeveelheid tussenstof of extracellulaire matrix (ECM). Deze extracellulaire matrix wordt door de kraakbeencellen wordt geproduceerd en bepaalt de eigenschappen van kraakbeen. Een belangrijk eiwit in deze extracellulaire matrix is collageen II; het geeft het kraakbeen zijn trekkracht geeft. Proteoglycanen, hydrofiele moleculen die water aantrekken in de matrix, zorgen voor de drukkracht van kraakbeen. Op basis van de moleculaire compositie van deze extracellulaire matrix wordt kraakbeen klassiek ingedeeld in 3 subtypes: hyalien (gewricht, neus), elastisch (oorschelp, strottenklepje) en fibreus (meniscus, tussenwervelschijven). Een vierde subtype kraakbeen kan ook gedefinieerd worden, hoewel het bestaan hiervan tijdelijk is: hypertroof of terminaal gedifferentieerd kraakbeen. Dit type kraakbeen is als het ware een tussenstation in een proces dat endochondrale verbening wordt genoemd; hierbij wordt kraakbeen omgezet in bot, bijvoorbeeld in de groeischijf. Alle lange beenderen in ons lichaam worden op deze manier gevormd tijdens de embryologie. Hierbij wordt aanvankelijk kraakbeen gemaakt, waarna de kraakbeencellen zich verder differentiëren (doorontwikkelen) tot hypertrofe kraakbeencellen. Hypertrofe kraakbeencellen produceren andere eiwitten (collageen X, VEGF en MMP13) dan gewone kraakbeencellen. Dat leidt tot mineralisatie, ingroei van bloedvaten en uiteindelijk tot de omzetting van het kraakbeen in bot.

Kraakbeen wordt in principe alleen tijdens de embryologie gevormd en het kan zich in het lichaam niet herstellen of regenereren. Kraakbenige defecten worden in de keel-, neus-, en oorheelkunde (KNO) daarom gereconstrueerd met lichaamseigen oftewel autoloog kraakbeen. Zo kan een neus gereconstrueerd worden met kraakbeen uit de oorschelp, of wordt een nieuwe oorschelp gemaakt met behulp van ribkraakbeen. Maar zeker bij grote of herhaalde ingrepen is de beschikbaarheid van autoloog kraakbeen beperkt. Tissue-engineering van autoloog kraakbeen, oftewel het in een laboratorium maken van kraakbeen van lichaamseigen cellen, zou daarvoor een goed alternatief kunnen zijn.

Verschillende cellen kunnen hiervoor gebruikt worden. De meest voor de hand liggende keuze is de kraakbeencel zelf. Nadat deze cellen uit het kraakbeen worden geïsoleerd, bijvoorbeeld uit de neus of het oor, kunnen ze in het laboratorium gestimuleerd worden om zich te vermeerderen (expanderen) totdat een voldoende groot celtaantal wordt bereikt. Deze geëxpandeerde chondrocyten hebben echter hun kraakbenige eigenschappen verloren en kunnen geen goede kraakbenige extracellulaire matrix meer produceren. Daarom moeten ze eerst opnieuw worden gestimuleerd (bijvoorbeeld met groeifactoren) om te redifferentiëren, om met andere woorden opnieuw “echte” kraakbeencellen te worden.

Een andere celsoort die gebruikt kan worden voor het tissue-engineering van kraakbeen is de volwassen stamcel die uit verschillende weefsels kan worden geïsoleerd. Omdat deze cellen geogst kunnen worden zonder een (nieuw) kraakbenig defect te maken en omdat zij zeer uitgebreid kunnen expanderen worden volwassen stamcellen als een zeer veelbelovende celsoort voor tissue-engineering gezien. Ten behoeve van dit proefschrift werden uit het beenmerg geïsoleerde volwassen mesenchymale stam cellen (BMSC)

bestudeerd. Bij de start van dit promotie onderzoek was reeds bekend hoe deze cellen gestimuleerd konden worden om kraakbeen te maken. Echter, het kraakbeen dat hierbij gevormd wordt is van het hypertrofe subtype en mineraliseert wanneer het in het lichaam wordt geïmplant, waardoor het niet geschikt is voor kraakbenige reconstructies.

Het doel van het onderzoek was om meer controle te krijgen over het proces van kraakbeen tissue-engineering met als uiteindelijk doel het maken van hyalien kraakbeen dat niet mineraliseert wanneer het in het lichaam wordt geïmplant. De achterliggende gedachte van dit promotie onderzoek is dat door bestudering van de ontwikkeling van kraakbeen in de embryologie, nieuwe inzichten verkregen kunnen worden die leiden tot meer controle over kraakbeen tissue-engineering.

In **hoofdstuk 2** wordt het onderzoek beschreven naar het gebruik van geëxpandeerde oor- en neuskraakbeencellen voor kraakbeen tissue-engineering. Zowel oor- als neuskraakbeencellen produceerden kraakbeen met collageen X, een teken van hypertrofie. Hoewel hypertrofie van kraakbeen kan leiden tot mineralisatie en verbening van het kraakbeen, mineraliseerden de verkregen kraakbenige constructen van oor- en neuskraakbeencellen niet. Dit werd getest in een in-vitro model en door de constructen in vivo in een muis te implanteren. Vervolgens werd een zogenoemde "DNA-microarray" uitgevoerd op beide celsoorten. Hierbij kunnen genen tegelijkertijd worden bestudeerd en wordt gemeten welke actief tot expressie komen. Dit geeft aan welke processen in de cel actief zijn. Door genen te bestuderen die significant verschillend tot expressie komen tussen oor- en neuskraakbeencellen komen we meer te weten over het fundamentele verschil tussen deze cellen. Er bleek geen verschil te zijn in expressie van genen waarvan reeds bekend was dat ze bepalen hoe goed een cel kraakbeen kan maken (collageen II, FGFR3, BMP2, ALK1). Desondanks viel wel iets anders op dat kan verklaren waarom oorkraakbeencellen meer kraakbeen kan maken dan neuskraakbeencellen. In oorkraakbeencellen is namelijk meer activiteit van de genen voor BMP5 en IGF1, groeifactoren met een stimulerende werking op kraakbeen, en minder activiteit van de genen voor MMP13 en ADAMTS5, enzymen die juist kraakbeen afbreken. Verder bleek dat ook geëxpandeerde chondrocyten, die de mogelijkheid kwijt zijn om uit zichzelf kraakbeen te maken, toch nog veel karakteristieken te hebben behouden van het kraakbenige subtype waaruit ze ooit zijn geoogst. Dit betekent dat wanneer voor het kweken van kraakbeen geëxpandeerde kraakbeencellen worden gebruikt van een donor-site van een ander kraakbenig subtype, het gekweekte kraakbeen ook moleculair zal verschillen van het kraakbeen wat gereconstrueerd moet worden.

In de volgende hoofdstukken is het onderzoek gericht op de uit het beenmerg geïsoleerde volwassen mesenchymale stamcellen (BMSC). Zoals eerder gezegd was reeds bekend dat in de huidige kraakbenige kweekprotocollen deze stamcellen hypertroof kraakbeen vormen en dat dit kraakbeen na implantatie in het lichaam mineraliseert. Dit proces van hypertrofie en mineralisatie van volwassen stamcellen wordt in de literatuur ook wel vergeleken met het proces van embryonale endochondrale verbening van de ledematen waarin hypertroof kraakbeen wordt omgezet in bot. Daarom worden in **hoofdstuk 3** deze

processen met elkaar vergeleken. In beide processen bleken drie achtereenvolgende stadia van kraakbeenontwikkeling aangetoond te kunnen worden: condensatie, differentiatie en hypertrofie. Vervolgens werd gekeken naar de aanwezigheid van de 3 verschillende subtypen fibroblast groei factor receptoren (FGFR). Deze receptoren op de celmembraan kunnen geactiveerd worden door groeifactoren waarna ze bepaalde processen in de cel aansturen. Juist deze FGFR werden bestudeerd, omdat bekend is dat mutaties in de onderliggende genen kunnen zorgen voor ernstige afwijkingen in de endochondrale verbening van de lange beenderen, wat kan leiden tot o.a. dwerggroei. Er bleek in beide processen een duidelijke stadiumspecifieke expressie van deze receptoren te zijn. Stimulering met groeifactoren van deze verschillende receptoren bleek in het algemeen een negatief effect te hebben op kraakbeenvorming van volwassen stamcellen. Het effect was echter wel afhankelijk van het subtype receptor dat werd gestimuleerd en in welke fase (condensatie, differentiatie of hypertrofie) dit precies gebeurde. Echter, een meer specifieke stimulatie van alleen FGFR2 beperkt tot de condensatiefase zou zelfs mogelijk stimulerend kunnen zijn voor de kraakbeenproductie. Hoewel men in tissue-engineering gewend is het effect van een stof gedurende de hele kweek te bestuderen, lijken de resultaten uit deze studie aan te tonen dat een meer stadiumspecifieke benadering aandacht behoeft omdat het de kans op succes vergroot.

In **hoofdstuk 4** hebben we deze stadiumspecifieke benadering daarom voortgezet. Dit keer werd het effect van TGF- β bestudeerd, de groeifactor die wordt gebruikt om volwassen stamcellen te stimuleren tot kraakbeenproductie. TGF- β kan via twee verschillende signaalroutes de cel stimuleren. In deze studie werd aangetoond dat in hyalien kraakbeen de signalering beperkt blijft tot de Smad2/3 route, terwijl in hypertroof kraakbeen signalering plaatsvindt via zowel de Smad2/3 als de Smad1/5/8 route. Door stadiumspecifiek de Smad2/3 en Smad1/5/8 route te remmen, kwamen we tot de conclusie dat beide routes in de vroege kraakbeenontwikkeling noodzakelijk zijn. In de late kraakbeenontwikkeling bleken deze routes echter verschillende functies te hebben. Door Smad2/3 te remmen werd verdere kraakbeenproductie geremd. Door Smad1/5/8 te remmen in de late kraakbeenontwikkeling werd de hypertrofie van volwassen stamcellen onderdrukt en werd er wel een kraakbenige extracellulaire matrix geproduceerd. Bovendien bleek ook dat de mineralisatie werd geremd in een in-vitro model.

Er bestaan 3 verschillende subtypes van TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3). Zoals eerder vermeld wordt TGF- β gebruikt om volwassen stamcellen te stimuleren tot kraakbeenvorming. In verschillende laboratoria worden echter verschillende subtypes gebruikt. Bovendien wordt in de literatuur vaak geen onderscheid gemaakt tussen deze subtypes wanneer resultaten van verschillende studies worden vergeleken. Daarom worden in **hoofdstuk 5** de verschillende TGF- β subtypes direct met elkaar vergeleken, waarbij de hoeveelheid kraakbeenproductie en de mate van hypertrofie en mineralisatie onderzocht werd. De keuze voor TGF- β subtype bleek niet bepalend voor de hoeveelheid kraakbeenproductie en er werd geen verschil gezien in de expressie van hypertrofie markers. Er werd wel een verschil gezien in de mate van mineralisatie; toediening van TGF- β 1 leidde tot significant minder mineralisatie dan TGF- β 3. Daarom lijkt voor verdere

experimenten TGF- β 1 het meest geschikt voor tissue-engineering van kraakbeen, terwijl TGF- β 3 meer geschikt is voor tissue-engineering van bot.

In **hoofdstuk 6** wordt een uitgebreide literatuurstudie beschreven, aangevuld met eigen data, om de vraag te beantwoorden of het sowieso mogelijk is om stabiel hyalien kraakbeen te maken uit volwassen stamcellen. Allereerst werd onderzoek gedaan naar hypertrofie en werd gezien dat dit in feite een gradueel proces is waarin kraakbeen zich in verschillende stappen ontwikkelt tot kraakbeen dat kan mineraliseren. In de huidige kraakbenige kweekprotocollen van volwassen stamcellen worden hypertrofie markers al gemeten voordat kraakbeenproductie heeft plaatsgevonden. Dit zou kunnen betekenen dat de uiteindelijke hypertrofie van volwassen stamcellen al in een zeer vroege fase wordt bepaald. De huidige kweekprotocollen en de methodes om hypertrofie te remmen komen voort uit kennis van endochondrale verbening van de lange beenderen. Het lot van deze kraakbeencellen is echter hypertrofie en mineralisatie. Daarom lijkt dit minder geschikt om de ontwikkeling van hyalien kraakbeen te bestuderen. Onderzoek naar de embryonale ontwikkeling van hyalien gewrichtskraakbeen laat zien dat al in een zeer vroege fase een subtiele balans nodig is van stimulerende en remmende factoren. Voor kraakbeen tissue-engineering zou dit kunnen betekenen dat het remmen van hypertrofie niet voldoende is om hyalien kraakbeen te maken, wanneer niet vanaf het begin van de kweek de juiste balans aan signalen wordt gegeven.

Hoewel de hypertrofie en daarmee de endochondrale verbening van volwassen stamcellen als nadelig wordt gezien in kraakbeen onderzoek, zou dit een alternatieve methode kunnen zijn om bot te maken. Bij bot tissue-engineering worden volwassen stamcellen in het algemeen direct gestimuleerd tot botvorming (intramembraneuze botvorming). Echter, in tegenstelling tot kraakbeen heeft bot behoefte aan bloedvoorziening en de resultaten van bot tissue-engineering zijn hierdoor teleurstellend. In **hoofdstuk 7** hebben we daarom bestudeerd of het mogelijk is om bot te maken van volwassen stamcellen via endochondrale verbening. Dit zou kunnen leiden tot een nieuwe aanpak van bot tissue-engineering, waarbij hypertroof kraakbeen in het lab gevormd wordt, wat vervolgens in het lichaam wordt geïmplanteed waar het ter plaatse op een natuurlijke manier wordt omgezet tot bot. In tegenstelling tot de volwassen stamcellen die direct tot bot werden gestimuleerd, produceerden de stamcellen die kraakbenig werden gestimuleerd verschillende moleculen die stimulerend zijn voor de vorming van bloedvaten. Volwassen stamcellen die direct tot bot werden gestimuleerd vormden een gemineraliseerd construct, echter de cellen in het construct overleefden implantatie in een muis niet. Volwassen stamcellen die kraakbenig werden gestimuleerd vormden hypertroof kraakbeen dat na implantatie in een muis vitaal bleef en waarin bloedvaten werden gezien. Er werd geen mineralisatie of botvorming in deze constructen gezien, waarschijnlijk omdat het kraakbeen niet voldoende lang geïmplanteed was. Deze gegevens laten echter wel zien dat hypertroof kraakbeen gevormd uit volwassen stamcellen in-vivo bloedvaten aantrekt waardoor het beter kan overleven. Inmiddels heeft vervolgonderzoek van onze onderzoeksgroep aangetoond dat langere in-vivo implantatie leidt tot daadwerkelijke omzetting van dit kraakbeen in bot.

Conclusie

In dit proefschrift wordt aangetoond dat geëxpandeerde oor- en neuskraakbeencellen kraakbeen vormen dat niet mineraliseert. Daarom zijn deze cellen veelbelovend voor de klinische toepassing van kraakbeen tissue-engineering. Dit in tegenstelling tot volwassen stamcellen die hypertroof kraakbeen maken dat na implantatie mineraliseert. Hoewel dit kan leiden tot een nieuwe aanpak voor tissue-engineering van bot, is de resulterende hypertrofie nadelig voor kraakbeen tissue-engineering. De kraakbeenontwikkeling van volwassen stamcellen verloopt via verschillende stadia (condensatie, differentiatie en hypertrofie) en lijkt op endochondrale verbening van de ledematen tijdens de embryologie. Daarom is een stadiumspecifieke benadering nodig om meer controle te krijgen over de kraakbeenvorming van volwassen stamcellen. De huidige kweekprotocollen en methodes om hypertrofie te remmen komen voort uit kennis van de ontwikkeling van de lange beenderen. Maar het lot van deze kraakbeencellen is hypertrofie en mineralisatie. Daarom lijkt dit eigenlijk geen geschikt model om de ontwikkeling van hyalien kraakbeen te bestuderen. Onderzoek naar de embryonale ontwikkeling van hyalien gewrichtskaakbeen laat zien dat al in een zeer vroege fase een subtiele balans nodig is van stimulerende en remmende factoren. Voor kraakbeen tissue-engineering zou dit kunnen betekenen dat het remmen van hypertrofie niet voldoende is om hyalien kraakbeen te maken, indien niet vanaf het begin van de kweek de juiste balans aan signalen wordt gegeven.

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PhD Portfolio Summary

Name PhD student: C.A. Hellingman	PhD period: 2006-2012
Erasmus MC Department: Otorhinolaryngology	Promotor(s): R.J. Baatenburg de Jong
Research school: -	Supervisor: G.J.V.M. van Osch

1. PhD training

	Year	Workload (Hours/ECTS)
General academic skills		
- Biomedical English Writing and Communication	2007	4
- Research Integrity (lectures only)	2008	
Research skills		
- Statistics	2008	2
In-depth courses (e.g. Research school, Medical Training)		
- Head and Neck Anatomy	2006/2010	2
Presentations		
		5
- Research group meeting Orthopaedics/Otorhinolaryngology (7x)	2006-2009	
- CTCR research group (6x)	2008-2009	
- Otorhinolaryngology department		
Research meeting (2x)	2009/2011	
Science-day (2x)	2006/2008	
- User committee Dutch Program of Tissue-Engineering (2x)	2007/2009	
International conferences – attendance only		
		5
- Tissue Engineering and Regenerative Medicine (TERMIS), Rotterdam, The Netherlands	2006	
- Dutch Program of Tissue Engineering (DPTE), Noordwijkerhout, The Netherlands	2006	
International conferences – poster presentation		
- Donor cell source for cartilage tissue engineering in otorhinolaryngology, European Society of Paediatric Otorhinolaryngology (ESPO), Budapest, Hungary	2008	
- Chondrogenesis begins with condensation: tissue engineering recapitulates embryonic development, ESPO, Budapest, Hungary and DPTE, Noordwijkerhout, The Netherlands	2008	
- Fibroblast Growth Factor Receptors in in-vitro and in-vivo chondrogenesis: tissue engineering recapitulates embryonic development, Gordon Conference on Craniofacial Morphogenesis and Tissue Engineering, Il Ciocco, Italy	2008	
- Fibroblast Growth Factor Receptors and chondrogenic phenotype of bone-marrow derived stromal cells, DPTE, The Netherlands	2008	
International conferences – podium presentation		
- Fibroblast Growth Factor Receptors in chondrogenesis, DPTE, Noordwijkerhout, The Netherlands	2007	
- Fibroblast Growth Factor Receptors in chondrogenesis, TERMIS, London, United Kingdom	2007	

- Fibroblast Growth Factor Receptors in chondrogenese, KNO vergadering, Nieuwegein, The Netherlands	2007	
- Fibroblast Growth Factor Receptors in chondrogenesis, ESPO, Budapest, Hungary	2008	
- Cartilage tissue engineering: comparing different cells sources, DPTE, Noordwijkerhout, The Netherlands	2008	
- Characterization of auricular and nasoseptal chondrocytes as donor cell sources for cartilage regenerative medicine, European Conference for Scientists and Plastic Surgeons, Rotterdam, TheNetherlands	2009	
- Smad signaling determines chondrogenic differentiation of bone-marrow derived mesenchymal stem cells, NVCB, Rotterdam	2009	
- Auriculaire en neusseptum chondrocyten als donor-cellen voor kraakbeen tissue-engineering, KNO vergadering, Nieuwegein, The Netherlands	2009	
International conferences – local organising committee		
- European Skull Base Society, Rotterdam, The Netherlands	2009	
Seminars and workshops		
- Workshops PhD-day	2008	
Didactic skills		
- Didactic skills (teach the teacher- preclinical)	2008	1
Other		
- Travel award TERMIS	2007	
- Science café	2007-2009	
- Attending literature meetings (once a week, one hour)		1
- Attending research group meetings (once a week, 2 hours)		1
- Attending research meeting otorhinolaryngology (once a month)		
2. Teaching activities		
	Year	Workload (Hours/ECTS)
Supervising practicals and excursions		
- Tutor first year medical student (including training)	2008	3
- Supervising 4rd year medical students ENT physical examination	2006-2009	
Supervising Master's theses		
- Fibroblast Growth Factor Receptor expression during in vitro redifferentiation of hyaline and elastic chondrocytes, Inez Slagt, Medical student	2007	5
- Transforming Growth Factor – beta: subtype dependent chondrogenic differentiation in mesenchymal stromal cell cultures, Froukje Cals, Medical student	2008-2009	5

Publications

Hellingman CA, van den Bergh WM, Beijer IS, van Dijk GW, Algra A, van Gijn J, Rinkel GJ. Risk of rebleeding after treatment of acute hydrocephalus in patients with aneurysmal subarachnoid hemorrhage. *Stroke* 2007; 38: 96-9.

Hellingman CA, Dunnebie EA. Cochlear implantation in patients with acute or chronic middle ear infectious disease: a review of the literature. *Eur Arch Otorhinolaryngol* 2009; 266: 171-6.

Farrell E, van der Jagt OP, Koevoet W, Kops N, van Manen CJ, **Hellingman CA**, Jahr H, O'Brien FJ, Verhaar JA, Weinans H, van Osch GJ. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? *Tissue Eng Part C Methods* 2009; 15: 285-95.

Hellingman CA, Koevoet W, Kops N, Farrell E, Jahr H, Liu W, Baatenburg de Jong RJ, Frenz DA, van Osch GJ. Fibroblast growth factor receptors in in vitro and in vivo chondrogenesis: relating tissue engineering using adult mesenchymal stem cells to embryonic development. *Tissue Eng Part A* 2010; 16: 545-56.

Hellingman CA, Verwiel ET, Slagt I, Koevoet W, Poublon RM, Nolst-Trenité GJ, de Jong RJ, Jahr H, van Osch GJ. Differences in cartilage forming capacity of expanded human chondrocytes from ear and nose and their gene expression profiles. *Cell Transplant* 2011; 20: 925-40.

Hellingman CA, Blaney Davidson E, Koevoet W, Vitters EL, van den Berg WB, van Osch G, van der Kraan PM. Smad signaling determines chondrogenic differentiation of bone-marrow derived mesenchymal stem cells: Inhibition of Smad 1/5/8P prevents terminal differentiation and calcification. *Tissue Eng Part A* 2011; 17:1157-67.

Cals FL, **Hellingman CA**, Koevoet W, Baatenburg de Jong RJ, van Osch GJ. Effects of transforming growth factor- β subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells. *J Tissue Eng Regen Med* 2011 [Epub ahead of print]

Hellingman CA, Koevoet W, van Osch GJ. Can one generate stable hyaline cartilage from adult mesenchymal stem cells? Accepted for publication in *J Tissue Eng Regen Med*.

Curriculum vitae

Catharine Antoinette (Katja) Hellingman werd geboren op 18 juli 1979 in het St. Mary's Hospital Vunapope te Kokopo, Papoea-Nieuw-Guinea. Dankzij het tijdsverschil werd haar geboorte al op 17 juli in Nederland gevierd. Zij groeide op in Bussum waar ze op de Hoeksteen basisonderwijs volgde. Vervolgens ging zij naar het St. Vitus College, waar zij in 1997 haar gymnasiumdiploma behaalde. Na afronding van het propedeuse examen farmacie werd zij in 1998 alsnog ingeloot voor de studie geneeskunde aan de Universiteit Utrecht. Tijdens haar studie heeft zij veel stages en co-schappen in het buitenland gedaan. Haar voorliefde voor KNO-heelkunde ontstond tijdens het betreffende co-schap in het Massachusetts Eye and Ear Infirmary in Boston, USA. Haar keuze co-schap doorliep zij op de afdeling KNO in het UMC Utrecht. Onder begeleiding van dr. Dunnebier deed zij tevens onderzoek naar cochleaire implantatie bij patiënten met otitis media. Eerder tijdens haar wetenschapsstage bij neurochirurgie werd haar belangstelling voor wetenschappelijk onderzoek al gewekt. Het genoemde onderzoek bij dr. Dunnebier heeft deze belangstelling verder versterkt. Beide onderzoeken leidden tot publicaties. Na het afronden van het artsexamen in 2005, werkte zij een half jaar als AGNIO Chirurgie in het Ziekenhuis Gelderse Vallei te Ede. Vervolgens aanvaardde zij in 2006 een functie als AGNIO op de afdeling KNO in het Erasmus MC te Rotterdam. Later dat jaar startte zij het wetenschappelijk onderzoek dat uiteindelijk leidde tot dit proefschrift. Dit onderzoek verrichtte zij onder leiding van prof. R.J. Baatenburg de Jong en dr. G.J.V.M. van Osch op de afdeling KNO in nauwe samenwerking met de afdeling Orthopedie. In 2009 keerde zij full-time terug naar de kliniek. Daar is zij nu werkzaam als AIOS KNO.

Dankwoord

Het is zover, mijn promotieonderzoek heeft uiteindelijk geleid tot een concreet boek. Maar ik ben me ervan bewust dat dit alleen tot stand kon komen door de inzet van en samenwerking met vele anderen. Ik wil hierbij graag al die mensen bedanken, die op zeer uiteenlopende manieren een bijdrage hebben geleverd aan mijn “boekje”.

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