THE EFFECTS OF BONE MORPHOGENETIC PROTEIN 6, DEXAMETHASONE AND HUMAN SERUM ON CHONDROGENIC DIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS *IN VITRO*

Master's Thesis
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ABSTRACT

Background and aims: Damaged articular cartilage is a common cause of pain, lowered mobility, lowered working capability and reduced quality of life. Injured articular cartilage hardly heals and the current treatments have only proven applicable for a fraction of patients, hence the great demand for a novel treatment. Mesenchymal stem cells from human adipose tissue are self-proliferative and they can undergo multilineage differentiation. In this study, we aim to examine the chondrogenic culture conditions and media compositions for adipose stem cells and to seek possible improvements.

Methods: Human adipose stem cells were chondrogenically differentiated with six media containing various combinations and doses of putative chondrogenic bioactive factors, namely TGF-β1, BMP-6, dexamethasone and human serum. After 2 and 4 weeks in three-dimensional high cell-density aggregate culture the expression of osteogenesis- and chondrogenesis related genes was analyzed with commercial Q-RT-PCR array plates. Histological stainings and immunohistochemistry were used to verify the accumulation of cartilaginous extracellular matrix.

Results: None of the media supplements proved to induce chondrogenesis exclusively. BMP-6 resulted in mixed chondrogenic and osteogenic differentiation, although a high dose of BMP-6 did suppress the hypertrophic marker *COL10A1*. Dexamethasone boosted the expression of a variety of ECM related genes, namely *COL1A2*, *COL3A1*, *COL11A1*, *COL12A1*, *COMP*, *SERPINH1* and *ALP*. When combined, a high dose of BMP-6 and dexamethasone resulted in putative adipogenic differentiation. Human serum proved unnecessary, or even detrimental for chondrogenesis.

Coclusions: We conclude that the growth factor combinations assayed here proved suboptimal for chondrogenesis; the cells underwent concomitant osteogenesis and even putative adipogenesis was observed. However, with further refinement of the chondrogenic culturing conditions the differentiation of human adipose stem cells should be even more controllable, and accordingly cartilage tissue engineering with adipose stem cells would seem feasible.

Key words: Tissue engineering, Mesenchymal stem cells, Chondrogenesis, TGF-beta superfamily proteins, Bone morphogenetic protein 6, Hypertrophy.

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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Nivelrustovauriot ovat yleisiä ja ne aiheuttavat kipua, liikunta- ja työkyvyn huonontumista sekä elämänlaadun heikkenemistä. Nivelrustovaurio paranee heikosti, eivätkä nykyiset hoidot sovellu kuin murto-osalle potilaista; kysyntä uudelle rustovaurionhoitomenetelmälle on erittäin suuri. Ihmisen rasvakudoksen mesenkymaaliset kantasolut ovat sekä jakautuvia että multipotentiaaleja. Tämän opinnäytetyön tavoite on tutkia ja kehittää rasvakudoksen kantasolujen rustoerilaistukseen sovellettavia elatusolosuhteita.

Tutkimusmenetelmät: Ihmisen rasvakudoksen kantasoluja rustoerilaistettiin kuudessa erilaisessa elatusliuoksessa. Elatusliuoksiin lisättiin seuraavia oletetusti rustoerilaistavia bioaktiivisia tekijöitä erilaisina yhdistelminä ja pitoisuuksina: TGF-β1, BMP-6, deksametasoni, ja ihmisseerumi. Soluja erilaistettiin 2 ja 4 viikkoa korkeassa solutiheydessä kolmiulotteisina aggregaatteina, minkä jälkeen rusto- ja luuerilaistukseen liittyvien geenien ilmentyminen analysoitiin Q-RT-PCR *array*-tekniikalla. Rustolle tyypillisen soluväliaineen muodostuminen arvioitiin histologisilla ja immunohistokemiallisilla värjäystekniikoilla.

Tutkimustulokset: Testatut bioaktiiviset tekijät eivät johtaneet yksinomaan solujen rustoerilaistumiseen. BMP-6 aiheutti sekä rusto- että luuerilaistumista, joskin suuri BMP-6-annos vaimensi hypertrofiamarkkerina tunnetun *COL10A1*:n ilmentymisen. Deksametasoni lisäsi useiden soluväliaineen tuottoon liittyvien geenien ilmentymistä, mukaan lukien *COL1A2*, *COL3A1*, *COL11A1*, *COL12A1*, *COMP*, *SERPINH1* ja *ALP*. Samanaikainen suuri BMP-6-annos ja deksametasoni johtivat ilmeisesti solujen rasvaerilaistumiseen. Ihmisseerumi puolestaan osoittautui tarpeettomaksi tai jopa haitalliseksi rustoerilaistukselle.

Johtopäätökset: Analysoidut erilaistusolosuhteet eivät osoittautuneet optimaalisiksi, sillä samanaikaista rusto-, luu- ja mahdollisesti jopa rasvaerilaistumista havaitiin. Toisaalta olosuhteita edelleen hienosäätämällä ihmisen rasvakudoksen kantasolujen erilaistumista voitaisiin kyetä hallitsemaan täsmällisemmin, jolloin rustovaurion kudosteknologinen hoito ihmisen rasvan kantasoluilla saattaisi olla mahdollista.

Avainsanat: Kudosteknologia, Mesenkymaaliset kantasolut, Rustoerilaistus, TGF-beta superperheen proteiinit, Luun morfogeneettinen proteiini 6, Hypertrofia.

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Abbreviations used in the text

3-D = Three-dimensional
AB = Alcian blue
ACI/ACT = Autologous chondrocyte implantation/transplantation
AGC = Aggrecan
ASC = Adipose stem cell
ALP = Alkaline phosphatase (liver/bone/kidney isoenzyme in this study)
BMSC = Bone marrow-derived mesenchymal stem cell
BMP-6 = Bone morphogenetic protein 6 (protein)
BMP-6= Bone morphogenetic protein 6 (gene)
BMPR = Bone morphogenetic protein receptor
CBFA1 = Core-binding factor α1
CD = Cluster of differentiation
COL1A1 = Collagen type 1 α-chain 1 -gene
COMP = Cartilage oligomeric matrix protein
CRTL1 = Cartilage link protein 1
C-terminal = Referring to the carboxyl terminus of a peptide sequence
CM = Chondrogenic medium
DAB = Diaminobenzidine
DEX = Dexamethasone
DMEM = Dulbecco's modified eagle medium
DPBS = Dulbecco's phosphate buffered saline
EGF = Epidermal growth factor
EM = Expansion medium
ER = Endoplasmic reticulum
ERK = Extra-cellular signal related kinase
FACIT = Fibril associated collagens with interrupted triple-helices
FACS = Fluorescence activated cell sorting
FBS = Foetal bovine serum
FGF = Fibroblast growth factor
GAG = Glycosaminoglycan
GOI = Gene of interest
hAC = Human articular chondrocyte
hASC = Human adipose stem cell
HS = Human serum

HSC = Haematopoietic stem cell

HSF1 = Heat-shock factor 1

IGF = Insulin-like growth factor

ISCT = International society for cellular therapy

MACI/MACT = Matrix-induced autologous chondrocyte implantation/transplantation

MACS = Magnetic activated cell sorting

MEKK1 = Mitogen-activated protein kinase kinase kinase 1

MSC = Multipotent stromal/stem cell

N-CAM = Neural-cell adhesion molecule

N-terminal = Referring to the amino terminus of a peptide sequence

P1 = Passage 1 (cell culture)

PBS = Phosphate buffered saline

PDGF-BB = Platelet-derived growth factor beta polypeptide

PG = Proteoglycan

PTHrP = Parathyroid hormone related protein

Q-RT-PCR = Quantitative real-time polymerase chain reaction

ROCK = Rho-associated, coiled-coil containing protein kinase

RUNX2 = Runt-related transcription factor 2

SARA = SMAD Anchor for receptor activation

sGAG = Sulphated glycosaminoglycan

SMAD = Combined acronym from *Caenorhabditis elegans* SMA-protein and *Drosophila melanogaster*' MAD-protein (mother against decapentaplegic).

SOX9 = Sex determining region Y - box 9

STRAP = Serine/threonine kinase receptor associated protein

TAK1 = TGF-β activated kinase 1

TB = Toluidine blue

TGF-β = Transforming growth factor β

TGF-βR = Transforming growth factor β receptor

TRAP-1 = TGF-βRI associated protein 1

TRIP-1 = TGF-βR interacting protein 1

Vol-% = Volume percentage

WNT-7 = Wingless-type MMTV integration site, member 7

1. Introduction

Articular cartilage defects affect a wide range of patients, especially the elderly, and the burden of damaged cartilage is great for both individual patients and for the society. Loss of joint functionality and reduced mobility quickly translate to diminished quality of life and considerable socio-economical costs due to lost work efficiency (Kiviranta and Vasara 2004; Goldring and Goldring 2007; Säämänen *et al.* 2008; Strauss *et al.* 2011). Damaged articular cartilage has limited healing capacity due to lack of vascularization and low number of tissue-residing cells, which furthermore lack extensive proliferative capabilities. Hence, articular cartilage defects tend to be degenerative and they predispose the joint to osteoarthritis. Surgical treatment options have provided some success in restoring joint function, but the newly formed repair tissue often has inferior biomechanical properties when compared to healthy articular cartilage (Kiviranta and Vasara 2004; Strauss *et al.* 2011; Dhinsa and Adesida 2012). What is more, these current treatment options have strict limitations concerning patient age, defect size and general joint condition. In relatively rare cases, osteoarthritis may lead to total joint replacement surgery.

The rationale of tissue engineering is to provide suitable chemical and mechanical cues for therapeutic cells, preferably within a biomaterial scaffold, to elicit formation of new tissue or regeneration of a damaged tissue. Stem cells from adult tissues provide an attractive means of regenerating damaged tissues. So far, bone marrow derived mesenchymal stem cells (BMSC) have been the cell of choice for musculoskeletal tissue engineering applications. However, adipose derived stem cells (ASC) have emerged as a fascinating alternative for BMSC. Adipose stem cells are easier and less painful to obtain from patients, and they display similar immunophenotype and differentiation capabilities with BMSC (Zuk *et al.* 2002; Winter *et al.* 2003; Huang *et al.* 2005; Hennig *et al.* 2007; Havlas *et al.* 2011). Here, hASC were cultured in the presence of various putative chondrogenic medium additives in order to obtain cartilage formation *in vitro*.

2. Review of literature

2.1 Articular cartilage

Three different types of cartilage can be distinguished by their molecular composition and mechanical properties: fibrous cartilage, elastic cartilage and hyaline cartilage. Fibrous cartilage is found in the *annulus fibrosus* of the intervertebral discs, the temporomandibular joint, the menisci, and the pubic symphysis. Elastic cartilage is found in the ears, nose, larynx and epiglottis. Hyaline cartilage is found in trachea, bronchi, larynx, nose, the rib cage, developing bones of the fetus, and, most importantly, in articulating joint surfaces.

2.1.1 Articular cartilage histology

The hyaline cartilage on articulating surfaces is a glassy, amorphous tissue lacking vasculature, neurons and lymphatic vessels. Articular cartilage is rich in extracellular matrix composed of collagen and glycosaminoglycans, whereas the cell content of articular cartilage is very low. The chondrocytes reside within cavities in the ECM, which are called *lacunae.* Occasionally two or more chondrocytes share a common *lacuna*, but mature chondrocytes are essentially non-dividing. Chondrocytes are commonly seen in columns; this is due to sequential cell divisions and minute ECM deposition during the last phases of growth, resulting in *lacunae* close together. In mature AC, chondrocytes rarely or never undergo cell division. Cells residing close to each other within lacunae separated only by thin ECM are called isogeneous groups, since they are usually derived from a common precursor. Usually a capsule of newly synthesized matrix surrounds each *lacuna*. (Ross *et al.* 1995; Young *et al.* 2006)

Chondrocytes represent the majority of the cartilage cell population. However, small quantities of mesenchymal progenitor cells capable of osteogenic, adipogenic and chondrogenic differentiation have been found in the superficial zone of human articular cartilage (Dowthwaite *et al.* 2004; Fickert *et al.* 2004; Hattori *et al.* 2007). The main purpose of articular cartilage is to allow joint motion: Highly specialized chemical composition and zonal arrangement permit virtually frictionless joint articulation, repetitive loading and distribution of load across the joint. (Ross *et al.* 1995; Young *et al.* 2006)

2.1.2 Cartilage during organogenesis

During organogenesis, hyaline cartilage molds (*anlagen*) of the vertebrae and the weight bearing bones of the skeleton are formed before endochondral ossification turns them into bone (Ross *et al.* 1995; Lewis Wolpert 2011). Development of a long bone begins with cells of mesenchymal origin condensing to form pre-cartilage aggregates. This aggregation of mesenchymal cells is supported by cell adhesion molecules N-cadherin and N-CAM (neural-cell adhesion molecule), (Tavella *et al.* 1994; Chimal-Monroy and de Leon 1999; Goldring *et al.* 2006), whose expression is stimulated by members of the TGF-β family of growth factors (Chimal-Monroy and de Leon 1999). Cells begin secreting cartilaginous ECM and are initially referred to as chondroblasts, but as they become surrounded by ECM they are called chondrocytes. The perichondrium (periosteum-to-be) forms a bony collar around diaphysis of the cartilage mold and subsequently the diaphysis itself begins to calcify. Chondrocytes become hypertrophic; they cause bone growth by enlarging and initiate mineralization by secreting ALP (alkaline phosphatase) and collagen type 10. Hypertrophic chondrocytes undergo apoptosis and cartilage ECM degrades (Kronenberg 2003). Simultaneously vasculature intrudes along with periosteal cells, resulting in replacement of cartilage ECM (collagen type 2) with bone ECM (collagen type 1). Vasculature also invades the ends of the bone, the upper and lower epiphysis. Epiphyseal discs (growth plates) are formed between the diaphysis and both ends of the bone. Hyaline cartilage in the epiphyseal discs continues to grow and undergo calcification, resulting in growth of long bones until early adulthood. However, only the epiphyseal cartilage of the growth plates is replaced by bone: a thin layer of articular cartilage is left at the ends of the bones to enable smooth joint articulation. The two tissues are inherently different: articular cartilage expresses tenascin-C and collagen type 12 and lacks the expression of matrilin-1, whereas the opposite is true in epiphyseal cartilage (Gregory *et al.* 2000). Endochondral ossification is depicted in Figure 1 below.

2.1.3 Control of chondrogenesis — SOX9

Transcription factor SOX9 (sex determining region Y - box 9) has been strongly associated with chondrogenesis during development, and it is required in multiple steps during chondrogenesis (Bell *et al.* 1997; Lefebvre *et al.* 1997; Ng *et al.* 1997; Zhao *et al.* 1997; Bi *et al.* 1999; Sekiya *et al.* 2000; Akiyama *et al.* 2002). SOX9 is expressed in cartilaginous tissues but not in hypertrophic chondrocytes (Bi *et al.* 1999; Akiyama *et al.* 2002; Kronenberg 2003). Lack of SOX9 in the limb buds results in severe defects of cartilaginous tissues and in those bony tissues, which normally derive from cartilage (Bi *et al.* 1999; Akiyama *et al.* 2002).

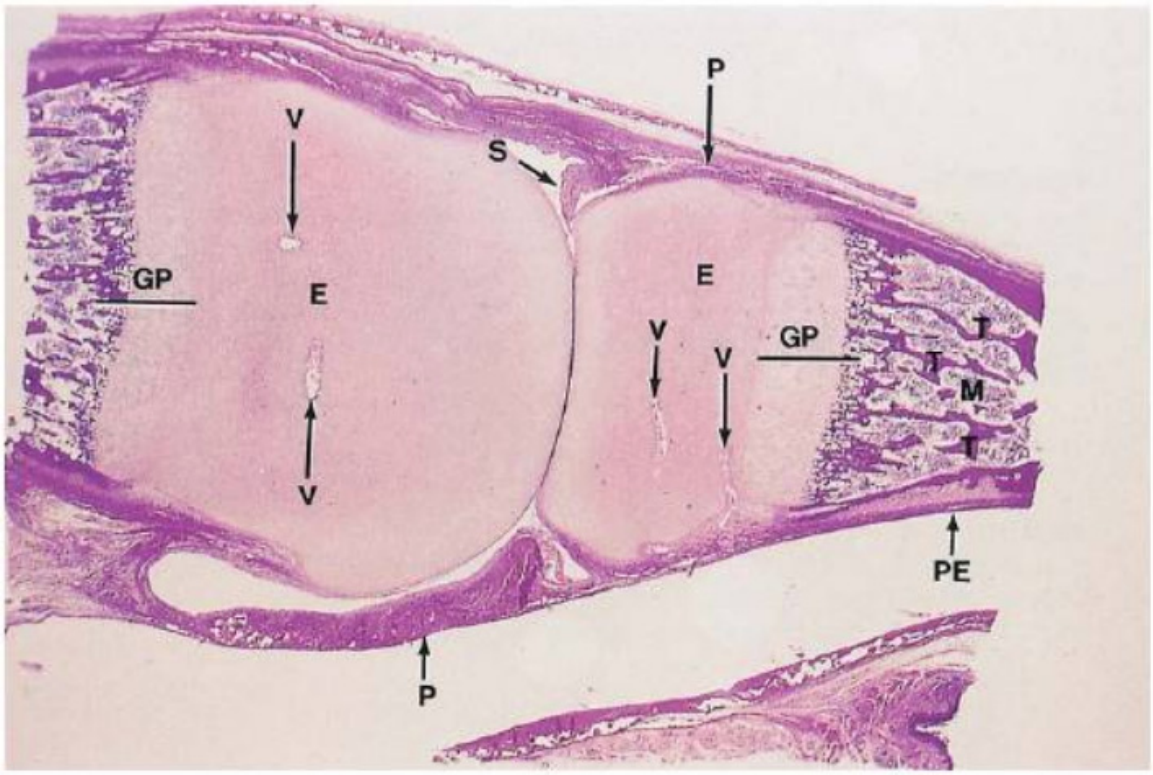

Figure 1 - Endochondral ossification of long bones. This histological section depicts the joint the development of long bones and the intervening joint. Abbreviations: GP = epiphyseal growth plate, T = mineralized tissue trabeculae, P = perichondrium, E = epiphyseal hyaline cartilage, PE = periosteum of diaphyseal bone, S = developing synovial capsule, V = invading blood vessels in otherwise hyaline cartilage. Figure from (Berman 2003).

SOX9 drives chondrogenesis by activating gene transcription. Most importantly, SOX9 directly binds to an enhancer sequence in *COL2A1* gene and activates its transcription (Bell *et al.* 1997; Lefebvre *et al.* 1997; Ng *et al.* 1997; Zhao *et al.* 1997). What is more, SOX9 seems to be essential for prechondral condensation and it also counteracts chondrocyte apoptosis in developing limbs and prevents chondrocyte hypertrophy (Bi *et al.* 2001; Akiyama *et al.* 2002). Furthermore, SOX9 induces the expression of *cartilage link protein 1* (*CRTL1*) (Kou and Ikegawa 2004) and *COL11A2* gene (Kronenberg 2003; Goldring *et al.* 2006), and seems to bind to and activate the promoter sequence of the gene coding aggrecan (Sekiya *et al.* 2000).

Other related transcription factors, namely SOX6 and a long form of SOX5 (L-SOX5) are coexpressed with SOX9 in chondrogenic locations during development and are involved in activating transcription of *COL2A1* (Lefebvre *et al.* 1998; Smits *et al.* 2001) *COL9A1*, *aggrecan* and *CRTL1* (Goldring *et al.* 2006). However, L-SOX5 and SOX6 expression seem to be under the control of SOX9 (Akiyama *et al.* 2002). Cotransfection of SOX5, -6 and -9 into human chondrocytes, BMSC or ASC

increased the cells' chondrogenic differentiation in 3-D culture, as seen by increased expression of components of cartilage ECM and decreased collagen type 10 expression (Kim and Im 2011; Yang *et al.* 2011). Despite being a commonly used chondrocytic marker gene, SOX9 is also expressed in non-cartilage locations. SOX9 is expressed in male gonads during testogenesis, and in other developing tissues during embryogenesis, including the heart, kidney, pancreas and neural crest, for example (Lefebvre *et al.* 1997; Akiyama 2008).

2.1.4 Control of chondrogenesis — RUNX2

Another transcription factor, runt-related transcription factor 2 (RUNX2, also known as CBFA1, core-binding factor α1) is closely related to the control of chondrogenesis and osteogenesis. RUNX2 is an essential transcription factor for osteoblast differentiation during development and for both intramembraneous and endochondral ossification (Komori *et al.* 1997; Otto *et al.* 1997). Its expression is localized in developing bones, the notochord and to the *nucleus pulposus* derived from the notochord (Otto *et al.* 1997) and in hypertrophic chondrocytes (Inada *et al.* 1999). Homozygous $RUNX2^{-/-}$ mice die due to respiratory failure just minutes after birth and show no bone formation and no mineralization, whereas cartilage formation remains normal (Komori *et al.* 1997; Otto *et al.* 1997). In comparison, heterozygous $RUNX2^{-/+}$ mice show delayed ossification and reduced size in certain bones (Komori *et al.* 1997; Otto *et al.* 1997). The growth plates of homozygous $RUNX2^{-/-}$ mice show no vascular invasion, ossification nor osteoblast differentiation, although slight calcification has been observed (Komori *et al.* 1997; Otto *et al.* 1997). Furthermore, the expression of alkaline phosphatase (ALP), an early marker of osteoblast differentiation, was essentially absent in RUNX2-null mice (Komori *et al.* 1997; Otto *et al.* 1997). The expression other bone-specific non-collageneous proteins osteopontin and osteocalcin was also diminished in $RUNX2^{-/-}$ mice (Komori *et al.* 1997; Inada *et al.* 1999).

In addition to blocking osteoblast development and bone formation, RUNX2 also affects chondrocyte maturation. Tissue-specific RUNX2-overexpression in chondrocytes has been shown to induce hypertrophic chondrocyte phenotype and endochondral ossification in transgenic mouse models (Takeda *et al.* 2001; Ueta *et al.* 2001; Kronenberg 2003). On the other hand, mouse models with dominant negative mutation of RUNX2 display delayed chondrocyte maturation and endochondral ossification, and maturational arrest to prehypertrophic collagen type 2 expressing phenotype (Inada *et al.* 1999; Ueta *et al.* 2001). Others have observed complete absence of chondrocyte maturation in mice

deficient in both RUNX2 and RUNX3 (Yoshida *et al.* 2004). It has been suggested that RUNX2 induces the expression of FGF18, which drives chondrocyte maturation (Hinoi *et al.* 2006). At the same time, RUNX2 expression seems to be under the control of Twist-1, which is expressed in the perichondrium and not by chondrocytes (Hinoi *et al.* 2006). Altogether, RUNX2 has a major role in effecting bone formation by controlling the differentiation and maturation of at least osteoblasts and chondrocytes and hence RUNX2 is commonly used as a marker of hypertrophic cartilage or early osteogenesis.

2.1.5 TGF-β superfamily growth factors

Signaling via TGF-β superfamily proteins controls the embryonic development of various tissues (Goumans and Mummery 2000) of which cartilage is the main interest of this study. To name but a few examples, TGF-β induces precartilage condensation (Chimal-Monroy and de Leon 1999; Tuli *et al.* 2003), maintains chondrocytic phenotype in articular cartilage (Karsenty and Wagner 2002) and upregulates cartilaginous ECM related genes in mesenchymal stem cells (Tuli *et al.* 2003). In addition, GDF-5 is a necessary cartilage growth regulator during joint formation (Merino *et al.* 1999; Karsenty and Wagner 2002; Lewis Wolpert 2011) and various BMPs are famous for their ability to induce virtually the whole sequence of endochondral ossification (Urist 1965; Karsenty and Wagner 2002).

TGF-β superfamily of growth factors encompasses structurally related proteins from TGF-β-, bone morphogenetic protein (BMP)-, growth- and differentiation factor (GDF)- and activin families. Cystine knot motif and dimer formation are common features of all TGF-β superfamily proteins. The TGF-β ligand binds to cell surface receptor resulting in receptor oligomerization. The type II receptors have a constitutively active kinase domain capable of phosphorylating Ser-, Tre- and Tyr- residues, whereas the type I receptors' kinase activity is not constitutive (Kang *et al.* 2009). The ligand-induced receptor oligomerization results in the phosphorylation of type I receptors by the type II receptors, presumably followed by subsequent conformational changes (Kang *et al.* 2009). Thereafter, intracellular receptor-activated SMAD-proteins (R-SMAD) become phosphorylated by the type I receptor, after which they form a complex with a common partner SMAD (SMAD-4). The complex then translocates to the nucleus to activate or inhibit the expression of targeted genes. Most often TGF-β results in phoshorylation of SMAD-2 and -3, whereas BMP results in phosphorylation of SMAD-1, -5, and -8, although some promiscuity is evident (Goumans and Mummery 2000; Derynck and Zhang 2003; Roelen and Dijke 2003).

Different TGF-β ligands' affinity towards different receptor types and subtypes differ considerably. For example, TGF-β signaling takes place principally via two type I receptors, namely T-βR1/ALK5 and ALK1 (activin receptor-like kinase) and one type II receptor, TGFβ-R2 (Derynck and Zhang 2003; Roelen and Dijke 2003). TGF-β initially binds to type II receptors to induce receptor oligomerization; in contrast, BMP ligands initiate receptor oligomerization by binding to type I receptors. BMP ligands have strongest affinity to ALK2, BMP-receptors B-R1A/ALK3, B-R1B/ALK6 (type I receptors), and B-R2 and activin receptors Act-R2, Act-R2B (type II receptors) (Derynck and Zhang 2003). Different receptors can mediate different kinds of cellular responses. B-R1B/ALK6 induces the initial cartilaginous skeleton formation during organogenesis, whereas
B-R1A/ALK3 is more localized in prehypertrophic chondrocytes, presumably controlling the rate of differentiation and chondocyte maturation (Zou *et al.* 1997).

The precise signaling with a limited amount of receptors and SMAD proteins is possible due to tight regulation by auxiliary proteins and by interaction with non-SMAD signaling cascades. In fact, TGF-β signaling is intricately regulated at multiple stages, and the cellular response is highly dependent on cell type and the signaling molecules' balance in the responding cell.

Firstly, various inhibitory proteins in the ECM, including noggin, chordin, DAN-family members (differential screening-selected gene aberrant in neuroblastoma), sclerostatin, follistatin and follistatin related protein can prevent the ligand—receptor interaction, and thus curb the TGF-β signaling (Roelen and Dijke 2003). Interestingly, BMP antagonist noggin effectively prevents mesenchymal condensation during chick limb development, inhibits chondrocyte proliferation and induces hypertrophic chondrocyte phenotype (Kronenberg 2003), and furthermore noggin deficiency causes cartilage overgrowth in mice (Karsenty and Wagner 2002).

Secondly, expression of the cognate receptors in different cell types may differ (Roelen and Dijke 2003). Moreover, accessory receptors, termed type III receptors, potentiate signaling by facilitating ligand binding (Roelen and Dijke 2003). Endoglin (CD105) is a type III receptor found in hASC, and it is tempting to speculate its role in hASC responsiveness to TGF-β signaling. During hypoxia, elevated endoglin expression supports wound healing and angiogenesis (Valluru *et al.* 2011).

Thirdly, SMURF-1 and -2 (SMAD ubiquitinylation regulatory factor) can interact with SMAD proteins and funnel either the associated receptors or the SMAD to degradation (Derynck and Zhang 2003; Roelen and Dijke 2003). Further control over TGF-β signaling is provided by inhibitory SMAD-proteins (SMAD-6 and -7) (Goumans and Mummery 2000; Derynck and Zhang 2003; Kang *et al.*

2009). On the other hand, auxiliary proteins such as SARA (SMAD anchor and receptor activation), Dab-2, Axin, TRAP-1 (TGF-βRI associated protein 1), endofin, TRIP-1 (TGF-βR interacting protein 1), STRAP (serine/threonine kinase receptor associated protein) and many others either facilitate or prevent R-SMAD recruitment to receptor complex and thus regulate the signaling (Derynck and Zhang 2003; Kang *et al.* 2009).

In addition to ligand-binding induced phosphorylation of the type I receptor by the type II receptor, both receptor types can be phosphorylated at auxiliary sites, regulating receptor activity and providing docking sites for proteins of other signaling cascades, such as the mitogen activated protein kinases (MAPK) (Kang *et al.* 2009).

Finally, TGF-β signaling can induce activity in kinases of other pathways (Figure 2), such as Erk-1/2 (extracellular signal-regulated kinase 1 or 2), p38 and JNK (c-Jun N-terminal kinase), TAK1 (TGF-β activated kinase 1/), MEKK1 (mitogen-activated protein kinase kinase kinase 1), and RhoA/ROCK (Rho-associated, coiled-coil containing protein kinase) (Derynck and Zhang 2003; Tuli *et al.* 2003; Goldring *et al.* 2006; Kang *et al.* 2009). *Vice versa*, SMAD can be phosphorylated by kinases from non-TGF-β pathways, such as those related to EGF signaling, hepatocyte growth factor signaling, Ras pathway kinases, calmodulin dependent kinase 2, protein kinase C, and kinases of the JNK- and Erk-pathways (Derynck and Zhang 2003). Furthermore, SMAD proteins can be phosphorylated at different sites, resulting in either activation or repression (Derynck and Zhang 2003; Kang *et al.* 2009).

2.1.6 Structure and function of articular cartilage

Articular cartilage ECM is composed of collagens, proteoglycans (PG) and glycosaminoglycans (GAG); the ECM accounts for roughly 95 *vol*-% of mature articular cartilage (Coates and Fisher 2010). In brief, collagen network provides the tensile strength while PG and GAG provide the osmotic properties needed for dynamic compressive properties. PG and GAG are entangled among the meshwork of collagen fibres. The dense and cross-linked collagen network restricts the hydration of PG and GAG to approximately 40—60 % (Getgood *et al.* 2009); in free solution the PG expand to five-fold larger volume (Heinegard and Oldberg 1989). Owing to these swelling properties, articular cartilage can withstand cyclic compressive loading, during which the synovial fluid seeps in and out of the tissue, providing an ingenious means of nutrient transportation (Heinegard and Oldberg 1989; Iozzo 1998; Chen *et al.* 2006; Getgood *et al.* 2009; Heinegard 2009; Coates and Fisher 2010).

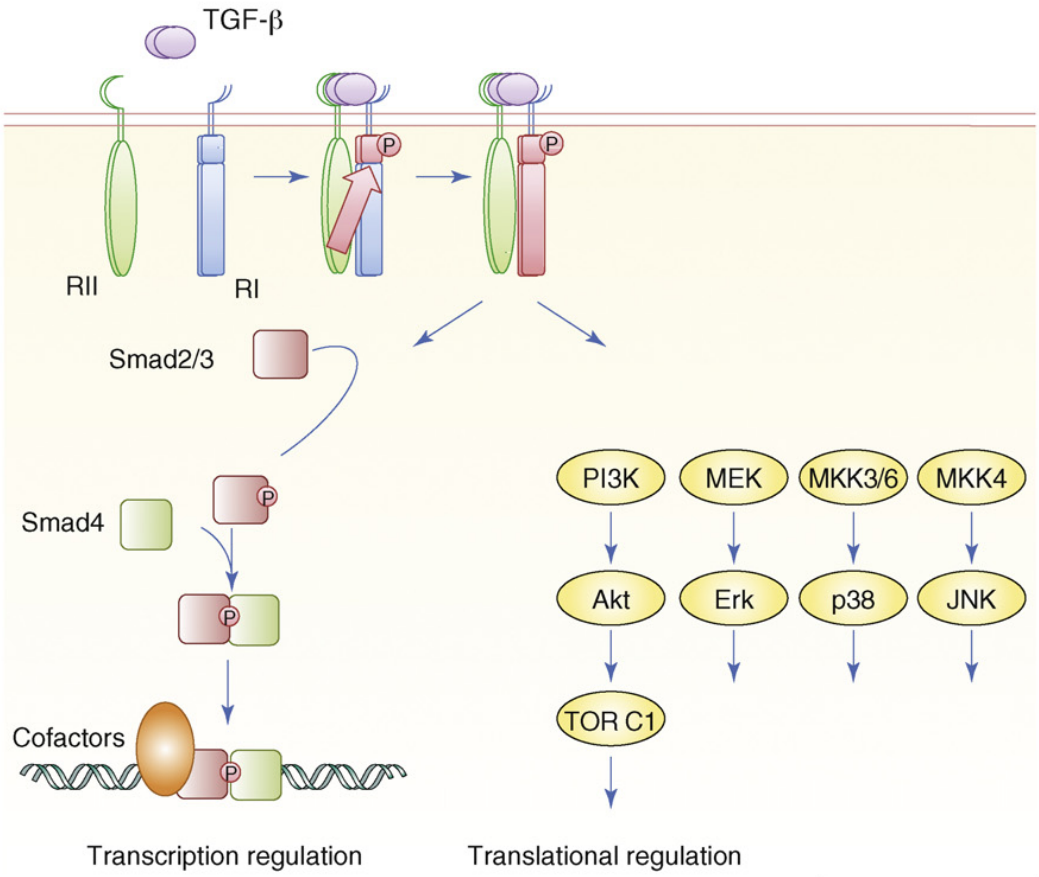

Figure 2 - Schematic illustration of TGF-β signaling. A TGF-β ligand binding initiates receptor oligomerization, resulting in intracellular kinase activity. Signal can be relayed via SMAD-dependent and non-SMAD pathways, and the non-SMAD pathways may affect SMAD signaling, as discussed in text. Abbreviations: P = phosphate, RI/II = TGF-β receptor I/II, PI3K = phosphatidylinositol 3-kinases, MEK = mitogen activated protein kinase kinase, MKK3/4/6 = mitogen activated protein kinase kinase 3/4/6, Akt = v-akt murine thymoma viral oncogene homolog, ERK = extracellular signal-regulated kinase, TOR C1 = transducer of regulated cAMP response element-binding protein 1, JNK = c-Jun N-terminal kinase. Figure from (Kang *et al.* 2009).

2.1.7 Zonal arrangement of articular cartilage

Collagens, GAG, PG and other components arrange the cartilage ECM in distinctive yet fully continuous zones with unique features, yielding the biomechanical properties of the AC. The zones are, from the synovial cavity towards the subchondral bone: tangential cartilage, transitional cartilage, radial cartilage and calcified cartilage (Figure 3). Furthermore, regions differing in molecular composition can be distinguished: the pericellular matrix directly around the chondrocytes, territorial matrix nearby the *lacunae*, and finally the less organized interterritorial matrix, which encompasses the most of the ECM (Ross *et al.* 1995, see also Figure 4). The supramolecular organization and zonal structure are, however, incompletely understood, let alone replicated in an experimental setting. Frankly, even the mature chondrocytes only possess limited capacity of renewing the zonal structure,

making articular cartilage defects prone to further degeneration. Furthermore, collagen turnover is recognized to be remarkably slow; estimates of total collagen turnover range from 100 years upward (Verzijl *et al.* 2000; Eyre *et al.* 2006). However, it has been proposed that chondrocytes be able to remodel the matrix compartment nearest to them (Eyre *et al.* 2006).

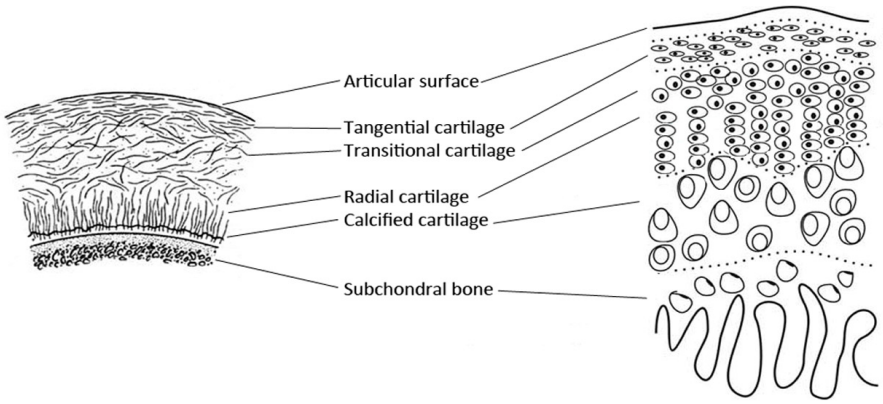

Figure 3 - Schematic figure displaying the zonal organization of mature articular cartilage. The arrangement of collagen fibres (left side) and the organization of chondrocytes (right side) in articular cartilage is presented. Note that in the deeper layers, both collagen fibres and chondrocyte columns are perpendicular to articular surface. In the middle zones the arrangement is more irregular, whereas near the surface the elongated cells and collagen fibres run parallel with the articular surface. See also Figure 4 for actual histological appearance of the cells. Figures modified from (http://www.orthoteers.org/%28S%28rdgkzc4y0loz2zy24ss3s2kh%29%29/mainpage.aspx?section=28&article=328 and http://www.bioscience.org/2003/v8/a/932/fulltext.asp?bframe=figures.htm&doi=yes, links checked 4.1.2012).

Closest to the articular cavity, the tangential zone is rich in collagen fibres running parallel to the joint surface, whereas GAG and water content are low (Eyre *et al.* 2006; Clouet *et al.* 2009; Coates and Fisher 2010). The tangential zone has the highest stiffness and tensile strength owing to the high collagen content (Clouet *et al.* 2009; Coates and Fisher 2010). The spindle-shaped cells in the tangential zone lie parallel to the joint surface, and they secrete lubricin (proteoglycan 4) to decrease friction (Getgood *et al.* 2009; Coates and Fisher 2010). In the next zone, the transitional zone, round chondrocytes and less organized fibrils of collagen types 2, -6, -9 and -11 are seen; collagen fibrils are bended and run more randomly than those in the tangential zone (Eyre *et al.* 2006; Clouet *et al.* 2009; Coates and Fisher 2010). Fibres containing collagen types 2, -9 and -11 are organized perpendicular to the joint surface in the radial zone; columns of rounded chondrocytes lie perpendicular to the joint surface as well (Ross *et al.* 1995; Clouet *et al.* 2009; Coates and Fisher 2010). Calcified cartilage is

made up of hypertrophic chondrocytes secreting collagen type 10 (Clouet *et al.* 2009; Coates and Fisher 2010). Calcified fibrils of collagen type 10 anchor the cartilage to the subchondral bone (Clouet *et al.* 2009; Coates and Fisher 2010). The total amount of GAG increases in the deeper layers compared to the surface-most layers, resulting in higher compressive modulus of the deep layers (Clouet *et al.* 2009; Coates and Fisher 2010).

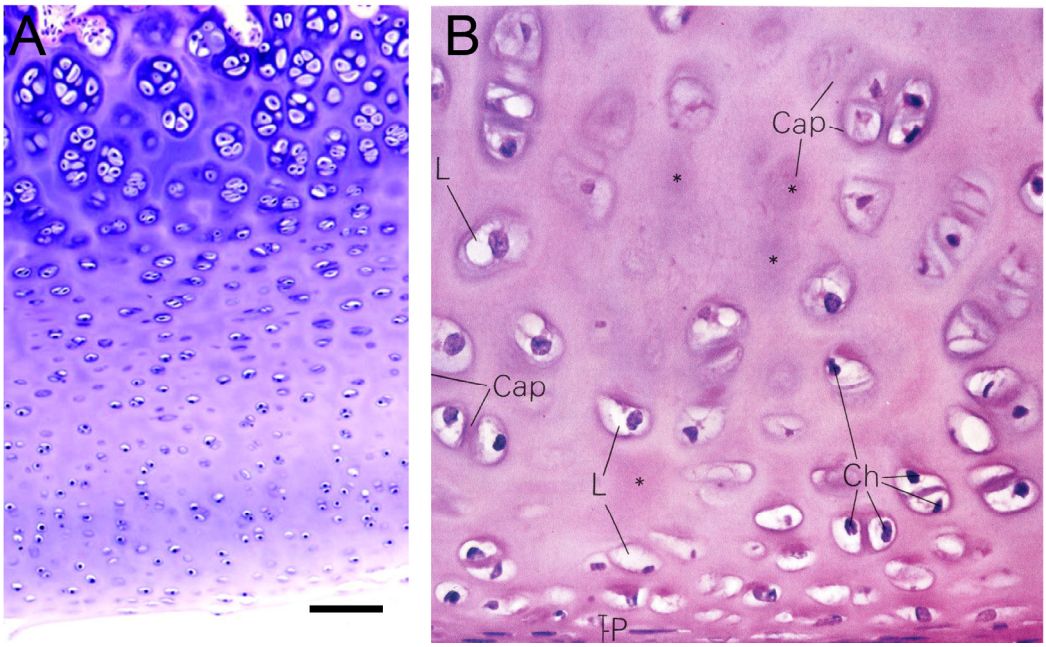

Figure 4 - Histological appearance of hyaline cartilage. Panel A: a histological section of bovine articular cartilage stained with toluidine blue displays two important things: Firstly, the zonal arrangement of cartilage, as also seen also in Figure 3. Secondly, the different staining properties of pericellular, territorial and interterritorial matrices can be observed. Scale bar: 100 µm. Figure modified from (Sunk *et al.* 2006). Panel B: a histological section of human trachea displaying the regional differences in the ECM. Ch = chondrocyte, L = lacuna, P = perichondrium, Cap = capsule, asterisk (*) = capsule without its lacuna included in the section. Figure modified from (Ross *et al.* 1995).

2.1.8 Collagen framework of the articular cartilage

Collagens are present throughout the body, providing mechanical integrity and support for tissues. There are various groups of collagens, namely the fibril forming collagens, the FACIT (fibril associated collagens with interrupted triple-helices) collagens, the short chain collagens, the microfibrillar collagens and the collagens of the basement membrane (Gelse *et al.* 2003), of which the first four bare most relation to cartilage. Although collagens vary in shape and size, they all contain the distinguished collagen triple-helical structure composed of three α-chains. The α-chains may be identical (homotrimeric collagens) or of different kinds (heterotrimeric collagens). Each α-chain contains several repeats of the "collageneous tripeptide", Gly-X-Y, where X and Y frequently correspond to proline or

hydroxyproline. Each α-chain forms an extended left-handed coil. Three of these coils form a supercoil by coiling around each other in a right-handed manner. The triple-helical structure is often referred to as the collagen monomer (Vanderrest and Garrone 1991; Gelse *et al.* 2003). Fibril forming collagen monomers of one or more types attach to each other in a staggered manner to form long collagen fibrils (see Figure 5). The proline and lysine residues in the α-chains may be hydroxylated to a varying degree, and they provide intermolecular hydrogen bonding to facilitate glycosylation and cross-linking of collagen fibrils (Vanderrest and Garrone 1991; Gelse *et al.* 2003). Also, hydroxylysine or -proline residues in cartilage contain plenty of glycosidically bound carbohydrates, which possibly control fibril diameter (Burgeson and Nimni 1992).

In addition to the triple helical domain, the N- and C-termini of the collagen fibril contain non-helical telopeptide- and propeptide domains. The C-propeptide seems to facilitate the initiation of the triple helix formation, while the N-propeptide apparently regulates the fibril diameter (Gelse *et al.* 2003). The non-helical telopeptides are involved in cross-linking the collagens together and to other macromolecules (Vanderrest and Garrone 1991; Gelse *et al.* 2003).

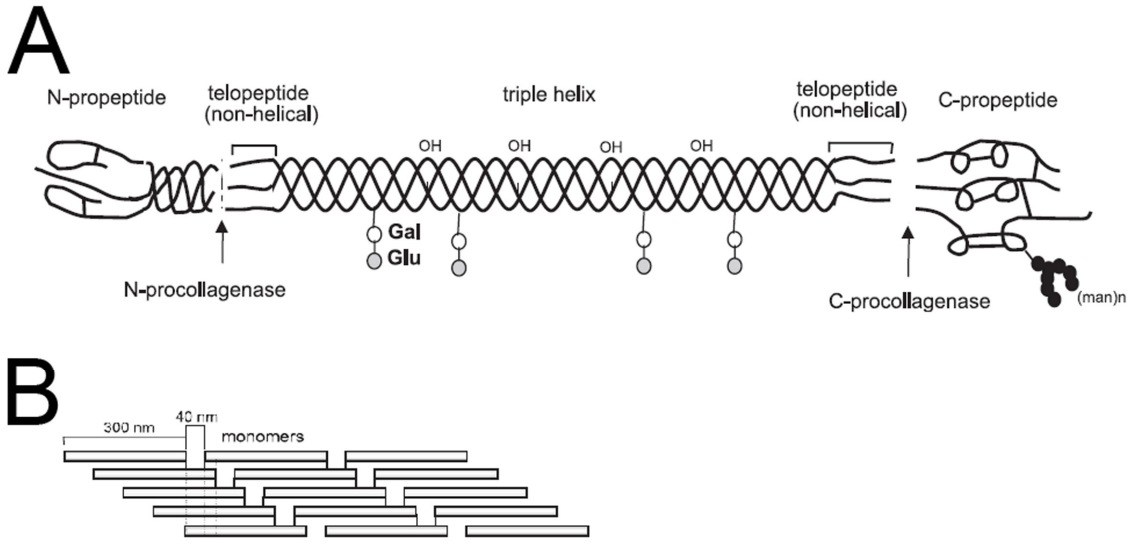

Figure 5 - Molecular organization of fibrillar collagen. A: The overall structure of a collagen monomer, where three α-chains coil around each other. B: The staggered organization of collagen monomers side-by-side and end-to-end gives rise to collagen fibrils. In articular cartilage, the fibrils are further arranged in a network-like anisotropic manner. Figure modified from (Gelse *et al.* 2003).

Roughly 25 % of cartilage wet weight (70 % dry weight) is collagen, and 80—95 % of that collagen is type 2 (Gelse *et al.* 2003; Eyre *et al.* 2006; Getgood *et al.* 2009). In healthy AC, highly cross-linked fibres composed of collagen types 2, -9 and -11 predominate, giving the articular cartilage

its unique properties (Eyre *et al.* 2006). Below, the collagen types related to cartilage are discussed in brief. Notice, that collagen antibodies recognize the fibrils formed by multiple triple-helical monomers, rather than individual gene-products. Hence, the conventional nomenclature is not suitable. Instead, the fibre made up from the translated products of *COL1A1* and *COL1A2* is referred to as "collagen type 1", and so on.

Collagen type 1. Fibril forming collagen type 1 is the most abundant type of collagen, and it can be found in skin, tendon, ligament, bone, fibrous cartilages and cornea (Burgeson and Nimni 1992; Gelse *et al.* 2003). Collagen type 1 is a heterotrimer made up of two α1-chains and one α2 chain (Gelse *et al.* 2003). Fibrils of collagen type 1 interact with other collagens, such as collagen type 3 in skin and type 5 in bone, tendon and cornea (Gelse *et al.* 2003). Collagen type 1 only exists in low amounts in the superficial layer of articular cartilage (Clouet *et al.* 2009). On the other hand, collagen type 1 is often seen in repair tissue following articular cartilage damage, but such repair tissue is fibrous cartilage instead of hyaline cartilage (Kiviranta and Vasara 2004; Strauss *et al.* 2011; Dhinsa and Adesida 2012).

Collagen type 2. Fibril forming collagen type 2 consists of three identical α-chains, which are similar to the α1-chains of collagen type 1 (Burgeson and Nimni 1992; Gelse *et al.* 2003). Collagen type 2 is the most prominent collagen in cartilage, but it is also present in the vitreous humor, the corneal epithelium and the *nucleus pulposus* of the intervertebral discs (Burgeson and Nimni 1992; Gelse *et al.* 2003). Collagen type 2 resembles the fibrillar structure of collagen type 1. However, collagen type 2 has higher hydroxylysine content and contains more O-linked glucosyl- and galactosyl residues, which support cross-linking between collagen fibrils and PG (Vanderrest and Garrone 1991; Gelse *et al.* 2003). Notably, two alternatively spliced isoforms of collagen type 2 exist: full-length type 2-A and a truncated type 2-B, lacking a cysteine-rich N-terminal domain composed of 69 amino acids (Sandell *et al.* 1991). Of these, type 2-A is mainly expressed in developing embryonic tissues, whereas type 2-B is considered a marker of mature cartilage (Sandell *et al.* 1991; Gelse *et al.* 2003).

Collagen type 3. Fibril forming collagen type 3 is a homotrimeric protein (Gelse *et al.* 2003) found to be associated with collagen types 1 and -2 (Eyre *et al.* 2006; Wu *et al.* 2010). In AC collagen type 3 fibres are cross-linked to each other and the telopeptides of collagen type 3 are cross-linked to the triple helical domain of collagen type 2, and *vice versa* (Wu *et al.* 2010). Based on these findings and the known role of collagen type 3 in skin healing (Witte and Barbul 1997), it has been proposed that

chondrocytes might secrete collagen type 3 in order to repair injured articular cartilage and to strengthen the damaged collagen type 2 network (Eyre *et al.* 2006; Wu *et al.* 2010). Whether or not an actual repair mechanism, collagen type 3 has been detected in osteoarthritic cartilage as well as in healthy cartilage (Aigner and McKenna 2002; Eyre *et al.* 2006).

Collagen type 6. Collagen type 6 is made up of three distinct α-chains (Burgeson and Nimni 1992; Gelse *et al.* 2003) and is found concentrated in the vicinity of the chondrocytes just around the *lacunae* (Eyre *et al.* 2006). This microfibrillar collagen seems to be quite ubiquitous and is found in most connective tissues, with the exception of bone (Burgeson and Nimni 1992; Gelse *et al.* 2003).

Collagen type 9. Collagen type 9 is a FACIT-type collagen, and the monomer is made up of three different α-chains (Gelse *et al.* 2003). This collagen is flexible due to interruptions in the triple-helical domain (Burgeson and Nimni 1992; Gelse *et al.* 2003; Eyre *et al.* 2006). Collagen type 9 interacts with collagen type 2, especially at the meeting points of two collagen type 2 fibrils, linking fibrils together via covalent cross-links (Burgeson and Nimni 1992; Gelse *et al.* 2003; Eyre *et al.* 2006). There are at least seven cross-linking sites on the collagen type 9 molecule (Eyre *et al.* 2006). Of note, one of the collagen type 9 α-chains contains a chondroitin sulphate side chain covalently linked to a serine residue, providing possible interactions with PG and GAG (Burgeson and Nimni 1992; Gelse *et al.* 2003).

Collagen type 10. Collagen type 10, a member of the short chain collagens, is a homotrimeric protein strongly associated with the onset of chondrocyte hypertrophy and calcification of cartilage matrix (Gibson and Flint 1985). Expression of collagen type 10 is restricted to the hypertrophic cartilage during endochondral ossification and to the zone of calcified cartilage separating articular cartilage from subchondral bone (Burgeson and Nimni 1992; Gelse *et al.* 2003; Kronenberg 2003). Type 10 collagen interacts with collagen types 2 and -11 in the calcified zone (Burgeson and Nimni 1992). Notably, collagen type 10 expression, matrix calcification and chondrocyte hypertrophy have also been shown to occur in late-stage osteoarthritis in humans (Fuerst *et al.* 2009). Due to its temporospatially localized expression, collagen type 10 can be used as a marker of chondrocyte hypertrophy and onset of mineralization.

Collagen type 11. Fibril forming collagen type 11 is a heterotrimer of three different α-chains (Burgeson and Nimni 1992), one of which is encoded by the same gene as the α-chain of collagen type 2, differing only in post-translational modifications (Gelse *et al.* 2003; Eyre 2004). Collagen type 11 is thought to function as a template for collagen type 2 and to limit the diameter of collagen type 2 fibrils (Gregory *et al.* 2000; Gelse *et al.* 2003; Eyre 2004; Eyre *et al.* 2006). Both collagen types 9 and -11 are found concentrated in newly synthesized thin fibres in the territorial matrix surrounding the *lacunae*, while less of these collagen types are present in more mature fibres (Eyre *et al.* 2006).

Collagen type 12. Heterotrimeric collagen type 12 is another member of the FACIT group: it contains a large non-collageneous domain (Eyre 2004) and it can be found in collagen type 1-expressing tissues and in articular cartilage (Gregory *et al.* 2001). Interestingly, collagen type 12 has been shown to be localized specifically in the articular cartilage and not in epiphyseal cartilage (Gregory *et al.* 2001). A role in stabilizing the organization of articular cartilage matrix has been suggested for collagen type 12, since it interacts with collagen type 2 fibres and with fibromodulin and decorin (Gregory *et al.* 2001). Of note, collagen type 12 is essential for osteoblast differentiation and bone ECM formation (Izu *et al.* 2011) and periodontal ligament formation (Reichenberger *et al.* 2000).

Collagen turnover and remodellation. The bulk collagen type 2 network is relatively stable, but some remodeling and turnover is believed to take place in the territorial and pericellular compartments nearest to the chondrocytes (Eyre *et al.* 2006). The presence of catabolic collagenase enzymes in articular cartilage is important for steady remodellation whereas elevated collagenase expression is related to osteoarthritic biochemical changes (Eyre *et al.* 2006). For example, MMP13 is secreted by chondrocytes in response to IL-1 stimulation (Eyre 2004; Eyre *et al.* 2006).

Serpin H1 chaperone protein. Serpin H1 (HSP47, heat shock protein 47) is a glycoprotein constitutively expressed in collagen producing cells' ER (endoplasmic reticulum) where it functions as a specific chaperone for triple helical collagens (Nakai *et al.* 1992), at least for types 1—5 (Nagata 1998; Nagata 2003; Mala and Rose 2010). Furthermore, *SERPINH1* is inducible by heat-shock factor 1 (HSF1) and by other transcription factors (Nagata 2003; Mala and Rose 2010). Suppressing *SERPINH1* results in diminished collagen production (Nagata 2003). Serpin H1 recognizes and binds to a Gly-X-Arg sequence in the properly coiled collagen α-chain, thereby preventing premature aggregation, easing triple helix formation and promoting the maturation of the collagen monomer (Nagata 2003;

Mala and Rose 2010; Nishikawa *et al.* 2010). Serpin H1 seems essential for bone formation: it is expressed in hypertrophic chondrocytes during endochondral ossification and mineralization (Loones and Morange 1998), whereas a mutation in *SERPINH1* is suspected to cause *osteogenesis imperfecta* ("brittle bone disease") in dogs (Drogemuller *et al.* 2009).

2.1.9 Proteoglycans and minor components of articular cartilage

Glycosaminoglycans in the form of proteoglycan aggregates constitute the second major component of cartilaginous matrix. The sulfated GAG carry a large negative charge, and are therefore chiefly responsible for the osmotic properties of cartilaginous tissue, which in turn provide this tissue its unique biomechanical properties. Here, the framework of proteoglycan aggregates and the role of other components are briefly described.

2.1.9.1 Aggrecan and proteoglycan aggregates

Hyaluronan is a large non-sulfated GAG that functions as a scaffolding molecule for proteoglycans in cartilage matrix (Roughley 2006). As indicated in Figure 6, hyaluronan forms the core of the proteoglycan aggregates. Aggrecan is a hyaluronan-binding modular core protein consisting of three functional domains. The N-terminal domain is responsible for hyaluronan binding, whereas the middle domain includes multiple binding sites for sulfated GAG (Iozzo 1998; Roughley 2006; Heinegard 2009). The C-terminal domain is believed to be related to post-translational modifications (Roughley 2006), lectin binding (Iozzo 1998), and EGF-repeat (Epidermal growth factor) interactions, providing a means of linkage to fibrillins, fibulins, and tenascins (Heinegard 2009). In the large GAG binding domain, chondroitin sulfate is most prominent, keratin sulfate being present in a restricted area nearest to the hyaluronan binding domain (Heinegard and Oldberg 1989; Iozzo 1998; Roughley 2006). In a PG-aggregate, approximately 100 chondroitin sulfate chains are attached to the GAG-binding domain of aggrecan (Heinegard and Oldberg 1989; Iozzo 1998; Heinegard 2009) and likewise, up to 100 aggrecan molecules bind to one hyaluronan molecule (Roughley 2006). The hyaluronan—aggregan binding can be strengthened by CRTL1, which interacts with both hyaluronan and aggrecan N-terminal domain (Heinegard and Oldberg 1989; Roughley 2006). The main function of these proteoglycan aggregates is to provide extremely high negative charge-density and hence an extremely high osmotic pressure in cartilage, which ultimately translates to the biomechanical properties of this tissue (Heinegard 2009), as explained above. The major proteoglycan aggrecan aside, other non-collageneous

components of cartilage matrix include small leucine-rich proteoglycans (SLRP), matrilins, COMP and other important components, which will be discussed next.

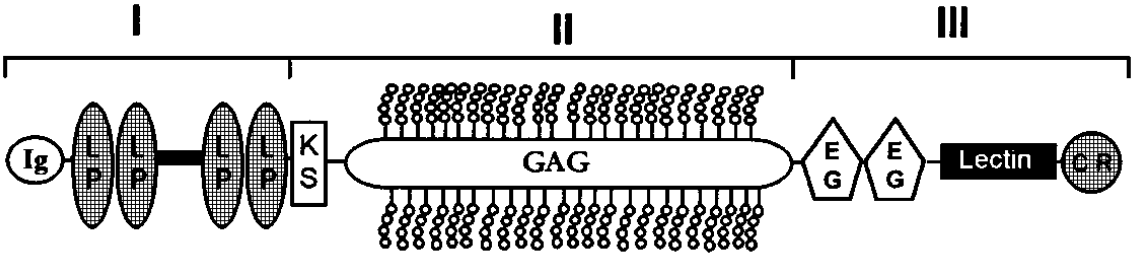

Figure 6 - The modular structure of aggrecan. This figure illustrates schematically the modular structure of aggrecan core protein. The Roman numerals indicate the proposed domains: I = N-terminal domain, II = middle domain, III = C-terminal domain. Symbols and abbreviations: Ig = hyaluronan binding immunoglobulin-type repeat, LP = link-protein type module, GAG = glycosaminoglycan-binding domain, EG = EGF-like module, Lectin = C-type lectin-like domain CR = complement regulatory protein, KS = keratan sulfate-attachment module. The glycosaminoglycan side chains are indicated by strings of small circles. Figure modified from (Iozzo 1998).

2.1.9.2 Small leucine-rich proteoglycans (SLRP)

The small leucine-rich proteoglycans found in cartilage ECM include decorin, biglycan (BGN), lumican, fibromodulin and chondroadherin (Iozzo 1998; Roughley 2006; Heinegard 2009). These SLRP belong to a large family of leucine-rich repeat proteins, which are not structurally related to aggrecan (Iozzo 1998; Roughley 2006). Briefly, SLRPs consist of a central domain of conserved leucine-rich repeats with flanking N-, and C-terminal domains, each containing disulphide bonds (Iozzo 1998; Roughley 2006; Heinegard 2009). Decorin and biglycan and have one and two, respectively, sites for N-linkage of chondroitin sulfate or dermatan sulfate chains at their N-termini (Iozzo 1998; Roughley 2006; Heinegard 2009). On the other hand, lumican and fibromodulin contain one to four N-linked keratin sulfate chains (Roughley 2006; Heinegard 2009). SLRP can control fibril diameter, protect fibrils from proteolytic degradation, cross-link the components of the cartilage ECM, control growth factor sequestering and link cells to the ECM (see Figure 7). This is due to SLRP's capability to interact with fibrillar (Heinegard and Oldberg 1989) as well as non-fibrillar collagen, fibronectin, elastin, integrins, growth factors such as FGF-2, IL-10, TGF-β and TNF-α (Iozzo 1998; Roughley 2006; Heinegard 2009). Although sometimes referred to as minor components, the SLRPs play many important roles in cartilage, and, from a stoichiometric point of view, are actually by no means minor compared to aggrecan (Heinegard and Oldberg 1989; Roughley 2006).

2.1.9.3 Matrilins

Matrilins are multimeric proteins composed of three or four subunits that contain von Willebrand motifs (Heinegard 2009). Matrilins interact with collagen triple helices, biglycan, decorin and COMP, thereby stabilizing the cartilage ECM (Heinegard 2009). Matrilin-3 is expressed in articular cartilage, whereas matrilin-1 is expressed in non-weight bearing cartilages (Heinegard 2009).

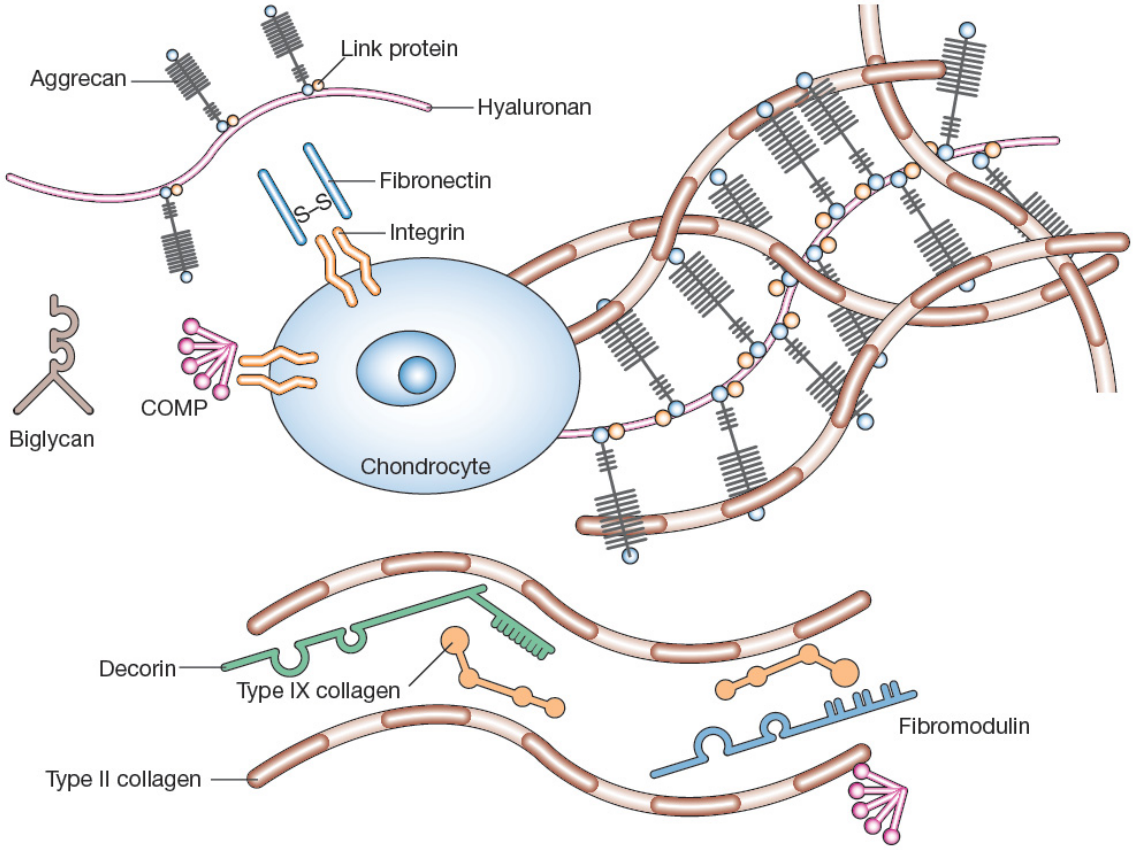

Figure 7 - Organization of the cartilage extra-cellular matrix. This schematic figure depicts the molecular organization and intermolecular interactions within cartilage ECM. See text for further discussion. COMP = cartilage oligomeric matrix protein. Figure from (Chen *et al.* 2006).

2.1.9.4 Cartilage oligomeric matrix protein (COMP)

COMP, also known as thrombospondin 5, consists of five identical sub-units linked together via an N-terminal coiled coil domain (Heinegard 2009). The C-terminal domains can bind to fibrillar collagen molecules and assist fibril formation or provide cross-linkage, whereas other domains interact with

non-fibrillar collagens and matrilins (Heinegard 2009). It seems that in osteoarthritis, elevated COMP expression coupled with decreased collagen expression results in a stoichiometric disorder, resulting in COMP occupying the binding sites of collagen fibrils and essentially inhibiting cross-linkage (Heinegard 2009). This provides one possible molecular explanation for poor repair capacity of articular cartilage. Under chondrogenic conditions *in vitro*, both hBMSC and hASC display highly elevated *COMP* expression (Winter *et al.* 2003).

2.2 Articular cartilage defects

Already some 250 years ago, William Hunter marvelled at the structure of articular cartilage and reported its troublesome inability to heal once injured (Hunter 1744). In addition to poor healing capacity, articular cartilage seems to be quite prone to injury. During knee surgeries of various reasons, AC-defects are found in over 60 % of patients, most often in the femoral condyle (Curl *et al.* 1997; Strauss *et al.* 2011). Cartilage defects can be a result of direct physical injury (Kiviranta and Vasara 2004; Goldring and Goldring 2007) or they can stem from aberrations in the ECM-encoding genes (Eyre *et al.* 2006). Because cartilage ECM experiences only slow turnover it accumulates age-related biochemical alterations, namely those related to oxidative stress and of advanced glycosylation end-products (Verzijl *et al.* 2000; Goldring and Goldring 2007). Cartilage defects are often graded by the deepness of the injury (see Table 1).

2.3 Surgical treatment of articular cartilage defects

Currently the clinically available treatment options for AC-defects include arthroscopic lavage and debridement (*i.e.* shaving chondroplasty), ACI/ACT (autologous chondrocyte implantation/transplantation), MACI/MACT (matrix-induced autologous chondrocyte implantation/transplantation), mosaicplasty, Pridie drilling, microfracture and various modifications of the above-mentioned, often including a biomaterial scaffold. These techniques are thoroughly discussed elsewhere; suffice to say none of them has proven universally applicable for cartilage regeneration, although excellent repair can be achieved in certain conditions (Kiviranta and Vasara 2004; Säämänen *et al.* 2008; Strauss *et al.* 2011; Dhinsa and Adesida 2012). Problems faced are manifold: poor integration to the surrounding host tissue often causes difficulties, as the repair tissue may detach if prematurely loaded (Vinatier *et al.* 2009; Strauss *et al.* 2011). Also, the resulting repair

tissue is often fibrous cartilage and displays inferior biomechanical properties compared to hyaline cartilage (Kiviranta and Vasara 2004; Strauss *et al.* 2011; Dhinsa and Adesida 2012). Especially large AC-defects, mirror-lesions, coexisting inflammation or osteoarthritis and elevated age make cartilage regeneration difficult (Kiviranta and Vasara 2004; Strauss *et al.* 2011). Lack of donor material and donor site morbidity limit the use of chondrocyte-based applications (Kiviranta and Vasara 2004; Dhinsa and Adesida 2012), although ACI is often considered the most established method for cartilage defect treatment. There have been some reports questioning the effectiveness of ACI compared to other methods (Knutsen *et al.* 2004; Knutsen *et al.* 2007). However, ACI, published already in 1994 by a Swedish group of researchers (Brittberg *et al.* 1994) is still seen as the gold-standard for regenerating hyaline-like cartilage (Peterson *et al.* 2002; Vasara *et al.* 2006; Strauss *et al.* 2011). The latest long-term follow-ups have shown good clinical and functional outcomes at 10—20 years in ACI treated patients (Peterson *et al.* 2010).

Table 1 - ICRS (International Cartilage Repair Society) grading of cartilage defects. The deepness of the defect determines the healing process: a deep defect will be infiltrated by mesenchymal stem cells from the bone marrow.

Grade 0	Normal appearing cartilage
Grade 1	Soft indentations and/or superficial cracks
Grade 2	Defect <50 % of the cartilage thickness
Grade 3	Defect >50 % of the cartilage thickness
Grade 4	Defect extends to or into the subchondral bone

2.3.1 Chondrocyte dedifferentiation

One of the drawbacks of ACI is that chondrocytes undergo dedifferentiation. During monolayer expansion chondrocytes immunophenotypic profile changes (Diaz-Romero *et al.* 2005) and the synthesis of cartilage ECM components, such as collagen types 2 and -9 and COMP decrease while collagen type 1 is accumulated (Zaucke *et al.* 2001; Schnabel *et al.* 2002; Darling and Athanasiou 2005a; Darling and Athanasiou 2005b). However, dedifferentiation is slowed down in a proper culture environment (Darling and Athanasiou 2005b), and dedifferentiated chondrocytes can be redifferentiated in suitable culture conditions in a bioreactor (Marlovits *et al.* 2003) or high cell-density 3-D culture (Schulze-Tanzil *et al.* 2002). Although some monolayer-induced phenotypic changes may be irreversible (Zaucke *et al.* 2001; Darling and Athanasiou 2005a).

2.4 Cartilage tissue engineering

The ultimate goal of tissue engineering is to regenerate or repair damaged tissues with combinations of cells, biomaterials and bioactive factors in order to improve heal or restore tissue function. Here, the current knowledge about the cells and bioactive factors used for cartilage tissue engineering is briefly presented.

2.4.1 Stem cells

Stem cells have the potential to undergo asymmetrical division resulting in self-renewal and differentiated cell progeny. In essence, one daughter cell maintains the stem cell pool while the other differentiates towards a specialized phenotype. Stem cells can be grouped according to their origin (to embryonic, foetal or adult stem cells) or based on their differentiation potential. Totipotent stem cells are described as being able to give rise to a whole organism, including all three embryonic germ layers, namely the ectoderm, mesoderm and endoderm, as well as the extraembryonic tissues, that is the trophectoderm. The fertilized egg and the cells of an early blastula are totipotent. Pluripotent stem cells, too, have the capacity to differentiate to all three germ layers found in the embryo, but they lack the ability to form the trophectoderm. Pluripotent stem cells can be acquired from the inner cell mass of the blastocyst, but they cannot give rise to a whole organism. Multipotent stem cells' differentiation capacity is even more restricted; they cannot differentiate to all three embryonic germ layers, but can give rise to several different cell types, usually of the same germ layer. Multipotent stem cells can be found in various tissues, and they are believed to facilitate turnover of old and damaged tissues; for example, haematopoietic stem cells (HSC) of the bone marrow continuously give rise to new red blood cells, which only have a lifespan of some 120 days. This knowledge is used when conducting bone marrow transplantations for leukemia patients after myeloablative radiation- or chemotherapy (Weissman 2000b; Weissman 2000a; Dreger *et al.* 2007). Unipotent stem cells are committed to give rise to a single cell type, thus providing a storage pool of rapidly renewing differentiated cells; the basal cells in the basal layer (*stratum basale*) of the skin epidermis functions as a stem cell pool for epidermal keratinocytes, for example.

2.4.1.1 Multipotent stem cells from adipose tissue

It seems multipotent stem cells can be isolated from virtually all post-natal tissues, of which bone marrow, trabecular bone, adipose tissue, skeletal muscle, neural tissues, periosteum, synovial membrane, teeth and peripheral blood provide some interesting examples (Weissman 2000b; Weissman

2000a; Chen *et al.* 2006; da Silva Meirelles *et al.* 2006; Horwitz 2006; Kern *et al.* 2006; Horwitz and Dominici 2008; Djouad *et al.* 2009; Mosna *et al.* 2010). These cells usually exhibit plastic adherence, self-renewal, a certain surface antigen profile of CD markers (cluster of differentiation) and differentiation potential towards multiple lineages (Horwitz *et al.* 2005; Dominici *et al.* 2006). Multipotent stem cells derived from both adipose tissue (ASC) and from bone marrow (BMSC) can be regarded as multipotent stromal cells (MSC), but a seed of confusion remains, since BMSC are often simply referred to as MSC in literature. Furthermore, ASC are known by multiple aliases, including AMSC (Adipose tissue-derived mesenchymal stem cells), ADAS cells (Adipose derived adult stromal cells), ATSC (Adipose tissue-derived stem cells), AMC (Adipose-derived mesenchymal cells), PLA (processed lipoaspirate), SVF cells (Stromal vascular fraction) and HADSC (Human adipose derived stem cells). Here, acronyms ASC and BMSC shall be used throughout the text.

Adipose tissue presents a readily accessible reservoir of multipotent stromal cells for tissue engineering purposes. Harvesting cells is minimally invasive and causes little pain or morbidity to the donor. Cells proliferate willingly and can be expanded in monolayer culture. Interest in hASCs was first evoked a decade ago when presence of self-renewing multipotent stem cells in human adipose tissue was shown in several publications (Katz *et al.* 1999; Halvorsen *et al.* 2000; Zuk *et al.* 2001; Zuk *et al.* 2002). Perhaps the most established method of isolating hASCs has been described by Zuk et al. (Zuk *et al.* 2001; Zuk *et al.* 2002). Briefly, minced adipose tissue is digested with collagenase, mature adipocytes are removed based on their buoyancy and red blood cells are removed based on their inability to attach to cell culture plastic. The resulting heterogeneous population of cells exhibits mainly fibroblast-like phenotype and can be expanded in monolayer culture with a stable cell division rate and low level of senescence (Zuk *et al.* 2001).

2.4.1.2 Immunophenotype of hASC

Generally hASC immunophenotype is confirmed with fluorescent activated cell sorting (FACS) at low passage. To be regarded as MSC, cells should express CD73, CD90 and CD105, and lack the expression of CD11b, CD14, CD19, CD34, CD45, CD79α, and HLA-DR (Dominici *et al.* 2006). However, the knowledge of hASC immunophenotype is still at its infancy and the guidelines may soon need updating. It has been noted that the set of surface markers for MSC described above remains ambiguous, as CD34, a common endothelial marker, for example, is invariably expressed in early passage ASC (Gimble *et al.* 2007; Gimble *et al.* 2011; Lindroos *et al.* 2011; Pachon-Pena *et al.* 2011).

Denominating a heterogeneous, unsorted cell population as stem cells is clearly a rational *faux pas*. However, it has been undisputedly shown that the heterogeneous population of hASC contains a subpopulation of multipotent mesenchymal stem cells capable of osteogenic, chondrogenic, adipogenic and myogenic differentiation under defined conditions (Zuk *et al.* 2002; Horwitz *et al.* 2005; Guilak *et al.* 2006). Later on the multilineage potential of hASC has been confirmed by other groups, and new lineage capabilities of hASC have also been described (Strem *et al.* 2005; Guilak *et al.* 2010; Lindroos *et al.* 2011). Thus, multilineage differentiation potential of this heterogeneous cell population is not merely due to presence of different kinds of cells with varying differentiation capabilities, nor due to contaminating stem cells stem cells from sources other than adipose tissue. Yet, as a non-uniform population, the multipotent stromal cells do not fulfill the criteria of stem cells *per se* (Horwitz *et al.* 2005). Make notice also of the fact that the exact origin of ASCs and other mesenchymal stem cells remains elusive. Recent publications postulated that a blood vessel-associated CD34+ cell population may represent the multipotent cells within the hASCs (Zimmerlin *et al.* 2010; Tallone *et al.* 2011).

Understandably, one of the hot topics in hASC research is the search for new and improved cell isolation methods in order to enrich the chondrogenic (or other lineage specific) differentiation potential by applying techniques such as FACS (fluorescence activated cell sorting) or MACS (magnetic activated cell sorting). Rada *et al.* sorted hASC with MACS for various cell surface antigens and compared the isolated subpopulations' relative gene expression levels of osteogenic, chondrogenic and stem cell markers. The results confirmed that multiple subpopulations with varying differentiation capabilities exist within the hASC population (Rada *et al.* 2010). In a related article Jiang *et al*. sorted hASCs with an antibody against CD105 and found that CD105+ cells exhibit stronger osteogenic and chondrogenic potential than do CD105- cells, while CD105- cells show signs of stronger adipogenic potential (Jiang *et al.* 2010). It was also shown that the CD105+ cells produce more homogeneous cartilage like neo-tissue in 3-D culture in polylactic acid coated polyglycolic acid scaffolds (Jiang *et al.* 2010). In register with all this, Rada *et al*. also reported enhanced chondrogenesis in CD105-enriched hASC as judged by expression levels of *COL2A1* and *SOX9* (Rada *et al.* 2010). In addition, similar results have also been obtained with CD105-enriched rat synovial stem cells (Qi *et al.* 2011). As noted above, CD105, endoglin, is an accessory receptor in TGF-b signaling. It seems tempting to speculate that a subpopulation expressing more CD105 may be more responsive to TGF-β, and thus more inducible to chondrogenesis with this growth factor.

2.4.1.3 Adipose-derived *vs.* bone marrow -derived mesenchymal stem cells

It has been demonstrated that BMSC and ASC represent two similar, yet clearly distinct populations of mesenchymal stem cells: Differences in the surface antigen profile (Gronthos *et al.* 2001; Zuk *et al.* 2002; Katz *et al.* 2005; Dominici *et al.* 2006) as well as in overall differentiation potential (Noel *et al.* 2008; Pachon-Pena *et al.* 2011) have been reported. Under currently established conditions, the chondrogenic differentiation potential of hASC has turned out inferior to, or at best, equivalent to that of hBMSC (Zuk *et al.* 2002; Winter *et al.* 2003; Huang *et al.* 2005; Im *et al.* 2005; Mehlhorn *et al.* 2006; Afizah *et al.* 2007; Hennig *et al.* 2007; Noel *et al.* 2008; Diekman *et al.* 2010b; Havlas *et al.* 2011; Hildner *et al.* 2011). In fact, a comparison of proliferative and differentiation capacities of donor matched MSC from various tissues found the synovium-derived hMSC to be superior compared to both hBMSC and hASC (Sakaguchi *et al.* 2005). Moreover, hMSC from both fibrous and adipose synovial tissue possess superior proliferative and differentiation potential compared to hASC from subcutaneous fat (Mochizuki *et al.* 2006). Finally, the adipogenic differentiation potential of hASC has been found superior compared to that of hBMSC (Sakaguchi *et al.* 2005; Pachon-Pena *et al.* 2011), whereas hBMSC seem to display stronger tendency towards hypertrophy and hence osteogenic differentiation (Mehlhorn *et al.* 2006; Noel *et al.* 2008; Diekman *et al.* 2010b). These results suggest that mesenchymal stem cells may have been precommitted towards a certain lineage due to their developmental history and the microenvironment they have resided in. However, the inferior chondrogenesis of hASC may well be overcome by optimizing the chondrogenic conditions (Hennig *et al.* 2007; Goldschlager *et al.* 2009; Kim and Im 2009a; Diekman *et al.* 2010b). Current chondrogenic conditions seem to be quite suboptimal indeed: One study reported a decline in the expression of chondrogenic marker genes after 2 and 3 weeks of culture, accompanied with increased *COL10* expression (Hamid *et al.* 2012).

Lastly, adipose tissue has a high concentration of mesenchymal stem cells compared to other tissues (Pittenger *et al.* 1999; Guilak *et al.* 2006; Kern *et al.* 2006; Jurgens *et al.* 2008). Thus, large numbers of ASC are easily accessible with minimal invasiveness and morbidity. It should be noted, however, that whatever the source tissue, the proliferative and multilineage differentiation capacities of mesenchymal stem cells may depend on donor age, isolation method and possible underlying diseases (Chen *et al.* 2006).

2.4.2 Chondrogenic differentiation of hASC *in vitro*

One of the most sought after applications of stem cell treatments is cartilage regeneration. The chondrogenic potential of hASC has been intensively studied during the first decade after the initial recognition of these cells. Chondrogenic differentiation of MSC from either bone marrow or adipose tissue is traditionally induced in high cell-density 3-D cell-aggregate culture. In essence, culturing cells in aggregates is believed to mimic the prechondral condensation events of embryonic cartilage development and provide cell-cell interactions needed for chondrogenic differentiation (Tavella *et al.* 1994; Johnstone *et al.* 1998; Chimal-Monroy and de Leon 1999; Sekiya *et al.* 2002). There are various methods for obtaining high cell-density aggregates suitable for chondrogenic differentiation. According to the micromass technique, a sitting drop of concentrated cell suspension results in a cell aggregate resembling prechondral condensation and capable of chondrogenic differentiation (Ahrens *et al.* 1977; Denker *et al.* 1995). Another common method to obtain cell aggregates is centrifuging cell suspension in a conical bottom tube to form a stable cell pellet, as described by Johnstone (Johnstone *et al.* 1998) and by others for hBMSC (Mackay *et al.* 1998; Sekiya *et al.* 2001a) and for hASC (Guilak *et al.* 2006). Entrapping chondrocytes (Guo *et al.* 1989) or hASC (Awad *et al.* 2004) within alginate beads has been successful in promoting chondrogenesis as well. However, culturing one aggregate per tube is quite labor intensive and may cause considerable costs in medium consumption. Hence, other methods of achieving 3-D aggregates more cost efficiently have been described.

Novel cell-aggregate culture systems have been developed to make high throughput applications more feasible and/or to solve the problems associated with nutrient gradients generated in macroscopic aggregates. For example, a method based on cell culture plates with microscopic wells has been described (Markway *et al.* 2010). This method produces smaller aggregates with larger surface area-to-volume ratio, which presumably translates to less problems with mass transport and more homogeneous cartilage like structure (Markway *et al.* 2010). Various methods of 3-D aggregate culture for hBMSC have been recently reviewed for osteogenic differentiation (Hildebrandt *et al.* 2011). Non-adherent multi-well culture system was deemed most convenient, since generating spheroids was highly successful and aggregate size could be controlled by adjusting the initial cell concentration (Hildebrandt *et al.* 2011).

With hBMSC, successful aggregate formation and subsequent chondrogenic differentiation has been described in a gravity driven sedimentation system on 96-well plates with conical wells (Welter 2007). No differences were reported between centrifuged and non-centrifuged aggregates, although it

was noted that free aggregation takes roughly 24 hours longer, and thus a lag phase may be seen in chondrogenesis (Welter *et al.* 2007). Cell spheroid culture system employing non-adherent multiwell plates has been previously described for human embryonic stem cells (Ng *et al.* 2005). Based on literature and own experience hASC were aggregated using a straightforward non-adherent 3-D culture system in the present study.

2.4.3 Induction of chondrogenesis with bioactive factors

Mere 3-D culture is not sufficient to induce chondrogenesis, though, and various inductive factors must be added to the culturing medium. At first, the chondrogenic conditions for ASC were essentially a reproduction of those developed for BMSC. However, it now seems that ASC require special conditions for optimal chondrogenesis. The most common bioactive factors used in chondrogenic media are discussed below.

2.4.3.1 Dexamethasone

Dexamethasone (DEX) is a fluorine containing synthetic glucocorticoid analog that was first included in chondrogenic cultures of BMSC at 100 nM (Johnstone *et al.* 1998; Mackay *et al.* 1998) and has been often included in chondrogenic media ever since. However, DEX is used at varying concentrations as an additive in various induction media for hASC, including chondrogenic, osteogenic, myogenic and adipogenic media (Zuk *et al.* 2002; de Girolamo *et al.* 2007; Vater *et al.* 2011).

With hBMSC, synergistic anabolic effects of DEX and TGF-β3 have been noted. Relative mRNA levels and protein synthesis rates of collagen types 2 and -11, COMP, aggrecan and dermatopontin were all upregulated in response to the combination of DEX and TGF-β3, demonstrating overall positive effects on chondrogenesis of hBMSC (Derfoul *et al.* 2006). However, others report that DEX represses the TGF-β induced chondrogenesis in rat articular chondrocytes (Miyazaki *et al.* 2000). On the other hand, DEX elicits contradictory responses in hASC; Firstly, DEX seems to increase DNA content in synergy with TGF-β1 (Estes *et al.* 2006a) and with TGF-β1 and ITS+1 (Awad *et al.* 2003). Secondly, DEX was noted to increase total protein synthesis when combined with TGF-β1 at 5—9 days of culture (Awad *et al.* 2003; Estes *et al.* 2006a). Further, when combined with TGF-β1, dexamethasone has been demonstrated to support 3-D spheroid formation of hASC (Huang *et al.* 2004; Kim and Im 2009a). However, DEX suppresses the TGF-β1-induced proteoglycan synthesis in early hASC culture (Awad *et al.* 2003), although *aggrecan* is upregulated with DEX (Diekman *et al.* 2010b). Finally, DEX may bring forth (unwanted) adipogenic

differentiation: A common expansion medium supplemented with 100 nM DEX has been shown to result in adipocytic phenotype and lipid vacuole formation in hASC *in vitro* (Arutyunyan *et al.* 2009). Similarly, addition of 100 nM DEX to osteogenic differentiation medium does not affect osteogenesis but results in coincident adipogenesis (Arutyunyan *et al.* 2009).

The precise mechanisms of action are currently not known, but DEX presumably activates transcription of genes containing glucocorticoid responsive promoter elements (Vater *et al.* 2011). Interestingly, DEX seems to induce chondrogenic differentiation in hASC via activation of *BMP-6* transcription and suppression of *COL10* (Diekman *et al.* 2010a). The authors reported a stimulative effect of DEX on endogenous *BMP-6* expression, which might have improved the chondrogenic differentiation of the cells to some degree in an autocrine fashion (Diekman *et al.* 2010a). Nonetheless, *COL1* expression was enhanced when DEX was combined with high-dose exogenous BMP-6, and GAG accumulation was restrained by DEX; the authors concluded that DEX has complex effect that strongly depend on and interact with other factors present (Diekman *et al.* 2010a). Furthermore, DEX has been shown to induce the expression of the transcription factor *SOX9* in a dose dependent manner in murine chondrocytes (Sekiya *et al.* 2001b). In addition, DEX was noted to increase the *COL2A1*-reporter construct expression (Sekiya *et al.* 2001b). The overall effects of DEX alone are far from being optimal in supporting chondrogenic differentiation, but when combined with other factors this steroid analog seems beneficial.

2.4.3.2 ITS+1 (insulin transferrin selenite)

ITS+1 is a commercial medium supplement that can be used as a serum replacement in order to avoid lot-to-lot variability. According to the manufacturer, ITS+1 contains 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, 0.5 μg/ml sodium selenite, 50 mg/ml bovine serum albumin and 470 μg/ml linoleic acid (Sigma-Aldrich). Compared to FBS (foetal bovine serum), ITS+1 was found more permissive to cell proliferation (Awad *et al.* 2003).

Structurally similar to insulin, IGF-1 can act as an anabolic factor on chondrocytes and it has been shown to preserve the chondrogenicity of chondrocytes during monolayer expansion (Shakibaei *et al.* 2006). Insulin and/or IGF-1 have been shown to support chondrogenic differentiation and cell proliferation in ATDC5, a murine chondrogenic cell line (Phornphutkul *et al.* 2006), and in other chondrogenic cell lines and primary cells (Phornphutkul *et al.* 2004), in rabbit ASC (An *et al.* 2010) as

well as in hASC (Kim and Im 2009a). At present, most chondrogenic media contain ITS+1 and hence insulin (Puetzer *et al.* 2010).

2.4.3.3 Ascorbic acid-2-phosphate

Ascorbic acid is an essential cofactor in collagen biosynthesis (Kao *et al.* 1976; David L Nelson 2008) and is therefore commonly included in chondrogenic media to augment collagen biosynthesis. Positive effects on chondrogenesis have been reported in different cell types. Ascorbate supported cartilage ECM production in ATDC5 murine cell line (Temu *et al.* 2010). In another study ascorbate was shown to support the biosynthesis of collagen types 2 and -10 and increase the ALP activity in chick embryo epiphyseal chondrocytes (Shapiro *et al.* 1991). In hBMSC, ascorbate can increase the cell proliferation as well as GAG and collagen synthesis (Choi *et al.* 2008). In hASC, ascorbate has been shown to support cell proliferation (Potdar and D'Souza 2010) and increase the synthesis rate of protein and PG (Awad *et al.* 2003).

Ascorbate can support the maturation and terminal differentiation of chondrocytes, leading to hypertrophy, collagen type 10 synthesis and ALP activity (Shapiro *et al.* 1991; Sullivan *et al.* 1994; Farquharson *et al.* 1998). Hence, ascorbate is frequently used in osteogenic differentiation media. On the other hand, human chondrocytes display hypertrophic phenotype and undergo calcification *in vitro* even in the absence of ascorbate in three-to-four weeks (Kirsch *et al.* 1992) whereas hASC cultured for two weeks under chondrogenic conditions with ascorbate do not display signs of hypertrophy (Wickham *et al.* 2003).

2.4.3.4 TGF-β

Although the molecular mechanisms remain partly elusive, it is well established that TGF-β isoforms exert strong chondrogenic stimuli on mesenchymal stem cells; only 6 out of 41 chondrogenic media reviewed recently did not contain any of these factors, and in these cases BMP-isoforms were included instead (Puetzer *et al.* 2010). Already in 1995 TGF-β1 was reported to stimulate accumulation of collagen type 2 and CRTL-1 accompanied with synthesis of PG in mouse a multipotent cell line (Denker *et al.* 1995). Similar chondrogenic effects have been described with rabbit BMSC in medium containing a combination of TGF-β1 and DEX (Johnstone *et al.* 1998). Later on, TGF-β1 has been reported to induce chondrogenesis in hBMSC (Barry *et al.* 2001; Weiss *et al.* 2010), and hASC (Zuk *et al.* 2001; Erickson *et al.* 2002; Zuk *et al.* 2002; Awad *et al.* 2003; Awad *et al.* 2004; Huang *et al.* 2004). A dose of 10 ng/ml is commonly used for hASC. A smaller concentration takes effect much

slower whereas a higher concentration becomes detrimental for chondrogenesis (Huang *et al.* 2004), although others have reported higher concentrations (25 ng/ml) being more effective in inducing chondrogenesis (Kim and Im 2009a). TGF-β1, TGF-β2 and TGF-β3 induce roughly similar chondrogenic effects on hASC *in vitro*, although some disagreement remains as to which is the most potent (Estes *et al.* 2006a; Kim and Im 2009b).

Not only does TGF-β induce cartilaginous ECM synthesis, but also seems to support precartilage condensation in mouse limb bud cells cultured in high cell-density by increasing cell—cell and cell—ECM interactions by upregulating N-cadherin, N-CAM (neural cell adhesion molecule), tenascin and fibronectin (Chimal-Monroy and de Leon 1999). Similarly, in human trabecular bone MSC, N-cadherin expression was induced with TGF-β (Tuli *et al.* 2003). With the aid of MAPK inhibitors, that study concluded that TGF-β induces the phosphorylation of Erk-1, p38 and JNK, which subsequently effect overt chondrogenesis: N-cadherin expression, elevated expression of *SOX-9, COL-10, COL-9 COL-2, IGF-1* and *aggrecan* combined with enhanced GAG accumulation (Tuli *et al.* 2003). The study also proposed that the aforementioned MAP kinases control WNT-7 (wingless-type MMTV integration site, member 7), which might be responsible for the progressive downregulation of N-cadherin; a transition necessary for the conversion from condensed cell mass into ECM producing mature chondrocytes (Tuli *et al.* 2003).

2.4.3.5 BMP-6

The acronym BMP, bone morphogenetic protein, was derived from early experiments demonstrating induction of ectopic bone formation (Urist 1965). Various BMPs may be beneficial for chondrogenesis when combined with TGF-β, although neither TGF-β nor any BMP alone seem to be sufficient. Combining TGF-β with BMP-4, -6, or especially BMP-2 resulted in enhanced chondrogenesis in hBMSC (Sekiya *et al.* 2005). In rabbit ASC, BMP-2 has been reported to support chondrogenesis by inducing collagen type 2 synthesis and GAG accumulation (Wei *et al.* 2006). In hASC, BMP-2 alone was noted to drive a mixed osteochondral differentiation and mineralization (Mehlhorn *et al.* 2007). However, BMP-2 in combination with TGF-β promoted more exclusive chondrogenesis as noted by increased *COL2* and *COMP* expression and elevated GAG amounts, although *COL10* expression persisted as well (Mehlhorn *et al.* 2007). It was suggested that hASC hypertrophy is controlled by two transcription factors, Dlx-5 and Msx-2. BMP-2 was noted to promote the expression of Dlx-5, which in turn stimulates *COL10*-, *RUNX2* and *ALP* expression, leading to mineralization and osteogenesis,

whereas TGF-β induced the expression of Msx-2, which antagonized the Dlx-5 effects, effectively preventing mineralization and osteogenesis (Mehlhorn *et al.* 2007).

On the other hand, BMP-6 has been recently high-lighted for its beneficial effects on chondrogenesis of hBMSC (Weiss *et al.* 2010) and hASC (Estes *et al.* 2006a; Estes *et al.* 2006b; Hennig *et al.* 2007; Diekman *et al.* 2010a; Diekman *et al.* 2010b). Of note, BMP-6 has been shown to induce chondrogenesis in hASC by upregulating expression of *aggrecan* and *COL2A1*, but also by suppressing *COL10A1*, thereby preventing hypertrophic phenotype at 7 days (Estes *et al.* 2006a) and even at 28 days (Estes *et al.* 2006b). These results of BMP-6 suppressing hASC hypertrophy while supporting chondrogenesis have been confirmed more recently (Diekman *et al.* 2010a; Diekman *et al.* 2010b). It seems the *COL10*-suppression is only obtained with a high dose of BMP-6, because others have reported *COL10* induction with a lower dose of BMP-6 in hASC (Hennig *et al.* 2007).

Of note, BMP-6 seems to overcome the inferior chondrogenic potential of hASC compared to hBMSC to some degree. It has been shown that, unlike hBMSCs, hASCs lack the expression of TGF-βR1, a key receptor involved in the signal transduction of TGF-β family proteins (Hennig *et al.* 2007). However, medium supplementation with BMP-6 induced the expression of TGF-βR1 in hASCs, thereby sensitizing the cells to TGF-β signaling. The combination of BMP-6 and TGF-β3, both 10 ng/ml, was found the most potent inducer of chondrogenesis in hASCs, although hypertrophy was evident both *in vitro* and *in vivo* (Hennig *et al.* 2007). In addition, others have reported similar effects on hASC with a combination of BMP-7 and TGF-β2 (Kim and Im 2009b). Intriguingly, BMP-6 is endogenously expressed in hypertrophic chondrocytes along with hypertrophic markers such as RUNX2, alkaline phosphatase and collagen type 10 (Kronenberg 2003; Goldring *et al.* 2006).

2.4.3.6 Serum and other factors

Common media supplements such as L-glutamine, penicillin and streptomycin are included in chondrogenic media routinely. Addition of FBS to the chondrogenic medium has been shown to increase collagen type 2 deposition (Diekman *et al.* 2010b), but a serum-free, or at least xeno-free option is more desirable from a clinical point of view (Heng *et al.* 2004; Gimble *et al.* 2011; Lindroos *et al.* 2011). To support the metabolism and cartilage ECM synthesis, nutrients such as sodium pyruvate and L-proline are usually added into chondrogenic media. Although beyond the scope of this study, it is worth mentioning that a bioreactor environment may be employed to specifically promote

cell differentiation and matrix production by mechanical stimulation or hypoxia (Altman *et al.* 2001; Darling and Athanasiou 2003; Chen *et al.* 2006; Sittichockechaiwut *et al.* 2009).

2.5 Future of hASC in cartilage defect treatment

A clinically applicable hASC treatment for cartilage defects is still out of reach. However, the aforementioned problem with chondrocyte dedifferentiation may be overcome to some degree by using human chondrocytes cocultured with hASC (Hildner *et al.* 2009; Lee and Im 2010). It was shown that a coculture (1:1) of chondrocytes and ASC results in cartilage formation comparable to that obtained with chondrocytes alone, and similar results were obtained for ASC cultured in chondrocyte conditioned medium (Lee and Im 2010). Chondrocyte conditioned medium and chondrocyte-hASC coculture have also been shown to result in enhanced chondrogenesis by others (Hwang *et al.* 2011). However, results of Lee and Im were only compared with human chondrocytes from osteoarthritic cartilage, and results of Hwang *et al.* only to sheep auricular chondrocytes. Also, Hildner and colleagues obtained inferior results with coculture compared to pure chondrocytes, although hASC seemed to contribute to cartilage related matrix production (Hildner *et al.* 2009). Taken together, it seems feasible to use a combination of hASC and chondrocytes for the ACI procedure in order to reduce the size of cartilage biopsies needed for treatment, but the culture conditions and ASC-chondrocyte ratio still need further optimization. Besides, hASC undergo phenotypical changes as well during monolayer-expansion; the expansion conditions may prime hASC towards a certain lineage, such as chondrogenesis (Estes *et al.* 2008).

In any case, safety becomes of utmost importance when using stem cell -based treatments for patients, especially for patients with non-lethal conditions. Contradictory results have been published regarding ASC safety. On one hand, ASC seem to promote the growth and invasiveness of pre-existing tumours (Muehlberg *et al.* 2009; Jotzu *et al.* 2011; Kucerova *et al.* 2011; Zimmerlin *et al.* 2011) but on the other hand suppression of tumour growth has also been reported for ASC (Kucerova *et al.* 2008; Cousin *et al.* 2009) and for BMSC (Mosna *et al.* 2010). So far, treating patients with cartilage defects using various ACI-like approaches with hBMSC have resulted in reasonable success with no serious problems (Wakitani *et al.* 1994; Wakitani *et al.* 2002; Wakitani *et al.* 2004; Kuroda *et al.* 2007; Wakitani 2007; Giannini *et al.* 2009; de Girolamo *et al.* 2010; Nejadnik *et al.* 2010; Kasemkijwattana *et al.* 2011; Wakitani *et al.* 2011).

3. Aims of the research

The aim of the present study was to evaluate the effects of DEX, human serum and BMP-6 on chondrogenesis of hASC. In order to further examine the effects of BMP-6 on hASC this growth factor was administered in two separate manners: a constant low dose or alternatively an initial high dose followed by constant low dose. Five different chondrogenic media compositions were used to induce differentiation of hASC during a culture period of 14 and 28 days. Quantitative real time reverse transcription PCR array was used to measure the relative expression levels of 84 genes related to osteo- and chondrogenesis. A selected subset of these results is presented in this thesis, based on main interests of the study. In addition, paraffin sections were stained for detection of extracellular matrix components characteristic to hyaline cartilage, namely sulfated proteoglycans, sulfated glycosaminoglycans and collagen types 1 and 2. In the long run, the aim is to understand and be able to administer the exogenous cues needed for successful chondrogenic differentiation of hASC. This knowledge is paramount in order to safely and efficiently regenerate damaged articular cartilage using the patient's own easily accessible stem cell reservoirs.

4. Materials and methods

4.1 Cell culture

Human adipose stem cells (hASCs) were isolated from subcutaneous mammary adipose tissue of three female donors, aged between 40 and 42 years. The cells were isolated from fresh tissue samples according to a protocol adapted from Zuk and colleagues (Zuk *et al.* 2001). Briefly, tissue samples were washed with Dulbecco's phosphate buffered saline, DPBS (Lonza, Basel, Switzerland) and homogenized with sterile scissors. Homogenate was mixed with equal volume of 1,5 mg/ml collagenase type 1 solution (Gibco, Invitrogen, Carlsbad, California, USA) in 50 ml falcon tubes and incubated for 90 minutes in a water bath at 37 ºC with continuous agitation. hASC were separated from mature adipocytes and connective tissue remnants by centrifugation at 600 g for 10 minutes. Contaminating red blood cells in the pellet were lysed by adding sterile water (Baxter, Deerfield, Illinois, USA) and mixing gently for one minute. After adding 18 ml of DPBS to restore isotonic saline concentration, the suspension was centrifuged at 600 g for 10 minutes. The resulting pellet was suspended in 10 ml of medium and passed through a 100-µm filter (Becton, Dickinson and company, Franklin Lakes, New Jersey, USA) to remove any remaining debris. The cells were then plated onto 75 cm^2 polystyrene cell culture flasks (Nunc, Roskilde, Denmark) in expansion medium containing DMEM/F-12 (Gibco, Invitrogen, Carlsbad, California, USA), 10 *vol*-% human serum (PAA Laboratories, Pasching, Austria), 1 *vol*-% GlutaMAX^{TM} (Gibco) and 100 units/ml of both penicillin and streptomycin (Lonza). The cells were expanded at 37 ºC humidified incubator with 5 % carbon dioxide atmosphere. After one day of culture the cells were washed with DPBS to remove any dead cells, debris or remaining lipids before changing medium. Hereafter half of the culture media was changed every three to four days (see Figure 8). Cells were grown near confluent, and then harvested with Tryple Select (Gibco, Invitrogen, Carlsbad, California, USA) for re-plating. Two of the three cell lines used in the study had been cryopreserved in gas phase liquid nitrogen storage system (MVE Biological Systems, Chart Industries, Garfield Heights, Ohio, USA) for approximately one year. Cryopreserved cells had been stored in freezing solution consisting of 10 vol-% dimethyl sulphoxide (Sigma-Aldrich, St. Louis, Missouri, USA) in human serum (PAA Laboratories). All cell lines were analyzed with FACS (fluorescence activated cell sorting) at passage 1 to ascertain the cell surface marker profile is in agreement with that published elsewhere for hASC (Gronthos *et al.* 2001; Katz *et al.* 2005; Dominici *et al.* 2006).

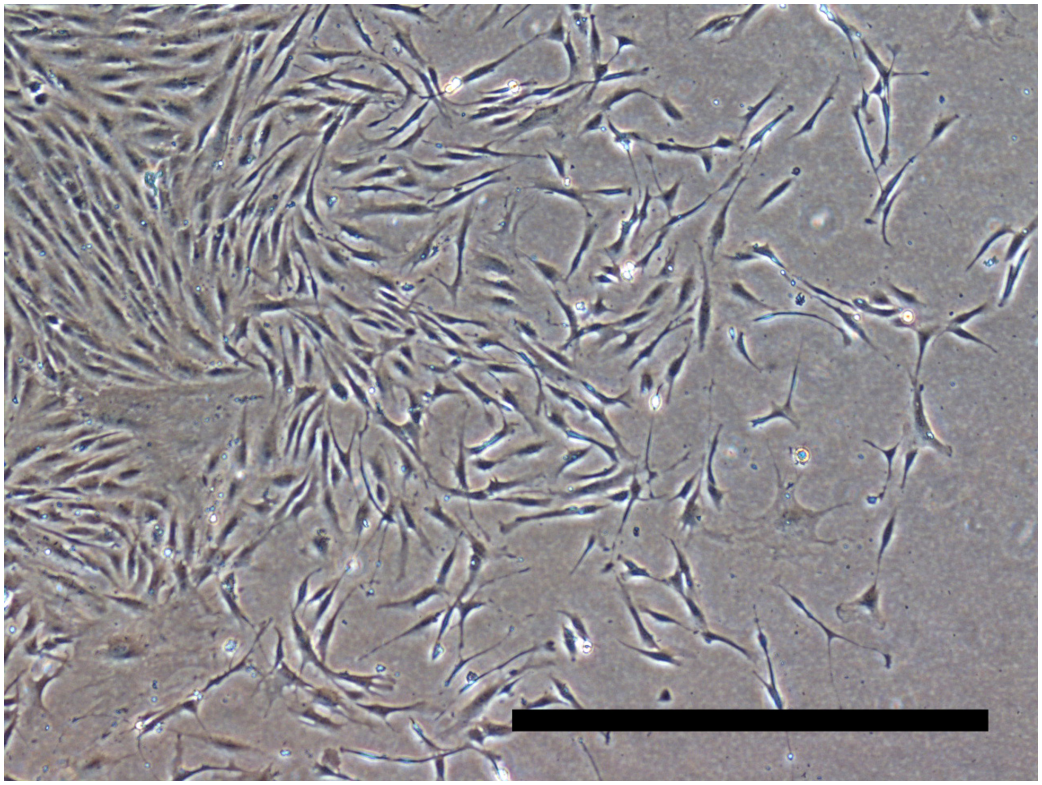

Figure 8 - hASC at passage 4 nearing confluence during monolayer expansion. A semi-heterogeneous population of fibroblast like cells along with more flattened cells is obtained with the isolation method described in text. Scale bar: 1 mm

The ASCs were monolayer-cultured until passage 4, then harvested with Tryple Select (Gibco), counted with haemocytometer and suspended in three different media; the aforementioned expansion medium, basal chondrogenic medium and basal chondrogenic medium with 1 vol-% human serum (PAA Laboratories), abbreviated EM, CM and CM-HS, respectively. The basal chondrogenic medium contains DMEM/F12 (Gibco), 1 % Glutamax (Gibco), 1 vol-% ITS+1 liquid media supplement (Sigma-Aldrich), 20 units/ml of both penicillin and streptomycin (Lonza), 50 µg/ml ascorbic acid-2-phosphate (Sigma-Aldrich), 55 µg/ml sodium pyruvate (Lonza) and 23 µg/ml L-proline (Sigma-Aldrich). Cells in EM, CM and CM-HS suspensions were plated on ultra-low attachment 24-well plates (Corning Life Sciences, Corning, New York, USA) in quantity of approximately 200 000 cells in 500 µl of medium per well. These ultra-low attachment plates have been coated with a hydrogel layer, which prevents cellular adhesion, thus making non-adherent culturing convenient. According to the manufacturer, the coating is very stable, non-cytotoxic, biologically inert and non-degradable.

The cell concentration was optimized for the ultra-low attachment plates with a pilot experiment; medium volumes of 1 ml and 500 µl and cell concentrations varying between 25 000 and

1 000 000 ml^{-1} were screened before the actual medium comparison study (see appendix A1). The cell concentration used here is in agreement with other published methods of producing 3-D cell aggregates from human mesenchymal stem cells (Johnstone *et al.* 1998; Mackay *et al.* 1998; Awad *et al.* 2004; Welter *et al.* 2007; Markway *et al.* 2010; Hildebrandt *et al.* 2011).

Table 2 - The media included in the study. The varying medium components and their concentrations in the chondrogenic media are presented in this table. All chondrogenic media also contain the components of basal chondrogenic medium, as described in text. Abbreviations: HS = human serum, DEX = dexamethasone, TGF-β1 = Transforming growth factor β1, BMP-6 = bone morphogenetic protein 6. See text for further discussion.

	HS	DEX	TGF-β1	BMP-6
Expansion medium				
A	10 vol-%	-	-	-
Chondrogenic media				
B	-	-	10 ng/ml	-
C	-	100 nM	10 ng/ml	10 ng/ml
D	-	100 nM	10 ng/ml	500 ng/ml for 2 changes, 10 ng/ml for the remainder
E	1 vol-%	-	10 ng/ml	10 ng/ml
F	1 vol-%	100 nM	10 ng/ml	500 ng/ml for 2 changes, 10 ng/ml for the remainder

After three days of non-adherent culture on ultra-low attachment plates the media were changed and bioactive factors were added to induce chondrogenesis. From here on the media were changed under microscope every third to every fourth day. Aggregates were maintained in static culture conditions for 14 and 28 days, counting from the addition of bioactive factors. Three bioactive factors were used in various combinations to study their putative effects on chondrogenesis of hASC; DEX (Sigma-Aldrich) in a concentration of 100 nM, TGF-β1 (Sigma-Aldrich) in a concentration of 10 ng/ml and lastly BMP-6 (R&D Systems, Minneapolis, Minnesota, USA) in either continuous low dose (10 ng/ml) or short-term high dose followed by a lower dose (500 ng/ml for the first two medium changes and 10 ng/ml thereafter). The abovementioned differentiating factors were all added in correct

combinations to the basal CM during media changes. The media compositions included in this study are presented below in Table 2.

4.2 RNA extraction

RNA samples were isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) and following the animal cell spin protocol in "RNeasy® Mini Handbook, 4th edition, April 2006". In brief, at each time point the media were aspired and aggregates washed with DPBS (Lonza). Aggregates from triplicate wells were pooled together to increase the RNA yield. Three wells added up to approximately 10 aggregates or so per sample. After DPBS aspiration the aggregates were suspended in lysis buffer included in the RNeasy kit (RNeasy buffer RLT), with 1 vol-% β-mercaptoethanol (Sigma-Aldrich) added to inhibit RNAase activity. Aggregates were homogenized in a reduced volume by pressing against the tube walls with pipette tip and pipetting gently up and down. Samples were purified from interfering DNA contamination with RNAase-free DNAase Set (Qiagen) according to the aforementioned protocol, after which RNA was eluted in RNAase free water from the RNeasy® Mini Kit. Following elution, a sample for concentration determination was taken to a separate tube, and the rest of the RNA was stored at -70 ºC for a maximum of two weeks before reverse transcription. The concentrations and purities of the RNA samples were measured with NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Average RNA yield was 50,4 ng/µl with standard deviation of 24,7 ng/µl, and hence the samples were diluted to same concentration, as described below. Absorbance at 260 nm divided by absorbance at 280 nm was 2,09 on average, with standard deviation of 0,2.

4.3 Reverse transcription

The reverse transcription and the Q-RT-PCR were performed according to the following protocol: "User Manual, RT2 profiler™ PCR Array System, part #1022A, version 4.26, May 17, 2010, SABiosciences". Briefly, the RNA samples were diluted in sterile water (B. Braun, Melsungen, Germany) to achieve 320 ng RNA per genomic DNA elimination reaction. (Note that DNAase treatment was included in both RNA isolation and reverse transcription steps.) For genomic DNA elimination, 2 µl of 5x Genomic DNA elimination buffer was mixed with the RNA sample in a total volume of 10 µl. Samples were incubated for 5 minutes at 42 ºC and then chilled on ice. Next, 10 µl of

reverse transcription cocktail (Table 3) was added to each sample and the samples were reverse transcribed to cDNA using the RT^2-First Strand Kit (SABiosciences, Qiagen, Hilden, Germany).

Table 3 - Reverse transcription reaction cocktail

5 x RT buffer (BC3)	4 µl
Primer & external control Mix (P2)	1 µl
RT Enzyme mix 3 (RE3)	2 µl
H_2O	3 µl
Total volume of RT cocktail	10 µl
320 ng of RNA after genomic DNA elimination	10 µl
Total volume of RT reaction mix	**20 µl**

Reverse transcription reactions were first incubated for 15 minutes at 42 ºC, then immediately heated to 95 ºC on a separate heat block for 5 minutes for enzyme inactivation. Next, 91 µl of water (B. Braun) was added to each reaction mix, and the cDNA products stored at - 20 ºC for up to four months. The temperatures in two heat blocks were set to match those suggested in the protocol with quicksilver thermometers.

4.4 Quantitative real-time PCR-array thermal cycling

Quantitative real time polymerase chain reactions (q-RT-PCR) were performed using RT^2-Profiler human osteogenesis PCR array plates (SABiosciences, Qiagen, Hilden, Germany) and RT^2- SYBR Green/ROX master mixes from the same company (SABiosciences, Qiagen, Hilden, Germany), see Table 4 for details of the PCR reaction mix. Plates were sealed with thin-walled optical cap strips provided with the array plates.

Table 4 - Quantitative PCR array reaction mix

RT^2- SYBR Green/ROX master mix	1350 µl
cDNA Reaction mix	102 µl
H_2O	1248 µl
Total volume of PCR reaction	**2700 µl**

Plates were briefly centrifuged to ensure all the liquid is at the bottom of the wells and that no interfering bubbles exist. The thermal cycling conditions used on ABI Prism sequence detection system model 7300 (Applied Biosystems, Foster City, California, USA) are described below.

1. 10 min pre heating at 95 ºC
2. 40 cycles of:
    a. 15 sec at 95 ºC
    b. 1 min at 60 ºC
3. Melting curve analysis (95 ºC 15 sec, 60 ºC 1 min, 95 ºC 15 sec)
4. Hold at 4 ºC

4.5 Q-RT-PCR data analysis background

An adaptation of the mathematical $2^{-\Delta\Delta Ct}$ model published by Livak and Schmittgen (Livak and Schmittgen 2001) was used for obtaining the relative expression levels of target genes. The method assumes roughly equal efficiencies for all primer pairs and all PCR reactions. In brief, the threshold cycle values of the genes of interest are normalized using a reference gene or -genes. The normalized threshold cycle values (ΔC_t values) are inversely proportional to the initial amount of the target sequence in a sample. The amount of target sequence is presumed to double in each cycle. While such optimal conditions may never be possible, the results will still be valid if the efficiencies of different primer pairs and different PCR reactions are equal enough, as they are according to the manufacturer. (Primer sequences could not be obtained from the manufacturer). Hence, the level of expression of any gene in any sample can be expressed as $2^{-\Delta Ct}$. Relative expression is obtained by dividing the experimental sample's level of expression with the calibrating sample's level of expression of a certain gene, i.e. $2^{-\Delta\Delta Ct}$.

4.6 The $2^{-\Delta\Delta Ct}$ method according to SABiosciences

The C_t thresholds were set comparable across all PCR array plates. This was done, as instructed by the manufacturer, by manually adjusting the C_t of the built-in positive PCR control samples on each plate to 18 cycles. Thereafter, all target gene threshold cycle (C_t) values within each plate were normalized

to varying cDNA amount by using the set of five reference genes, namely *β-2-microglobulin*, *hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase* and *β-actin*. The ΔC_t values were then calculated by subtracting the average of the five reference genes' C_t from the C_t of the gene of interest (GOI) on the same plate (*formula 1*).

$$\Delta C_t = C_t \text{ (GOI)} - \text{average } [C_t \text{ (reference)}] \qquad \textit{formula 1}$$

$\Delta\Delta C_t$ values were then obtained by subtracting the ΔC_t value of the calibrating sample from the ΔC_t value of another sample. In this study, the normalized C_t values ($=\Delta C_t$) for each GOI were separately calibrated to the 2-week sample cultured in the basal chondrogenic medium (medium B in Table 2). Each cell line was calibrated separately, and all samples, including 4-week samples, were calibrated to the same two-week basal chondrogenic sample (*formula 2*). Samples cultured in expansion medium resulted in poor RNA yield and quality, and were therefore not used as calibrators, as was initially planned. Instead, these samples were calibrated to basal chondrogenic medium along with all the other samples.

$$\Delta\Delta C_t = \Delta C_t \text{ (experimental sample)} - \Delta C_t \text{ (calibrator sample)} \qquad \textit{formula 2}$$

Relative expression levels were obtained by calculating $2^{(-\Delta\Delta Ct)}$, assuming overall comparable PCR efficiencies across all primer pairs. Samples used as calibrators will get a value of 1 by definition, since $-\Delta\Delta C_t$ will be zero. Calculations were performed with SABiosciences web based program and manually checked. The genes analyzed and discussed in this study are listed in Table 5. The full list of genes analyzed on the Q-RT-PCR array plate is given in the appendix.

4.7 Biochemical stainings and immunocytochemistry

At each time point, the media were aspired and aggregates washed with PBS. Aggregates were fixed overnight in 4 % para-formaldehyde in PBS solution at room temperature and then transferred to 70 % ethanol. Samples were dehydrated using a series of increasing ethanol concentrations and xylene before embedding in paraffin. For all staining procedures, 5 µm sections were taken and sections of rabbit trachea were used as positive controls.

Table 5 - List of genes of interest. (For full list of genes analyzed, see appendix A2).

Abbreviation	Gene name	Alternate gene name(s)
	Chondrogenic marker genes	
BGN	Biglycan	DSPG1, PG-S1, PGI, SLRR1A
COL11A1	Collagen, type 11, alpha 1	CO11A1, COLL6, STL2
COL12A1	Collagen, type 12, alpha 1	BA209D8.1, COL12A1L, DJ234P15.1
COL2A1	Collagen, type 2, alpha 1	ANFH, AOM, COL11A3, MGC131516, SEDC
COL3A1	Collagen, type 3, alpha 1	EDS4A, FLJ34534
COMP	Cartilage oligomeric matrix protein	EDM1, EPD1, MED, MGC131819, MGC149768, PSACH, THBS5
SOX9	SRY (sex determining region Y)-box 9	CMD1, CMPD1, SRA1
	Osteogenic marker genes	
ALP	Alkaline phosphatase, liver/bone/kidney	AP-TNAP, FLJ40094, FLJ93059, HOPS, MGC161443, MGC167935, TNAP, TNSALP
COL10A1	Collagen, type 10, alpha 1	-
COL1A2	Collagen, type 1, alpha 2	OI4
RUNX2	Runt-related transcription factor 2	AML3, CBFA1, CCD, CCD1, MGC120022, MGC120023, OSF2, PEA2aA, PEBP2A1, PEBP2A2, PEBP2aA, PEBP2aA1
SERPINH1	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	AsTP3, CBP1, CBP2, HSP47, PIG14, PPROM, RA-A47, SERPINH2, gp46
	TGFβ signaling related genes	
BMP1	Bone morphogenetic protein 1	FLJ44432, PCOLC, PCP, TLD, pCP-2
BMP2	Bone morphogenetic protein 2	BMP2A
BMP4	Bone morphogenetic protein 4	BMP2B, BMP2B1, MCOPS6, ZYME
BMP6	Bone morphogenetic protein 6	VGR, VGR1
TGFβ1	Transforming growth factor, beta 1	CED, DPD1, TGFB, TGFbeta
TGFβR1	Transforming growth factor, beta receptor 1	AAT5, ACVRLK4, ALK-5, ALK5, LDS1A, LDS2A, SKR4, TGFR-1
TGFβR2	Transforming growth factor, beta receptor II (70/80kDa)	AAT3, FAA3, LDS1B, LDS2B, MFS2, RIIC, TAAD2, TGFR-2, TGFbeta-RII
	Housekeeping genes	
B2M	Beta-2-microglobulin	-
HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT, HPRT
RPL13A	Ribosomal protein L13a	-
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, MGC88685
ACTB	Actin, beta	PS1TP5BP1

The presence of glycosaminoglycans (GAG) was visualized with Alcian blue (Sigma Aldrich) and Toluidine blue (Sigma Aldrich) stains. Alcian blue (AB) specifically stains sulphated GAG at low pH (Lev and Spicer 1964). Nuclear fast red stain (Biocare medical, California, USA) was used as a counter stain with Alcian blue. Toluidine blue (TB) stains sulphated GAG pinkish or purple by metachromasia, while other cellular components remain various shades of blue (Shepard and Mitchell 1976; Schmitz *et al.* 2010).

For antigen retrieval the sections were incubated for 30 min at 37 ºC in 0,2 % trypsin (Sigma Aldrich) and additionally for 30 min at room temperature in a mixture of 0,125 U/ml chondroitinase ABC (Sigma Aldrich) and 0,725 U/ml hyaluronidase (Sigma Aldrich). The presence of collagen fibres was detected with antibodies for collagen type 1 (Sigma Aldrich) and collagen type 2 (Developmental Studies Hybridoma Bank, Iowa, USA) using 1:2000 and 1:1000 dilutions, respectively. Antibody labelling steps and wash steps were performed in a humid chamber and in washing racks, respectively. Primary antibodies were labelled with a biotinylated secondary antibody (Vector laboratories, California, USA, cat. BA-2001) diluted 1:500. After the secondary antibody binding a complex of avidin and biotinylated peroxidase (Vector laboratories) was incorporated to the secondary antibody to enhance antigen detection according to manufacturer's instructions. A 3,3'-diaminobenzidine (DAB) based chromogen kit (Invitrogen) was used for colour reaction. Control sections were made by substituting the secondary antibody with plain antibody dilution solution free of any antibodies.

4.8 Statistical analysis

The quantitative real time Q-RT-PCR results were checked for statistical significance with SPSS 19 statistics software (IBM Corporation, Armonk, New York, United States). Results of both time points were analyzed separately with non-parametric two-sided Kruskal-Wallis test. After performing Kruskal-Wallis test, the adjusted P values of the pairwise comparisons between two groups were used as measures of significance.

4.9 Ethical concerns

All patients have given approval for research use of their tissue samples under informed consent. The study was performed with the authorization from Pirkanmaa hospital district's ethical committee (permission number R03058).

4.10 Computer software used

Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, California, USA), Applied Biosystems 7300 System software (Life Technologies Corporation, Carlsbad, California, USA), EndNote 9 (Thomson Reuters, New York, New York, USA), GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, California, USA), IBM SPSS Statistics 19 (IBM Corporation, Armonk, New York, United States), Microsoft Office tools 2003 (Microsoft Corporation, Redmond, Washington, USA).

5. Results

5.1 Surface antigen profile of the cell lines

FACS-results at P1 (see Table 6) confirmed that all hASC cell lines used in this study exhibited a surface antigen profile similar to that described in literature (Gronthos *et al.* 2001; Katz *et al.* 2005; Dominici *et al.* 2006; Lindroos *et al.* 2011).

Table 6 - Comparison of cell surface marker expression in this study and in literature. * Numbers correspond to percentage of cells expressing protein on their surface. ** Mean percentage of cells expressing protein on their surface ± standard error mean. *** Mean percentage of cells expressing protein on their surface ± standard deviation. **** Symbols refer to the markers' expression frequency on cells' surface; + ≥ 95%, - ≤ 2%.

	CD marker										
	14	**19**	**34**	**45**	**49d**	**73**	**90**	**105**	**106**	**HLA-ABC**	**HLA-DR**
Cell line *											
#1	8,3	1,4	14,4	4,2	24,1	92,2	98,5	83,1	0,5	11,8	2,7
#2	1,9	0,7	17,8	2,5	34,5	97,7	99,3	86,4	1,7	18,9	1,0
#3	1,3	0,4	10,8	1,7	63,4	99,4	99,8	95,6	0,9	58,7	0,4
Gronthos *et al.* 2001 **	0		28±13	0	9±2			36±9		93±3	1±0
Katz *et al.* 2005 ***					78±20		98±1		1±2	97±2	1±1
Dominici *et al.* 2006 ****	-	-	-	-		+	+	+			-

5.2 Non-adherent culture system

Plating 200 000 hASC in 500 µl on non-adherent wells resulted in approximately 2—4 large aggregates and several smaller cell bundles in all tested media. During the first few days of culture the aggregates were smaller in size and greater in numbers (termed pre-aggregates), but gradually adhered to each other. Aggregates in chondrogenic media had a diameter of roughly 1 mm, but the aggregates in expansion medium were considerably smaller (see Figure 9).

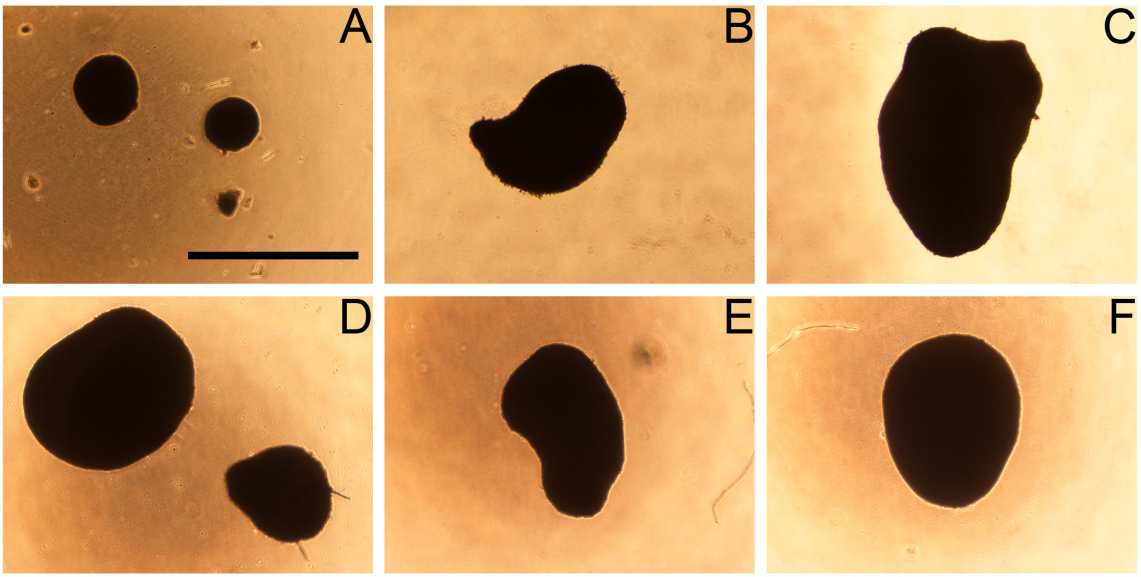

Figure 9 - hASC at P5 in different culture media after 27 days of non-adherent culture. Figures of one representative cell line are shown. Media compositions A to F are those presented in Table 2 and in text. Scale bar 1 mm.

5.3 Biochemical stainings

Results of different media compositions are presented here using the nomenclature shown in Table 2. Representative staining images of each culture condition are shown below.

5.3.1 Alcian blue staining and Toluidine blue staining

Toluidine blue staining was shown to function properly in rabbit trachea slides where GAG was easily distinguishable by pinkish or purple colour (see appendix A3). In expansion medium at 14 days only faint evidence of GAG was seen mainly in the middle of the aggregates with toluidine blue (TB) and alcian blue (AB) stainings. In the expansion medium by 28 days there seemed to be no positive staining whatsoever. The basal chondrogenic medium B resulted in strong and uniform positive staining with both AB and TB at 14 days. At 28 days, the positive staining was somewhat fainter and masked by increase in cell content. In medium C at 14 days, the positive staining with AB and TB was intermediate, while the overall structure seemed quite dense with ECM. At 28 days, the positive staining with AB and TB seemed to decrease slightly.

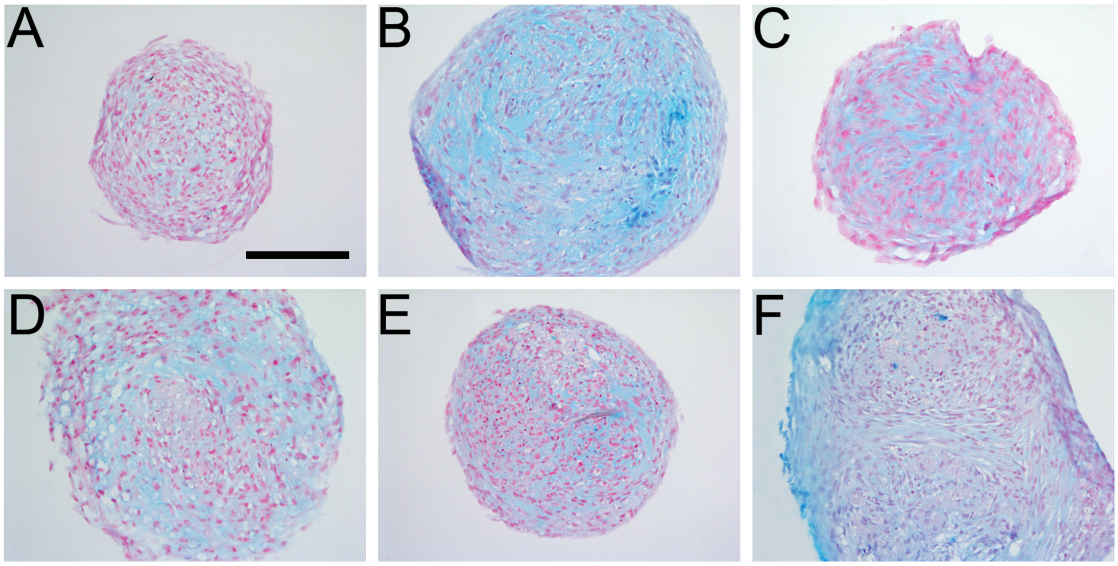

Figure 10 - Alcian blue staining at 14 days. Media compositions A to F are those presented in Table 2 and in text. Scale bar: 200 µm

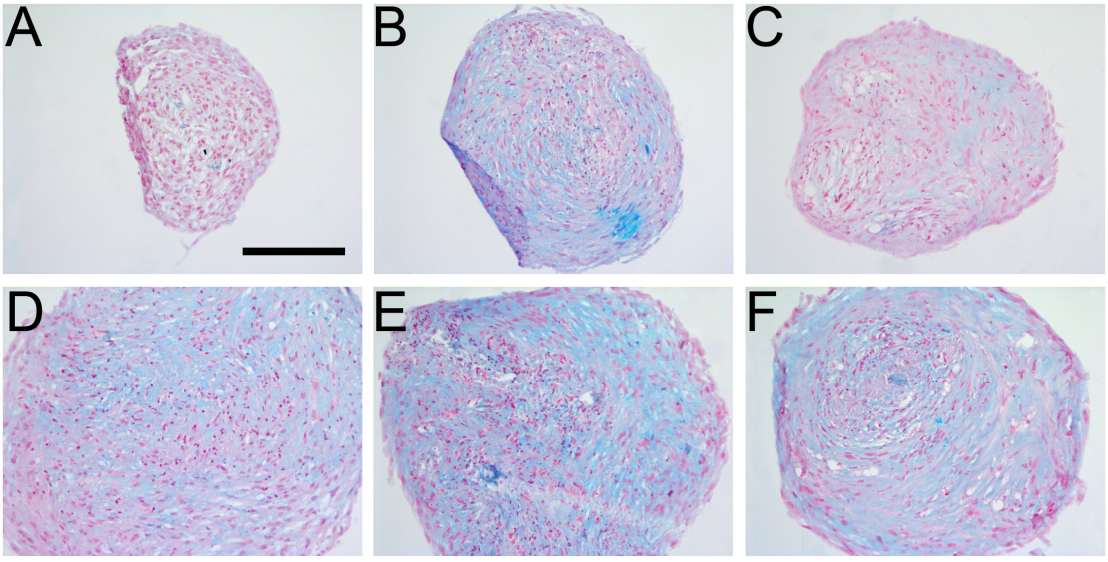

Figure 11 - Alcian blue staining at 28 days. Media compositions A to F are those presented in Table 2 and in text. Scale bar: 200 µm

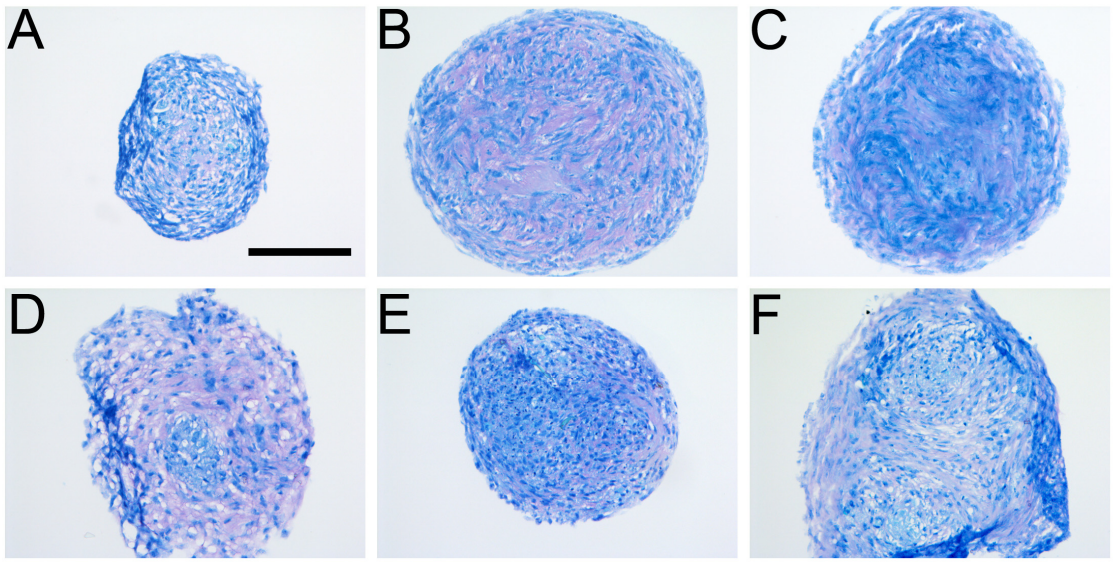

Figure 12 - Toluidine blue staining at 14 days. Media compositions A to F are those presented in Table 2 and in text. Scale bar: 200 µm

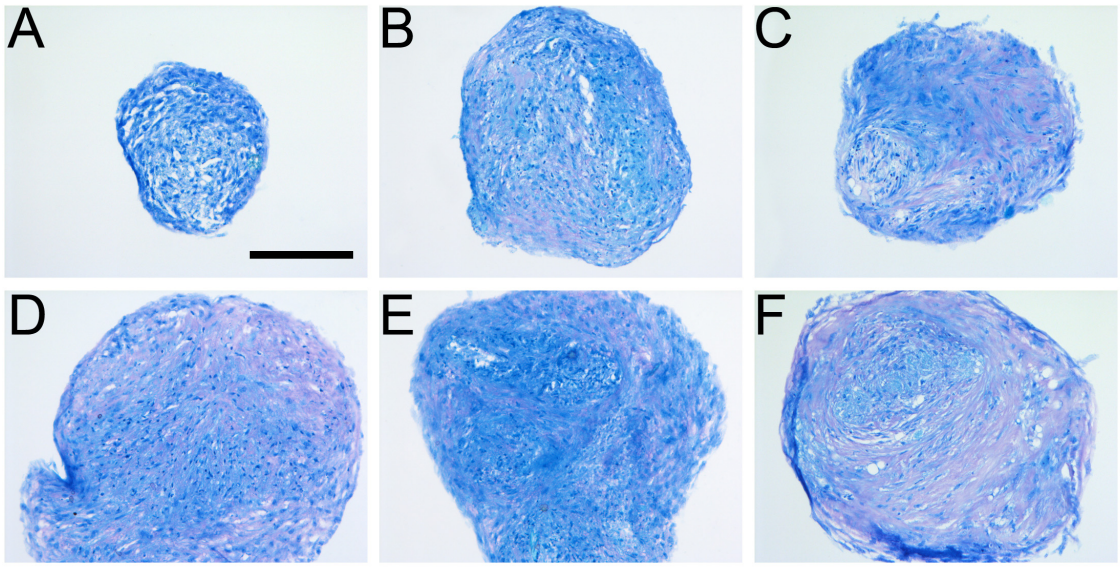

Figure 13 - Toluidine blue staining at 28 days. Media compositions A to F are those presented in Table 2 and in text. Scale bar: 200 µm

Strong positive staining with both AB and TB was seen in medium D at 14 days, although the aggregates seemed somewhat spongy. By 28 days, the sponginess was diminished but strong positive staining with AB and TB remained. Especially by 14 days, the positive staining in medium D seemed to focus on the outer edges of the aggregates, although there were some discrepancies between the three cell lines. Aggregates in medium E at 14 days stained positively with AB and TB rather uniformly, with slightly more staining on the outer edges. By 28 days, AB gave an intermediate staining rather uniformly, while TB staining seemed more concentrated in the periphery of the aggregates. At 14 days in medium F the positive staining with both AB and TB was quite strong. At 28 days the staining seemed to get stronger still. As with medium D, the positive staining was strongest in the periphery of the aggregates. By 14 days two aggregates in medium F seemed to have fused together; positive staining with AB and TB were seen mainly following the outer edges of the two smaller aggregates. By 28 days, both AB and TB gave overall strong positive staining in medium F, most intensively in the outer edges of the aggregates. Dead or dying cells are represented by the small intensely red (AB, Figures 10 and 11) or intensely blue (TB, Figures 12 and 13) spots of clustered DNA. At 14 days, these spots were mainly seen in medium E and to a lesser degree in media B and F. At 28 days, however, the spots were seen in all media (Figures 10—13 and data not shown). Interestingly, spongy or porous appearance caused by lipid vacuole-like structures was observed in many sections (Figures 10—13). These vacuoles were seen especially in media A, D and F (Figure 14), and to a lesser degree in medium C, while the least vacuoles were seen in media B and E. The vacuoles were observed similarly at both timepoints and they were spread uniformly throughout the affected aggregates. The actual nature of these vacuoles was not confirmed.

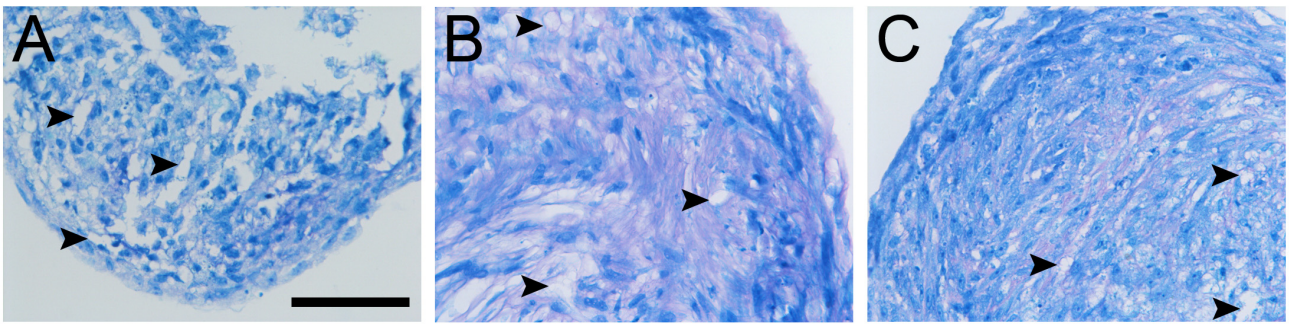

Figure 14 - Toluidine blue staining of vacuole-rich cell aggregates. Figures A, B and C correspond to media compositions A (14 days), D (14 days) and F (28 days), respectively. Arrowheads indicate putative lipid vacuole structures. Scale bar: 100 μm

5.3.2 Immunocytochemical stainings for collagen types 1 and -2

Both primary antibodies and the antibody detection procedure functioned properly, as was verified by positive control sections of rabbit trachea (see appendix A3). Presence of collagen type 1 was confirmed in aggregates cultured in any chondrogenic conditions and even in those cultured in expansion medium, albeit to a lesser degree (Figures 15 and 16). Collagen type 2 was absent in most samples, and found only in sporadic manner in a few aggregates under chondrogenic conditions. Thus, the selected staining figures shown for collagen type 2 are not representative across all three cell lines.

At 14 day time point, staining for collagen type 1 was seen in all chondrogenic conditions and in expansion medium (Figure 15). Expansion medium only resulted in faint staining across the aggregate. Clustered staining throughout the aggregate was seen in basal chondrogenic medium B. Staining was strong but somewhat localized to the aggregate periphery in media C, D and F. Notice that staining in medium F appears weaker merely due to the different staining intensities between two consecutive sets of staining; when compared to the negative control of this staining set, medium F gave a clear positive signal (data not shown). In medium E, areas of intense staining extended into the core of the aggregate.

At 28 days, a similar expression pattern was repeated. Expansion medium failed to support even collagen type 1 deposition, whereas all chondrogenic media maintained collagen type 1 accumulation. Basal chondrogenic medium resulted in strong staining throughout the aggregate with a few more intensively stained areas in aggregate periphery. In media C and F collagen type 1 accumulation occurred inside the aggregate and was no longer restricted to aggregate periphery, although the core still stained less intensively in both conditions. Staining in medium D was moderate and still quite localized to the aggregate periphery. Medium E resulted in the most consistent staining for collagen type 1 throughout the aggregate.

Staining for collagen type 2 was, in most cases, indistinguishable from that seen on negative controls slides (data not shown). In few isolated cases, positive staining was seen concentrated to the edges and in the periphery of the aggregates. Positive staining for collagen type 2 in media B, C and D can be seen in figure 17. Notice that this figure is not representative across all the samples, but merely an exemplary figure.

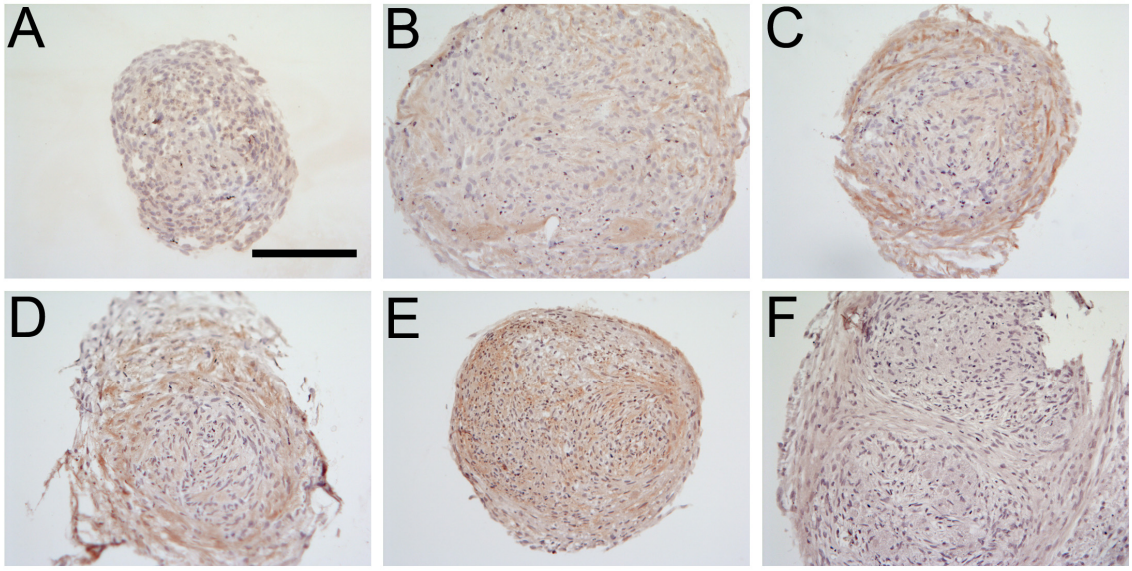

Figure 15 - Collagen type 1 staining at 14 days. Media compositions are those presented in Table 2 and in text. Scale bar: 200 µm

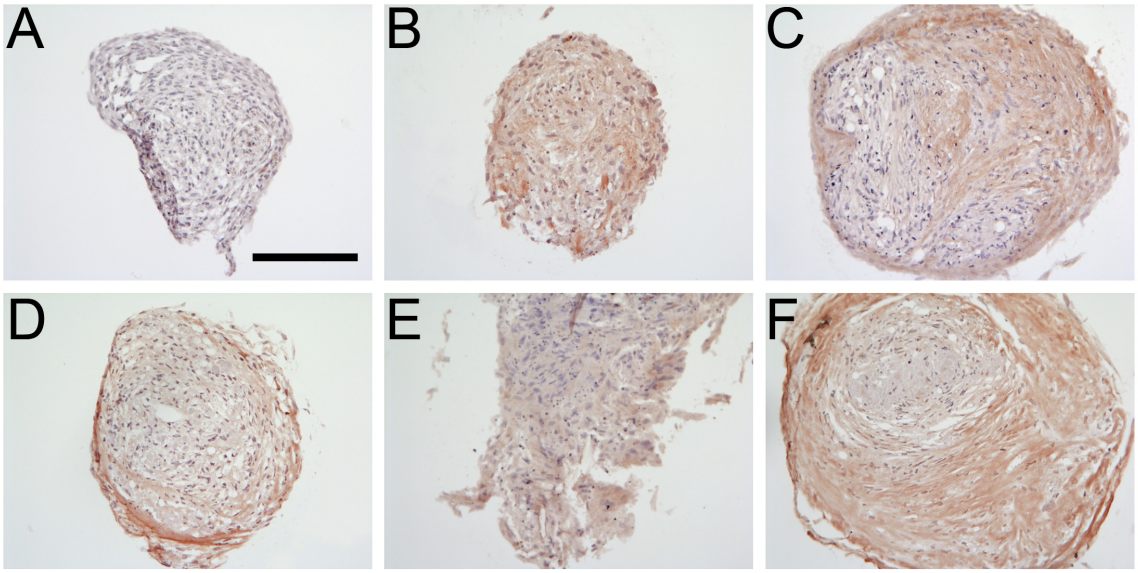

Figure 16 - Collagen type 1 staining at 28 days. Media compositions are those presented in Table 2 and in text. Scale bar: 200 µm

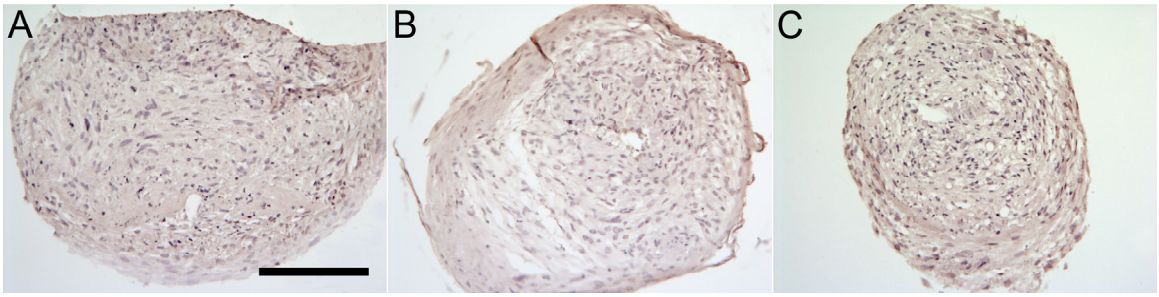

Figure 17 - Collagen type 2 staining. Collagen type 2 staining was weak in all samples, but some conditions resulted in faint positive staining. Figures A, B and C represent media B (14 days), C (14 days) and D (28 days), respectively. Media compositions are those presented in Table 2 and in text. Scale bar: 200 μm.

5.4 Quantitative real-time PCR array results

Selected results from the quantitative real time RT-PCR array results are presented here in groups of related marker genes, namely chondrogenic marker genes, osteogenic marker genes and genes related to TGF-β family signaling. Results are presented in floating bar graphs, where top and bottom ends of a bar represent the highest and the lowest values, respectively. The line dividing the bar represents the median value, which in this data set corresponds to the third measured value. Numbering next to peak values indicates the cell line, pointing out the cases where one cell line outperforms the other two. Numbering of the cell lines is consistent throughout the RT-PCR results section. When appropriate, the calculated P values are indicated above the floating bar figures for statistically significant differences and for nearly significant differences.

5.4.1 Chondrogenic marker genes

Any chondrogenic medium (media B to F) resulted in an increase in relative expression of collagen genes *1A1, 1A2, 3A1, 5A1, 10A1, 11A1 and 12A1* when compared to the sample cultured in expansion medium. Similarly, *COMP* and *BGN* were upregulated by most chondrogenic media. On the other hand, the transcription factor *SOX9* remained almost unaffected.

5.4.1.1 *COL2A1*

Relative expression of *COL2A1* varied considerably; one cell line displayed over 10 000-fold upregulation in medium D compared to basal chondrogenic medium (see Figure 18). The C_t for *COL2A1* ranged from 21 to "undetermined", indicating highly variable transcription of this gene. Although the biological variation here was noteworthy, the media C and D seemed to induce the greatest *COL2A1* upregulation. It seemed that the presence of BMP-6 in basal CM upregulated

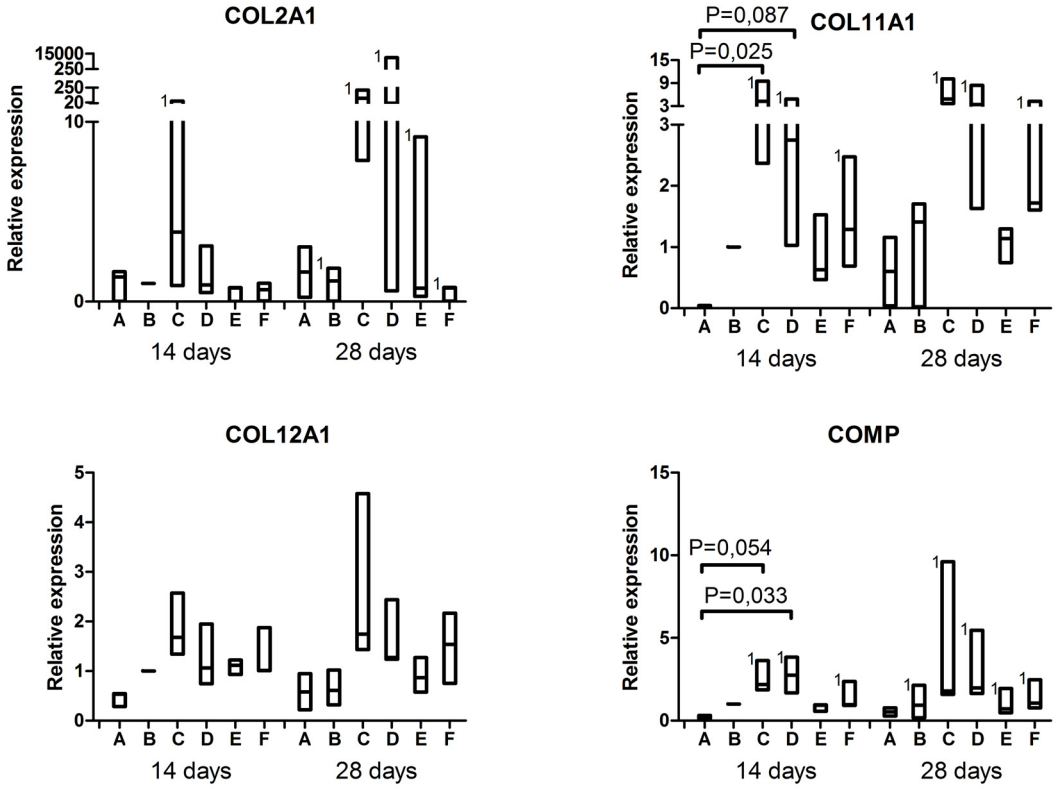

Figure 18 - Relative expression of chondrogenesis related genes, panel A. The panel presents the relative expression of the genes of interest (GOI). Variability in input cDNA was normalized against five housekeeping genes. Basal chondrogenic medium (medium B) at 14 days was used as a calibrating sample within each cell line for each GOI, giving a value of 1 by default for medium B in each graph. See text for detailed description.

COL2A1 at constant low dose or at short-term high dose, but HS, on the contrary, dampened the upregulation. Also of note, the first cell line represented the highest value in the majority of the cases, most interestingly in medium C at 14 days and in media C, D, and E at 28 days.

5.4.1.2 *COL11A1*

The expression of *COL11A1* seemed to be supported by DEX: media lacking DEX (A, B, E) displayed lower expression than those containing DEX (C, D, F). These differences were quite apparent at 28 days, but a similar trend was seen already at 14 days (see Figure 18). Interestingly, DEX accompanied with a constant low dose of BMP-6 (medium C) resulted in overall higher *COL11A1* expression with less variance between the cell lines than did the short-term high dose of BMP-6 with DEX (medium D). The only significant difference (P=0,025) was observed between expansion medium A and chondrogenic medium with DEX and a low dose of BMP-6 (medium C).

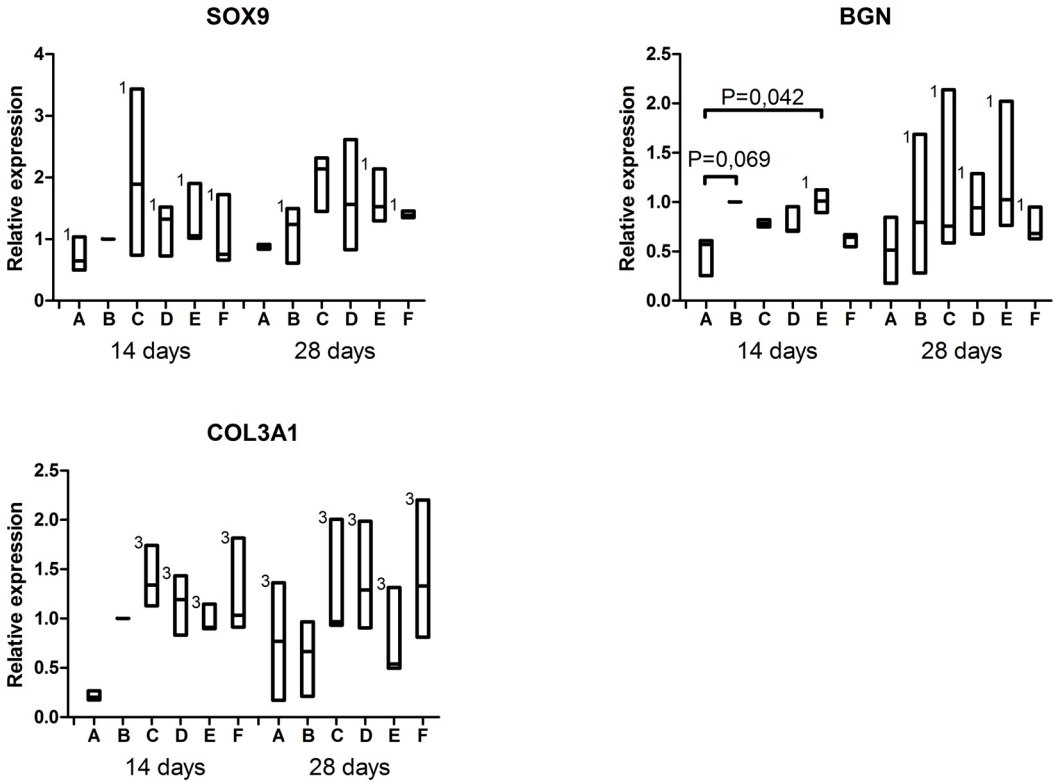

Figure 19 - Relative expression of chondrogenesis related genes, panel B. The panel presents the relative expression of the genes of interest (GOI). Variability in input cDNA was normalized against five housekeeping genes. Basal chondrogenic medium (medium B) at 14 days was used as a calibrating sample within each cell line for each GOI, giving a value of 1 by default for medium B in each graph. See text for detailed description.

5.4.1.3 *COL12A1*

All chondrogenic media upregulated *COL12A1* at 14 days compared to expansion medium, but at 28 days, the upregulation was clearest in chondrogenic media containing DEX and BMP-6. The only significant difference was that between expansion medium and chondrogenic medium C (P=0,025) at day 14. Quite remarkably, the strongest upregulation was obtained with a constant low dose of BMP-6 combined with DEX (medium C); both higher dose of BMP-6 (medium D) and lack of DEX with an addition of human serum (medium E) diminished the upregulation. The first cell line was responsible for the highest relative expression in most media at 14 and at 28 days (see Figure 18).

5.4.1.4 Cartilage oligomeric matrix protein (*COMP*)

COMP seemed to be upregulated in all chondrogenic media compared to expansion medium. The upregulation was further supported by the presence of DEX in media C, D and F. However, 1 % HS

seemed to suppress *COMP* expression in media E and F. Note also that by day 14 in media A, C, E and F, and by day 28 in media B, C, D, E and F the first cell line was responsible for all the peak values of *COMP*. This taken to account, the effects of CM, HS and DEX mentioned above are quite clear at 14 and 28 days (see Figure 18). Significant difference was seen chondrogenic medium D versus expansion medium A (P=0,033), and a nearly significant difference in chondrogenic media C versus expansion medium A (P=0,054).

5.4.1.5 *SOX9*

At 14 and especially at 28 days *SOX9* seemed to be somewhat upregulated by any chondrogenic medium. At 14 days the first cell line in media A, C, E and F accounted for the peak values (see Figure 19).

5.4.1.6 Biglycan (*BGN*)

The strongest biglycan (*BGN*) expression was observed in chondrogenic media with no DEX, namely media B and E at 14 days (see Figure 19). There was a statistically significant difference between the expansion medium A and the chondrogenic medium E (P=0,042), and a nearly significant difference between medium A and chondrogenic medium B (P=0,069).

5.4.1.7 *COL3A1*

All chondrogenic media upregulated *COL3A1* at 14 days, but at 28 days the effect was seen most notably when DEX was present. It should be noted that the third cell line, which was responsible for the highest relative *COL3A1* expression in media C, D, E and F at both 14 and 28 days, was the only "fresh" cell line that had not been cryopreserved before use (see Figure 19).

5.4.2 Osteogenic and hypertrophic marker genes

All chondrogenic media also induced expression of *COL1A1, COL1A2*, *COL10A1*, *SERPINH1* and *RUNX2*. Upregulation of *ALP* was also seen in certain chondrogenic conditions, most notably when using a non-cryopreserved cell line.

5.4.2.1 *COL1A2*

At 14 days *COL1A2* was upregulated by all chondrogenic media, with statistical significance between expansion medium A and chondrogenic medium C (P=0,019) and a nearly significant difference between expansion medium A and chondrogenic medium D (P=0,054).

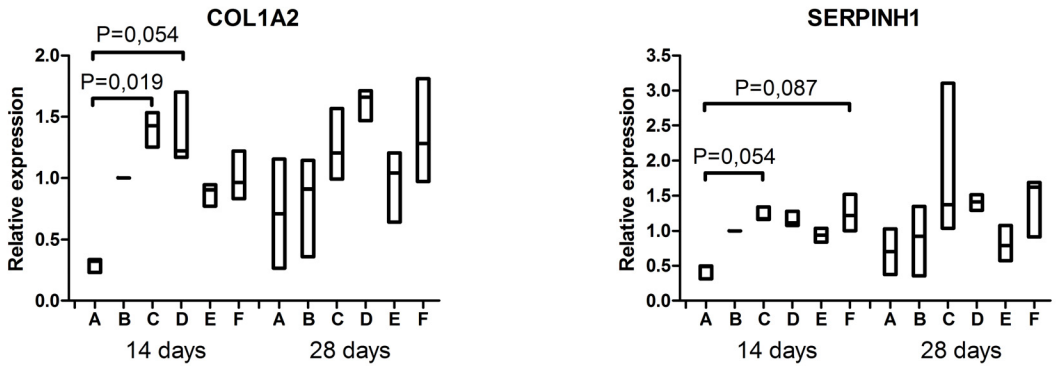

Figure 20 - Relative expression of osteogenesis related genes, Panel A. The panel presents the relative expression of the genes of interest (GOI). Variability in input cDNA was normalized against five housekeeping genes. Basal chondrogenic medium (medium B) at 14 days was used as a calibrating sample within each cell line for each GOI, giving a value of 1 by default for medium B in each graph. See text for detailed description.

At 14 and 28 days *COL1A2* seemed to be upregulated with the combination of any dose of BMP-6 and DEX and downregulated by human serum (see Figure 20). These effects can be seen by comparing media C, D and F to medium E, and by comparing medium F to medium D, although biological variance greatly masks the differences at 28 days. Similar results were seen with the gene encoding another α-chain, *COL1A1* (data not shown).

5.4.2.2 *SERPINH1*

SERPINH1 was also upregulated by any chondrogenic medium at day 14. Interestingly, at 28 days a slight upregulation of *SERPINH1* was seen in media C, D and F, all containing DEX. Viewed in this light, a subtle positive effect of DEX on *SERPINH1* can be seen already at 14 days, where media C, D and F showed the highest relative expression levels of *SERPINH1* (see Figure 20). Nearly significant differences were seen between media A and medium C (P=0,054) and to a lesser degree between medium A and medium F (P=0,087).

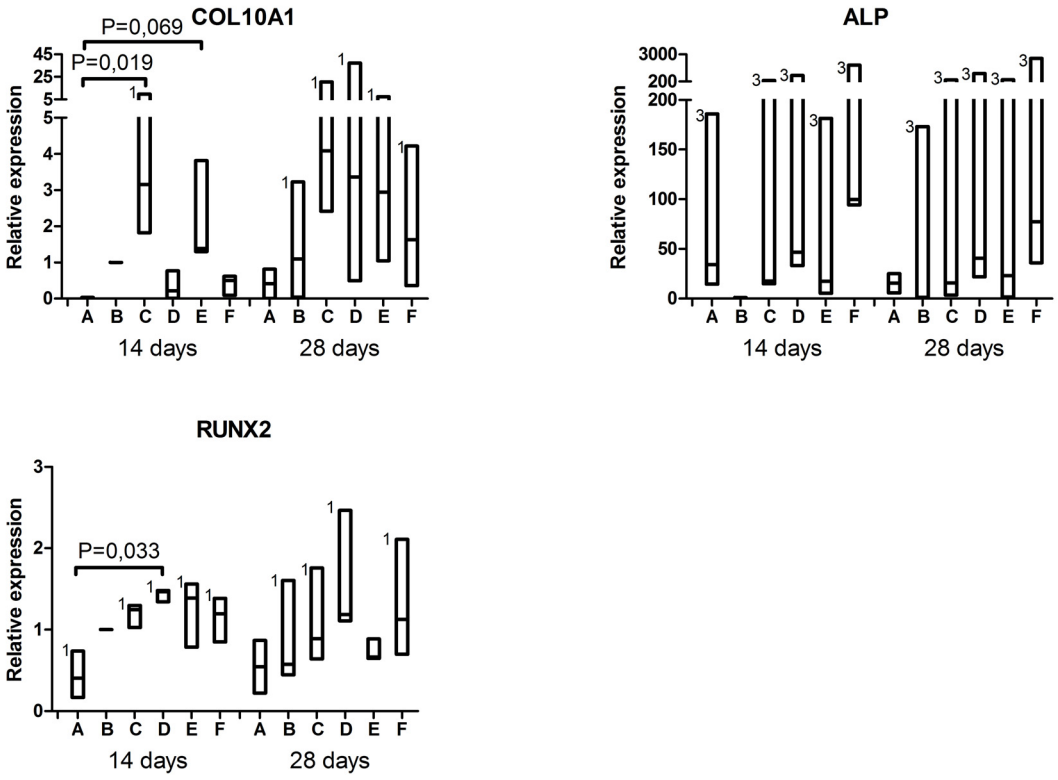

Figure 21 - Relative expression of osteogenesis related genes, Panel B. The panel presents the relative expression of the genes of interest (GOI). Variability in input cDNA was normalized against five housekeeping genes. Basal chondrogenic medium (medium B) at 14 days was used as a calibrating sample within each cell line for each GOI, giving a value of 1 by default for medium B in each graph. See text for detailed description.

5.4.2.3 *COL10A1*

Expression of *COL10A1* was enhanced by all chondrogenic media at 14 and at 28 days compared to expansion medium. The relative expression of *COL10A1* was strongly downregulated by short-term high dose BMP-6 at 14 days as seen by the differences between media C and D, and between media E and F (see Figure 21).On the other hand, a constant low dose of BMP-6 allowed higher *COL10A1* expression (media C and E) compared to basal CM (medium B). In fact, statistical significance was observed between expansion medium A and chondrogenic medium C (P=0,019) and to a lesser degree between expansion medium A and chondrogenic medium E (P=0,069). The *COL10A1*-suppression caused by initial high dose of BMP-6 had practically faded away by day 28.

5.4.2.4 Alkaline phosphatase (*ALP*)

The third cell line displayed the highest relative expression of alkaline phosphatase (*ALP*) in all chondrogenic conditions tested here, scoring values roughly a 100 times larger than the other two cell

lines. The third cell line had not been cryopreserved, but was instead used fresh in this study. When the third cell line and the other two were analyzed separately, it seemed clear that BMP-6 upregulated *ALP* at 14 days and at 28 days in a dose-dependent manner. In addition, in the absence of DEX in media B and E *ALP* expression was lowered at 14 days. Finally, plain expansion medium resulted in relative *ALP* expression greater than that achieved in basal CM at 14 days (see Figure 21).

5.4.2.5 *RUNX2*

RUNX2 expression was elevated in all chondrogenic conditions, and there was a statistically significant difference between the expansion medium A and chondrogenic medium D (P=0,033). Compared to basal chondrogenic medium B at 14 days, chondrogenic medium C caused elevated expression of *RUNX2*, and chondrogenic medium D even more so. Similar steady rise was seen also at 28 days. 1 % HS seemed to decrease *RUNX2* expression at both time points (see Figure 21).

5.4.3 TGF-β and BMP signaling

In the genes encoding TGF-β signaling pathway ligands and receptors, the overall responses to different chondrogenic media were very subtle. Of note, the expansion medium supported higher expression of *BMPs -2, -4,* and *-6* than did any chondrogenic medium, although there were no statistically significant differences.

5.4.3.1 *BMP-4*

BMP-4 expression seemed to follow an interesting pattern; at 14 days, expansion medium A and chondrogenic medium C (containing DEX and a low dose of BMP-6) displayed the strongest expression, although variance between donors was large. Compared to medium C, the exclusion of DEX and BMP-6 (medium B), inclusion of high-dose BMP-6 (medium D), or replacing DEX with 1% HS (medium E) all resulted in considerably lower endogenous *BMP-4* expression. At 28 days, the pattern remained nearly the same, although endogenous *BMP-4* was induced in one cell line by medium B as well. (See Figure 22)

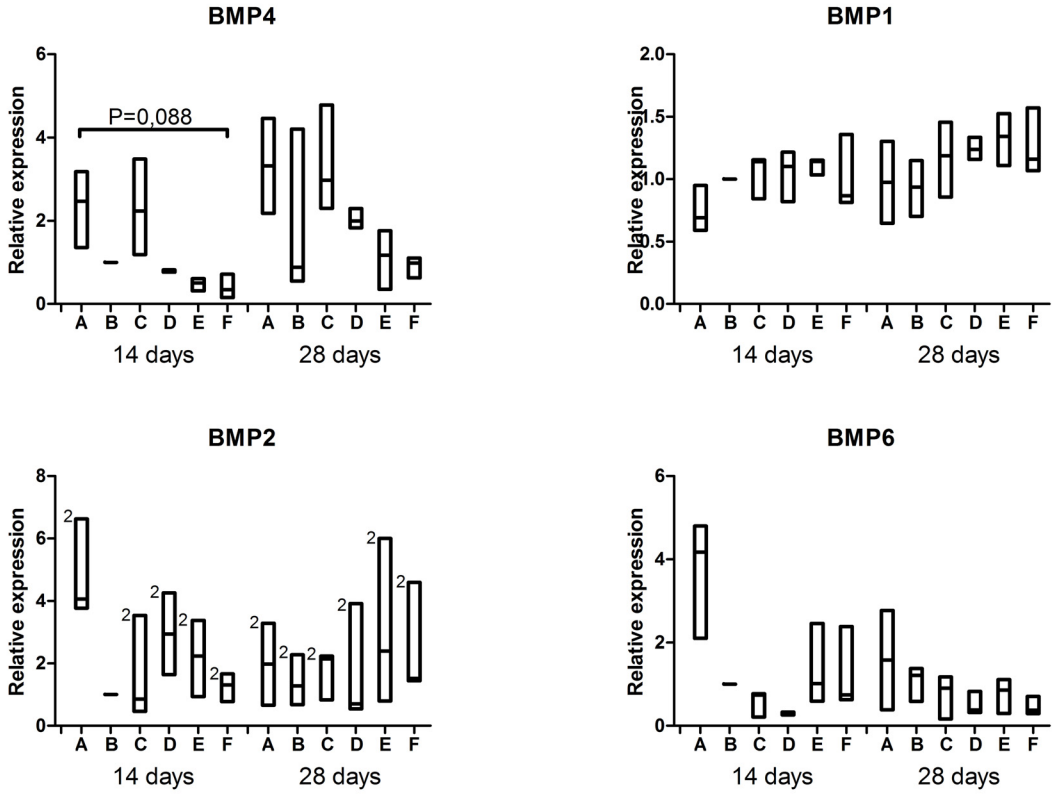

Figure 22 - Relative expression of TGF-β signaling related genes, Panel A. The panel presents the relative expression of the genes of interest (GOI). Variability in input cDNA was normalized against five housekeeping genes. Basal chondrogenic medium (medium B) at 14 days was used as a calibrating sample within each cell line for each GOI, giving a value of 1 by default for medium B in each graph. See text for detailed description.

5.4.3.2 *BMP-1*

At 14 days, the relative expression level of *BMP-1* was slightly higher in all chondrogenic media compared to expansion medium. However, the differences were less clear at 28 days. Overall, *BMP-1* expression seems to remain at a rather constant level. (See Figure 22)

5.4.3.3 *BMP-2*

Conversely to *BMP-1*, the relative expression of *BMP-2* was highest in expansion medium at 14 days, but differences level off at 28 days. Interestingly, the second cell line displayed strongest *BMP-2* expression across all conditions. (See Figure 22)

5.4.3.4 *BMP-6*

Similarly to *BMP-2, BMP-6* was expressed strongly in expansion medium at 14 days, whereas no differences could be seen at 28 days timepoint (See Figure 22).

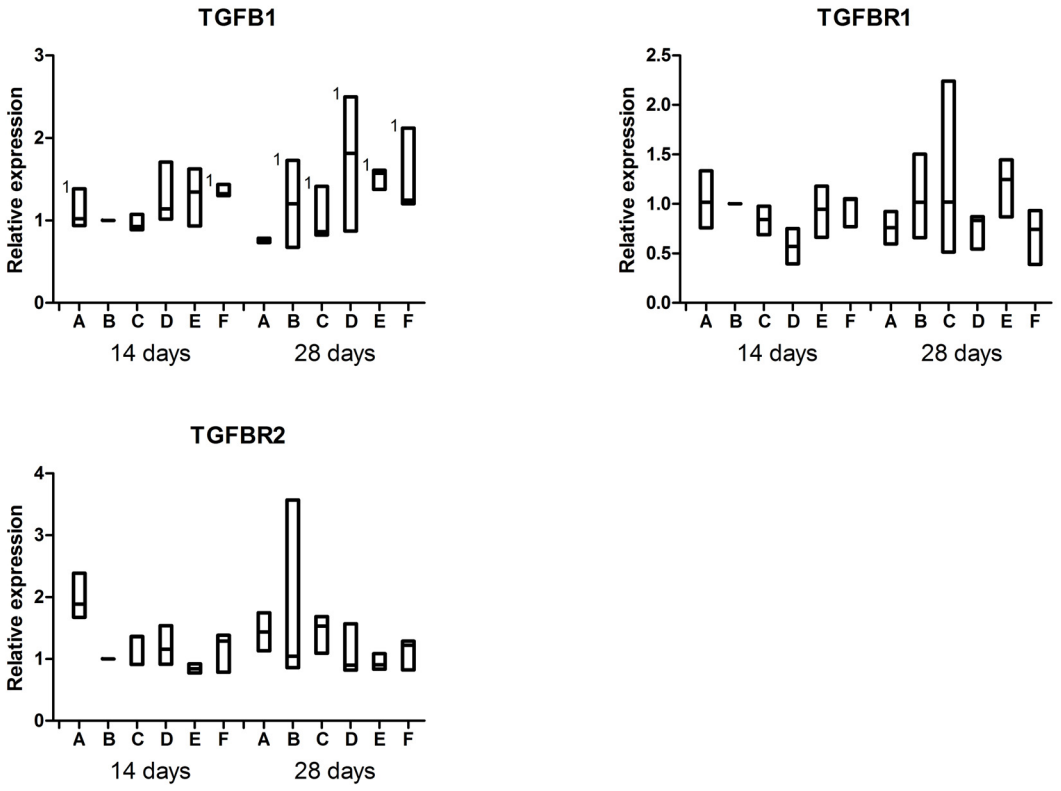

Figure 23 - Relative expression of TGF-β signaling related genes, Panel B. The panel presents the relative expression of the genes of interest (GOI). Variability in input cDNA was normalized against five housekeeping genes. Basal chondrogenic medium (medium B) at 14 days was used as a calibrating sample within each cell line for each GOI, giving a value of 1 by default for medium B in each graph. See text for detailed description.

5.4.3.5 *TGF-β1*

At 14 days, *TGF-β1* expression remained rather stable across all conditions. At 28 days the expression of endogenous *TGF-β1* seemed to be upregulated by chondrogenic media, especially in the first cell line (See Figure 23).

5.4.3.6 *TGFβR1* and *TGFβR2*

In a similar way with genes encoding TGF-β-ligands, the expression of the genes encoding TGF-receptors also remained rather unaltered by various chondrogenic cues. Even TGF-β receptor 1 (*TGFβR1*, See Figure 23) displayed little or no response to BMP-6 or any other factor at either time point. Furthermore, expression of TGF-β receptor 2 (*TGFβR2*, See Figure 23) was decreased by all chondrogenic media at 14 days.

6. Discussion

This study was aimed to refine and extend the knowledge of the effects of various bioactive factors on hASC cultured in chondrogenic 3-D aggregate system. The objective was to test various combinations of different inducing factors. This study also assessed the feasibility of generating cell aggregates of human adipose stem cells cultured on non-adherent multi-well plates.

6.1 Surface antigen profile of the cell lines

The overall surface antigen profile of the hASC was in line with previous publications (Gronthos *et al.* 2001; Katz *et al.* 2005; Dominici *et al.* 2006). Of note, CD34 was expressed in all cell lines. CD34 is absent in hBMSC, but commonly expressed in hASC (Gimble *et al.* 2007; Gimble *et al.* 2011; Pachon-Pena *et al.* 2011) and is routinely detected in early passage hASC in our laboratory (Lindroos *et al.* 2011).

6.2 Effects of TGF-β1 and basal chondrogenic medium

Collagen type 1 expression accompanied with fibrocartilaginous phenotype is commonly seen following chondrogenic differentiation with exogenously added TGF-β family growth factors. Here, *COL1A2* expression was seen in all chondrogenic conditions and at 4 weeks in expansion medium as well (see Figure 20). Immunochemical stainings confirmed collagen type 1 deposition in all media, although staining was generally more intensive in chondrogenic conditions (see Figures 15 and 16). On the other hand, *COL2A1* expression was highly varying and nearly no positive immunochemical staining was seen. However, accumulation of GAG was seen in all chondrogenically differentiated samples, despite considerable biological variation (Figures 10—13). All this taken together, it seems hyaline-like cartilage or fibrous cartilage can be obtained with relative ease, whereas generating hyaline cartilage *in vitro* remains debatable.

6.3 Effects of dexamethasone

DEX is commonly used as a promotive factor, to enhance differentiation towards one lineage or another. The effects of this synthetic corticosteroid on chondrogenesis are rather controversial. Despite all contradictions, DEX was still included in 28 out of 41 chondrogenic differentiation media for BMSC and ASC reviewed recently (Puetzer *et al.* 2010). In line with the previous inconsistencies, our results show mixed effects of DEX on hASC. DEX was involved in the upregulation of *COL11A1*, *COL3A1*, *COL12A1*, *COL1A2*, *COMP* and *SERPINH1*, whereas *BGN* expression was suppressed by DEX.

6.3.1 Dexamethasone and chondrogenesis

DEX seems to promote hASC viability for at least 2 weeks, since dead cells occurred mostly in the histological sections in DEX-free media. Others have published similar observations with hASC; a combination of DEX and FGF-2 increased the proliferation of hASC (Lee *et al.* 2009), whereas a combination of DEX and TGF-β increased cell viability and protein synthesis rate in hASC (Awad *et al.* 2003; Estes *et al.* 2006a). However, high protein synthesis rate does not correlate with high levels of *COL2A1* mRNA or collagen type 2 protein (Estes *et al.* 2006a). In fact, *COL2A1* expression is downregulated in hASC by DEX, but this downregulation can be rescued with other media components (Diekman *et al.* 2010a). Nevertheless, DEX may promote chondrogenesis indirectly. For example, 100 nM DEX has been shown to upregulate endogenous expression of *BMP-6* gene in hASC (Diekman *et al.* 2010a). In accordance, DEX stimulates *BMP-6* transcription and translation in hBMSC (Liu *et al.* 2004) and other glucocorticoid analogues can induce the same effects on rat foetal calvarial cells (Boden *et al.* 1997). Furthermore, DEX promotes expression of *SOX9* transcription and translation in mouse chondrocytes in early (1—2 days) culture (Sekiya *et al.* 2001b).

The highest *BGN* expression was seen in chondrogenic media lacking DEX. This suggests DEX may have suppressed *BGN* expression induced by other components of the chondrogenic medium. Others have also reported overall suppression of GAG accumulation with DEX (Awad *et al.* 2003; Diekman *et al.* 2010a). On the other hand, we report that TGF-β1 combined with BMP-6 and DEX caused the greatest *COMP* upregulation. *COMP*-induction in hASC with a combination of BMP-6 and TGF-β3 has been noted previously (Hildner *et al.* 2010).

Compared to basal chondrogenic medium, DEX further upregulated *COL1A2* expression. This induction was seen with the combination of DEX and any dose of BMP-6, whereas others have

reported *COL1A1* induction only with DEX and a high-dose BMP-6 (Diekman *et al.* 2010a). Our data also suggest DEX induces the expression of *SERPINH1*, a heat-shock protein assisting in the biosynthesis of collagens. Thus, one may speculate that the collagen-inducing effects of DEX are, at least partly, due to the induction of *SERPINH1*.

6.3.2 Dexamethasone and adipogenesis

It seems DEX resulted in lipid droplet-like structure formation within the aggregates, which was further aggravated by a high dose of BMP-6. However, the identity of these putative lipid vacuoles could not be confirmed since cryo-samples were not prepared for Oil red-O staining. Others have reported small quantities of lipid droplet-like structures in hASC aggregates cultured in commercial chondrogenic medium presumably containing DEX (Hildner *et al.* 2010). In murine ASC, DEX supports adipogenic differentiation and lipid vacuole formation when combined with vitamin D or retinoic acid in osteogenic differentiation medium (Malladi *et al.* 2006). It has been previously noted that hASC cultured under osteogenic conditions with 100 nM DEX can easily switch to adipogenic differentiation if ERK signaling cascade is inhibited chemically (Liu *et al.* 2009). Zuk and coworkers have reported successful adipogenesis of hASC in the presence of DEX, whereas hASC osteogenesis was noted to be hindered by DEX (Zuk *et al.* 2002). Other groups have noticed that DEX supports adipogenic differentiation and lipid vacuole formation in hASC in expansion medium and in osteogenic medium, although simultaneous osteogenesis takes place in the latter (Arutyunyan *et al.* 2009). Similar findings were reported by Lee and coworkers, who obtained enhanced osteogenic and adipogenic differentiation with hASC expanded in medium containing a combination of DEX and FGF-2 (Lee *et al.* 2009).

6.4 Effects of BMP-6

Administration of exogenous BMPs to hASC has yielded beneficial, albeit controversial effects on chondrogenic and on osteogenic differentiation. Concerning hASC chondrogenesis, perhaps the most interesting findings circle around BMP-6, the growth factor used in the present study.

It has been shown that, unlike hBMSC, hASC lack a TGF-β receptor 1 (TGFβR1) and therefore respond weakly to chondrogenic stimuli of TGF-β3 (Hennig *et al.* 2007). However, adding 10 ng/ml BMP-6 in the culture medium induces TGFβR1 in hASC, thereby strengthening their TGF- β responsiveness and chondrogenic potential (Hennig *et al.* 2007). In the present study, no apparent

effects of BMP-6 on *TGFβR1* could be seen (see Figure 23). This discrepancy may be at least partially explained by the differences in the expansion media used prior to chondrogenic induction. The expansion medium of Hennig *et al.* contained 2 % foetal calf serum, 2*10-8 M dexamethasone, 10-7 M ascorbic acid-2-phosphate, 5 µg/ml insulin, 5µg/ml transferrin, 5µg/ml selenous acid, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml recombinant human EGF and 10 ng/ml recombinant PDGF-BB (Platelet-derived growth factor beta polypeptide). One may speculate that the additional bioactive components in the expansion medium primed the hASC for BMP-6 signaling and chondrogenesis in the study of Hennig *et al.*, whereas a simple expansion medium failed to prime the hASC in the present study.

DEX has been shown to decrease the expression of the hypertrophic marker collagen type 10 (Estes *et al.* 2006a; Diekman *et al.* 2010a), but we did not observe any clear effects on *COL10A1* expression with DEX. However, we do confirm the *COL10A1* -suppression with BMP-6 that has been reported previously with hASC (Estes *et al.* 2006a; Estes *et al.* 2006b; Diekman *et al.* 2010a; Diekman *et al.* 2010b). At 14 days, the initial high dose of BMP-6 was found to strongly suppress the expression of *COL10A1* compared to other chondrogenic media (see Figure 21). At 28 days of culture, the initial downregulation of the short-term high-dose BMP-6 had faded away. This shows that the initial high dose of BMP-6 combined with DEX was not sufficient to suppress *COL10A1* for three weeks, and that a continuous high dose may be required for *COL10A1* -repression in hASC. Although not analyzed here, the effect of the short-term high-dose of BMP-6 might have been even more evident at an earlier time point. In comparison to hASC, stimulation of *COL10A1* has been seen in hBMSC after a constant high dose of BMP-6 (Sekiya *et al.* 2002) or BMP-2 (Sekiya *et al.* 2005). This discrepancy might be explained with the inherent differences between these cells: hBMSC seem to be more prone to hypertrophy and mineralization than hASC (Mehlhorn *et al.* 2006; Noel *et al.* 2008; Diekman *et al.* 2010b).

Strong *COL1A1* upregulation is usually only seen with high-dose of BMP-6 combined with DEX (Estes *et al.* 2006a; Diekman *et al.* 2010a), but we report induction of *COL1A2* and *COL1A1* with constant low dose of BMP-6 in combination with DEX as well. Although it seems clear DEX and BMP-6 synergistically induce *COL1A1* expression in hASC, it has also been postulated that BMP-6 only has a modest additional effect on *COL1A1* expression compared to the upregulation achieved with basal CM (Hildner *et al.* 2010).

Others have reported that *COL2A1* induction in hASC requires both TGF-β1 or -3 and BMP-6 addition in the differentiation medium (Estes *et al.* 2006a; Hennig *et al.* 2007; Diekman *et al.* 2010a; Hildner *et al.* 2010). We found that the regulation of *COL2A1* was highly donor dependent, as seen by the variance of the expression of this chondrogenic marker gene. Although no systematic pattern could be deduced, it can be noted that *COL2A1* was highly upregulated only in chondrogenic conditions containing both TGF-β1 and BMP-6 (see Figure 18). This poor response may be due to lack of growth factors in the expansion medium, since the abovementioned reports have all employed more or less complex expansion media before differentiation.

Aggrecan induction and GAG deposition in hASC with BMP-6 has been reported (Estes *et al.* 2006a; Diekman *et al.* 2010b; Hildner *et al.* 2010). We did not quantitatively analyze the GAG amounts, but the biochemical stainings for GAG (Figures 10—13) suggest that short-term high-dose BMP-6 may support GAG accumulation. However, the basal CM with no BMP-6 also resulted in plentiful positive staining, thereby questioning the need of this growth factor.

6.5 Short-term high-dose growth factor administration

Some studies suggest short-term high dose- or cycling growth factor administration as viable options for chondrogenic differentiation of hASC (Diekman *et al.* 2010a) and for hBMSC (Indrawattana *et al.* 2004; Henrionnet *et al.* 2010), whereas administering a constant high dose of BMP-6 to hASC results in fibrocartilaginous phenotype (Estes *et al.* 2006b). In this study, only an initial high dose of BMP-6 was administered in order to avoid the detrimental effects of constant high dose of BMP-6 on chondrogenesis. The results were compared to constant low dose of BMP-6 to observe what benefits and/or disadvantages are obtained.

We confirm here that the effects of BMP-6 of hASC are highly dose dependent. The abovementioned *COL10A1* repression was achieved with an initial high dose of BMP-6 but not with a constant low dose, which is in line with previous reports (Estes *et al.* 2006a; Estes *et al.* 2006b; Hennig *et al.* 2007; Kim *et al.* 2008; Diekman *et al.* 2010a; Diekman *et al.* 2010b). A constant low dose of BMP-6 allowed high *COL10A1* expression, as has been seen in previous reports (Hennig *et al.* 2007; Kim *et al.* 2008), although this seems to be highly donor dependent (Hildner *et al.* 2010).

On the other hand, osteogenic marker genes *ALP* and *RUNX2* were induced in a seemingly dose-dependent manner by BMP-6 with clear increase in expression in the initial high-dose treatment

group. Conversely, *COL1A1*- and *COL1A2*-upregulation was roughly the same in media containing a constant low dose or initial short-term high-dose of BMP-6. Taken together, administering BMP-6 as a short-term high-dose does not exclude its detrimental effects, and furthermore the beneficial effects fade away soon after the dosage is lowered. It would be interesting to measure the gene expression levels at more frequent time points.

6.6 Effects of human serum

Human serum contains a plethora of growth factors and hormones, all possibly affecting cell growth and differentiation. Although not certain, it may be speculated that FGF-2 within the serum is responsible for some of the effects seen here. FGF-2 is commonly used in expansion media for hASC to increase cell proliferation (Estes *et al.* 2006a; Estes *et al.* 2006b; Mehlhorn *et al.* 2007; Diekman *et al.* 2010a; Diekman *et al.* 2010b). FGF-2 in expansion medium has been shown to promote proliferation and potentiate subsequent chondrogenic differentiation in FGF-2-free chondrogenic medium in mouse ASC (Chiou *et al.* 2006) and in hBMSC (Solchaga *et al.* 2005). Nevertheless, FGF-2 added to chondrogenic medium has been shown to hamper chondrogenesis in hASC (Hildner *et al.* 2010). Whatever the exact cause, we did observe faster aggregate formation in chondrogenic media containing 1 % human serum. In line with this, enhanced cell mediated contraction has been reported with hASC in alginate beads (Diekman *et al.* 2010b).

In this study, human serum did repress the DEX- and BMP-6-induced expression of *COL1A2* to a level comparable to that of the basal chondrogenic medium (see Figure 20). In a similar manner, *COMP* expression induced by other media components was severely reduced by human serum (see Figure 18). *COL2A1, COL12A1* and *RUNX2* seemed to be downregulated as well, but the biological variance in these cases was much greater.

6.7 Effects of cryopreservation

The relative expression of *ALP* was strikingly higher in one cell line over the other two in all condition (see Figure 21), and this was the only “fresh”, non-cryopreserved line of the three. Albeit insignificant, this result gives an opportunity for speculation whether cryopreservation hampers the osteogenic potential of hASC or not. No interference with proliferation or differentiation have been reported with

cryopreserved hASC (Gonda *et al.* 2008; Liu *et al.* 2008) nor cryopreserved hBMSC (Kotobuki *et al.* 2005). However, lack of consensus remains since cryopreservation was recently shown to hamper proliferation as well as osteogenic and adipogenic differentiation of hASC *in vitro* and *in vivo* (James *et al.* 2011). Among other osteogenic markers, ALP-activity was markedly diminished as a result of cryopreservation although it was noted that addition of osteogenic bioactive factors rescues the diminished osteogenic potential to some degree (James *et al.* 2011).

6.8 High cell-density 3-dimensional culture

The aggregate formation method described in this study produced nearly spheroid hASC aggregates of constant size, roughly 1 mm in diameter. However, considerable differences in aggregate size were seen, even within one culture well. In addition, aggregate structure and morphology seemed to be somewhat inconsistent, as was seen in the histologically stained sections. Considerable structural differences were observed between the layers of the aggregates (core versus surface), possibly stemming from nutrient and/or oxygen gradient. For example, histological sections at 2 weeks in media D and F and at 4 weeks in media C and F displayed obvious differences between aggregate surface and core. Generating small microaggregates of hBMSC (approximately 170 cells/microaggregate) has proven beneficial by not only reducing gradients between core and surface, but also by increasing chondrogenic differentiation (Markway *et al.* 2010).

We observed lipid droplet-like structures within the aggregates, especially in media containing DEX. It might be speculated that hASC be predisposed to adipogenesis, since they do display stronger adipogenic potential than do hBMSC (Sakaguchi *et al.* 2005; Pachon-Pena *et al.* 2011). However, the initial cell density of the aggregates might have been sub-optimal for chondrogenesis, thereby favoring adipogenic differentiation. Without centrifugation, it takes longer to reach high cell density, but whether this affects chondrogenesis or adipogenesis remains debatable (Welter *et al.* 2007).

All difficulties aside, this method is noticeably less labour intensive than producing single aggregates in conical tubes, and thus makes large-scale culturing more feasible, as has been noted elsewhere with similar techniques (Welter *et al.* 2007; Hildebrandt *et al.* 2011). One inevitable problem of macroscopic aggregate culture is the limited diffusion of oxygen and nutrients from the culture medium to the aggregate's core. The method described here initially produces a vast number of smaller aggregates (termed *pre-aggregates*) that seem more consistent and may be more suitable for high cell-

density tissue engineering. The suitability of pre-aggregates for osteochondral tissue engineering is being tested with biomaterial scaffolds. The gradients between the core and the surface may be further minimized by decreasing the aggregate size, or by employing dynamic compressive loading in a bioreactor to support efficient fluid perfusion throughout the aggregate (Darling and Athanasiou 2003).

6.9 Array-technology as a tool for evaluating differentiation

Array-technology provides a powerful means to obtain a large amount of information about the cells undergoing differentiation. In this study, we used the SABiosciences PCR array for human osteogenesis to get an overview of the transcriptome of hASC during chondrogenic differentiation. The same array has been used to assess the chondrogenic differentiation of hBMSC in presence of TGF-β and BMP-2 elsewhere (Henrionnet *et al.* 2010). In that article, TGF-β was found to upregulate the expression of *COL2A1*, *COMP*, *COL10A1* over 1000-fold, and *BMP-2*, *COL11A1*, *SOX9* and *BGN* to a lesser degree (Henrionnet *et al.* 2010). It was also noted that replacing TGF-β with BMP-2 halfway through the 4-week culture period resulted in greater *ALP* expression and increased *COL2A1* expression (Henrionnet *et al.* 2010). These results are generally in line with the data presented here (see Figures 18—23, with the intriguing exception of *BMP-2*, which we found downregulated in all media containing TGF-β (see Figure 22). However, not only is the tissue of origin different, but also the components of the culture media differ considerably between the aforementioned article and the present study.

In another similar venture, a microarray setting was used to compare donor matched bovine chondrocytes and BMSC (Huang *et al.* 2010). This study analyzed the differences in the transcriptomes of chondrogenically differentiated bovine BMSC and chondrocytes. After 28 days of chondrogenic differentiation BMSC had failed to replicate the chondrocyte transcriptome. At 28 days, 324 genes were misregulated more than 2-fold in BMSC compared to chondrocytes and 18 of these misregulations were confirmed with Q-PCR (Huang *et al.* 2010). It was subsequently confirmed that some of the remaining differences may be temporal; BMSC may express certain genes at a later timepoint (Huang *et al.* 2010).

6.10 Differences between the three cell lines

The Q-PCR array results taken together, the first cell line showed greatest expression of chondrogenic marker genes *COL2A1*, *COL11A1*, *SOX9*, *BGN*, *COMP* and *COL12A1*, as well as osteogenic marker genes *RUNX2* and *COL10A1*. On the other hand, the non-cryopreserved third cell line outperformed the other two cell lines in *ALP* expression levels in virtually all conditions and had the highest expression of *COL3A1*. The second cell line showed strongest relative expression levels of *BMP-2*. While many struggle with large biological variations, one group has suggested an extended passaging would decrease donor-to-donor variability and increase chondrogenic potential of hASC (Estes *et al.* 2006b). Alternatively, several groups have employed FACS or MACS to enrich a mixed cell population based on surface marker profile. These have found that hASC (Rada *et al.* 2008; Jiang *et al.* 2010) or rat synovial stem cells (Qi *et al.* 2011) enriched for CD105 show increased chondrogenic potential. No cell sorting was performed in this study but, quite surprisingly, the most chondrogenic cell line displayed the lowest amount of CD105 at P1.

6.11 Preventing hypertrophy and mineralization

Hypertrophy and subsequent calcification of the tissue engineered cartilage pose major problems. We confirmed BMP-6 as a potential hypertrophy-preventing agent, but there are other ways of avoiding unwanted hypertrophy. PTHrP (parathyroid hormone related protein) maintains proliferation of chondrocytes and delays hypertrophy during organogenesis (Vortkamp *et al.* 1996; Karsenty and Wagner 2002; Kronenberg 2003); hence it has been suggested as a hypertrophy preventing agent in chondrogenic media. In one study, PTHrP was shown to increase GAG accumulation and expression of *COL2A1* while suppressing *COL10A1* and RUNX2 in both hASC and hBMSC (Kim *et al.* 2008). However, another study with hBMSC demonstrated that PTHrP prevents not only hypertrophy, but chondrogenic differentiation as well, as deduced from the suppression of *COL2A1* and *COL10A1* (Weiss *et al.* 2010). These discrepancies may be due to different timepoints of administration of PTHrP or the use of different PTHrP- fragments.

One group has transplanted chondrogenically differentiated hBMSC and human articular chondrocytes (hAC) in mice (Pelttari *et al.* 2006). They concluded hAC-pellets maintained a chondrocytic phenotype, whereas hBMSC-pellets underwent mineralization and vascularization

reminiscent of endochondral ossification (Pelttari *et al.* 2006). Others have reported prominent differences between the transcriptomes of the articular cartilage and growth plate cartilage, latter of which is destined to ossification (Yamane *et al.* 2007). Hence, one may speculate that the cartilage obtained with stem cells *in vitro* might also be destined to undergo hypertrophy and subsequent ossification. Somehow, the cells need to be persuaded to embrace an articular phenotype.

6.12 Methodological considerations and limitations of the study

Due to limited resources, the media compositions had to be narrowed down to five chondrogenic media and expansion medium. Alas, all possible combinations of the three bioactive factors could not be fitted in this study, and thus the results do not provide a systematic analysis of the effects of BMP-6, DEX and human serum. Thus, care should be taken when effects on gene expression are denoted to one factor or another. However, valuable information of the effects of these factors and their combinations can still be deduced from the data presented here, although pin-pointing the most effective growth factor combination was not possible. Also, notice that hASC cultured in expansion medium were used as control with the intention of bringing forth the effects of varying culture conditions and media additives. Ideally, the results should be compared against healthy human primary articular chondrocytes, or against fibrous chondrocytes. What is more, the results were compared neither with day-0 controls nor with 2-D monolayer controls, which could have provided additional information about the success of the 3-D culture method. Finally, it must be recognized that the PCR data may not reflect the actual synthesis rate and accumulation of the corresponding gene products.

6.13 Summary of the discussion and conclusions

Figure 24 offers an oversimplified summary of the results of this study from the point of view of chondrogenic differentiation. In brief, the positive effects of TGF-β1 on chondrogenesis are generally taken for granted, and this was the case in the present study as well. When TGF-β1 was combined with other factors, synergistic effects were noted in the relative expression of some chondrogenesis related genes, namely *COL11A1*, *COL2A1* and *COMP*. However, the same was true with hypertrophy related genes, namely *COL1A1* and *COL1A2*, *ALP* and *COL10A1*.

BMP-6 proved to be a promising inducer of chondrogenesis, and dose-dependent effects could be seen on certain genes' relative expression. Most importantly, the initial high dose of BMP-6 clearly suppressed the expression of *COL10A1* for 2 weeks. On the downside, BMP-6 stimulated the relative *ALP*- and *RUNX2* expression in a seemingly dose dependent manner, and upregulated *COL1A1* and *COL1A2* in synergy with DEX. Nevertheless, the relative expression of *COL2A1* was increased substantially in conditions containing BMP-6, although in a haphazardous manner.

The overall status of DEX remains controversial, but based on the results presented here, DEX seems unnecessary if not detrimental for chondrogenesis of hASC. DEX was found to boost the expression of multiple genes, including *COL1A2*, *COL1A1, COL3A1*, *COL11A1*, *COL12A1*, *COMP*, *SERPINH1* and *ALP*, while repressing *BGN*. Of note, in the presence of DEX the hASC aggregates were more loose and spongy, and this observable fact was more pronounced if DEX and BMP-6 were combined. Instead of using DEX in the chondrogenic medium, it might be better suited for expansion medium.

Human serum seems appropriate for expansion medium, but adding human serum to chondrogenic culture medium remains questionable. No clear benefits were seen in gene expression or histology with 1 *vol*-% human serum addition to chondrogenic medium. Of note, the relative expression of *COMP* and *COL1A1* and *COL1A2* seemed to be suppressed by presence of human serum. Still, initial cell aggregation took place somewhat faster, making medium changes more convenient. We hypothesized this to be due to adhesive factors within the serum assisting the formation of cell aggregates.

	Cryo-preservation	BMP-6	BMP-6-ST	Dexamethasone	Human serum
Chondrogenic markers	Repress *COL3A1*	Induce *COL2A1* Induce *COL12A1*	Induce *COL2A1* Repress *COL12A1*	Induce *COL11A1* Induce *COMP* Induce *COL12A1* Repress *BGN* Induce *COL3A1*	Repress *COMP* Repress COL2A1 Repress COL12A1
Osteogenic markers	Repress *ALP*	Induce *COL10A1* Induce *COL1A2* Induce *ALP* Induce *RUNX2*	Repress *COL10A1* Induce *COL1A2* Induce *ALP* Induce *RUNX2*	Induce *COL1A2* Induce *SERPINH1* Induce *ALP*	Repress *COL1A2* Repress *RUNX2*
Other			Lipid vacuoles	Adipogenesis Lipid vacuoles Promote viability	Promote aggregation

Figure 24 - Summary of the effects of various bioactive factors. Colours are used to highlight the given effects' importance for fulfilling the aim of this study, namely chondrogenic differentiation of human adipose stem cells. Color annotation: blue = beneficial for chondrogenesis, red = detrimental for chondrogenesis. This diagram provides an oversimplified summary of the results. For in-depth analysis, refer to text.

6.14 Future perspectives

The results of this study provided interesting information about the effects of various putative chondrogenic factors on hASC. However, many questions were left unanswered. In the future research the dosage, combination and time of administration of bioactive factors must be further refined to obtain optimal chondrogenesis. Culturing hASC in 3-D aggregates of smaller size appears to be highly advantageous, as does introducing a co-culture system with chondrocytes. Sorting hASC based on their immunophenotype prior to differentiation could be used to isolate a population capable of superior chondrogenesis. In addition, the interaction with a complex 3-D environment provided by a natural or synthetic biomaterial scaffolds might provide essential chondrogenic cues for hASC. Finally, transferring the abovementioned culturing techniques and media compositions into a dynamic bioreactor environment could further improve the quality and functionality of the engineered cartilage.

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8. Appendix

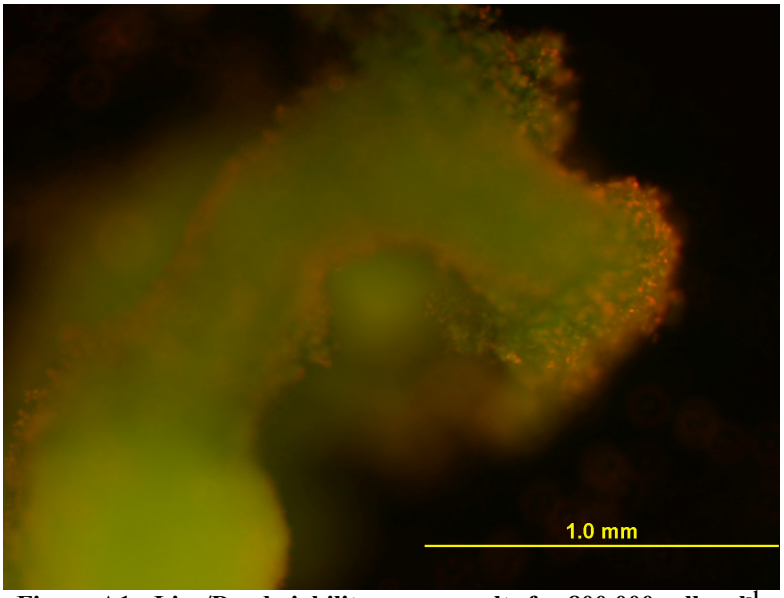

Figure A1 - Live/Dead viability assay results for 800 000 cells ml^{-1}.

8.1 Cell concentration optimization for non-adherent culture

In brief, adipose stem cells isolated with the protocol described in text were cultured until passage 3. Cells were harvested and plated on ultra-low attachment 24-well plates (Corning Life Sciences) in concentrations of 2,5*10^4, 5,0*10^4, 1*10^5, 2*10^5, 3*10^5, 4*10^5, 5*10^5, 6*10^5, 8*10^5 and 1*10^6 cells per ml. Total volumes of 0,5 and 1 ml of expansion medium or basal CM without TGF-β1 were studied. Cells were cultured for 14 days and the media were changed every third to every fourth day. The cell viability was evaluated with Live/Dead viability assay, which employs ethidium homodimer 1 (EthdH-1) and calcein AM (CaAM) stains to distinguish live and dead cells (Molecular Probes, Invitrogen, Carlsbad, California, USA). EthdH-1 forms a red fluorescent complex with nucleic acids that are released from the nuclei as a result of cell death. The non-intact membranes of dead cells allow diffusion of the dye, staining dead cells red. Conversely, CaAM is cleaved by esterase activity inside live cells to a fluorescent form which gets trapped inside the cells, whereas dead cells lack both enzymatic activity and intact plasma membranes; therefore only live cells stain green. After washing with DPBS, cell aggregates were incubated for 45 min in a staining solution containing 0,5 µM of both dyes. Results were analyzed with Olympus IX51 fluorescent microscope (Olympus, Shinjuku, Tokyo, Japan). Overall, aggregate formation was successful in all conditions tested. However, multiple

macroscopic, spheroid cell aggregates were most reproducibly obtained with total cell amount of 200 000 cells in 0,5 ml of medium, whereas a higher cell concentration resulted in deformed and squashy cell mass with considerable red staining in Live/Dead viability assay (See figure A1).

8.2 Full gene list of SAB osteogenesis Q-RT-PCR array plate

Table A1 - Full list of genes analyzed with the SABiosciences human osteogenesis Q-RT-PCR array.

Abbreviation	Gene name	Alternate gene name(s)
AHSG	Alpha-2-HS-glycoprotein	A2HS, AHS, FETUA, HSGA
ALPL	Alkaline phosphatase, liver/bone/kidney	AP-TNAP, FLJ40094, FLJ93059, HOPS, MGC161443, MGC167935, TNAP, TNSALP
AMBN	Ameloblastin (enamel matrix protein)	-
AMELY	Amelogenin, Y-linked	AMGL, AMGY
ANXA5	Annexin A5	ANX5, ENX2, PP4
BGLAP	Bone gamma-carboxyglutamate (gla) protein	BGP, OC, PMF1
BGN	Biglycan	DSPG1, PG-S1, PGI, SLRR1A
BMP1	Bone morphogenetic protein 1	FLJ44432, PCOLC, PCP, TLD, pCP-2
BMP2	Bone morphogenetic protein 2	BMP2A
BMP3	Bone morphogenetic protein 3	BMP-3A
BMP4	Bone morphogenetic protein 4	BMP2B, BMP2B1, MCOPS6, ZYME
BMP5	Bone morphogenetic protein 5	MGC34244
BMP6	Bone morphogenetic protein 6	VGR, VGR1
CALCR	CALCITONIN RECEPTOR	CRT, CTR, CTR1
CD36	CD36 molecule (thrombospondin receptor)	CHDS7, FAT, GP3B, GP4, GPIV, PASIV, SCARB3
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	CAD11, CDHOB, OB, OSF-4
COL10A1	Collagen, type X, alpha 1	-
COL11A1	Collagen, type XI, alpha 1	CO11A1, COLL6, STL2
COL12A1	Collagen, type XII, alpha 1	BA209D8.1, COL12A1L, DJ234P15.1
COL14A1	Collagen, type XIV, alpha 1	UND
COL15A1	Collagen, type XV, alpha 1	FLJ38566
COL1A1	Collagen, type I, alpha 1	OI4
COL1A2	Collagen, type I, alpha 2	OI4
COL2A1	Collagen, type II, alpha 1	ANFH, AOM, COL11A3, MGC131516, SEDC
COL3A1	Collagen, type III, alpha 1	EDS4A, FLJ34534
COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)	-
COL5A1	Collagen, type V, alpha 1	-
COMP	Cartilage oligomeric matrix protein	EDM1, EPD1, MED, MGC131819, MGC149768, PSACH, THBS5
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	GMCSF, MGC131935, MGC138897
CSF3	Colony stimulating factor 3 (granulocyte)	G-CSF, GCSF, MGC45931
CTSK	Cathepsin K	CTS02, CTSO, CTSO1, CTSO2, MGC23107, PKND, PYCD
DMP1	Dentin matrix acidic phosphoprotein 1	ARHP, ARHR, DMP-1
DSPP	Dentin sialophosphoprotein	DFNA39, DGI1, DMP3, DPP, DSP, DTDP2
EGF	Epidermal growth factor (beta-urogastrone)	HOMG4, URG
EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	ERBB, ERBB1, HER1, PIG61, mENA
ENAM	Enamelin	ADAI, AI1C, AIH2

FGF1	Fibroblast growth factor 1 (acidic)	AFGF, ECGF, ECGF-beta, ECGFA, ECGFB, FGF-alpha, FGFA, GLIO703, HBGF1
FGF2	Fibroblast growth factor 2 (basic)	BFGF, FGFB, HBGF-2
FGF3	Fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2) oncogene homolog)	HBGF-3, INT2
FGFR1	Fibroblast growth factor receptor 1	BFGFR, CD331, CEK, FGFBR, FLG, FLJ99988, FLT2, HBGFR, KAL2, N-SAM, OGD
FGFR2	Fibroblast growth factor receptor 2	BEK, BFR-1, CD332, CEK3, CFD1, ECT1, FLJ98662, JWS, K-SAM, KGFR, TK14, TK25
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	FLT, VEGFR1
FN1	Fibronectin 1	CIG, DKFZp686F10164, DKFZp686H0342, DKFZp686I1370, DKFZp686O13149, ED-B, FINC, FN, FNZ, GFND, GFND2, LETS, MSF
GDF10	Growth differentiation factor 10	BMP-3b, BMP3B
ICAM1	Intercellular adhesion molecule 1	BB2, CD54, P3.58
IGF1	Insulin-like growth factor 1 (somatomedin C)	IGF1A, IGFI
IGF1R	Insulin-like growth factor 1 receptor	CD221, IGFIR, IGFR, JTK13, MGC142170, MGC142172, MGC18216
IGF2	Insulin-like growth factor 2 (somatomedin A)	C11orf43, FLJ22066, FLJ44734, INSIGF, pp9974
ITGA1	Integrin, alpha 1	CD49a, VLA1
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	BR, CD49B, GPIa, VLA-2, VLAA2
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	CD49C, FLJ34631, FLJ34704, GAP-B3, GAPB3, MSK18, VCA-2, VL3A, VLA3a
ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	CD11B, CR3A, MAC-1, MAC1A, MGC117044, MO1A, SLEB6
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	CD29, FNRB, GPIIA, MDF2, MSK12, VLA-BETA, VLAB
MINPP1	Multiple inositol polyphosphate histidine phosphatase, 1	DKFZp564L2016, HIPER1, MINPP2, MIPP
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	SL-2, STMY2
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	CLG4, CLG4A, MMP-II, MONA, TBE-1
MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)	CLG1, HNC, PMNL-CL
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	CLG4B, GELB, MMP-9
MSX1	Msh homeobox 1	HOX7, HYD1, STHAG1
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	DKFZp686C01211, EBP-1, KBF1, MGC54151, NF-kappa-B, NFKB-p105, NFKB-p50, p105, p50
PDGFA	Platelet-derived growth factor alpha polypeptide	PDGF-A, PDGF1
PHEX	Phosphate regulating endopeptidase homolog, X-linked	HPDR, HPDR1, HYP, HYP1, PEX, XLH
RUNX2	Runt-related transcription factor 2	AML3, CBFA1, CCD, CCD1, MGC120022, MGC120023, OSF2, PEA2aA, PEBP2A1, PEBP2A2, PEBP2aA, PEBP2aA1
SCARB1	Scavenger receptor class B, member 1	CD36L1, CLA-1, CLA1, MGC138242, SR-BI, SRB1
SERPINH1	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	AsTP3, CBP1, CBP2, HSP47, PIG14, PPROM, RA-A47, SERPINH2, gp46
SMAD1	SMAD family member 1	BSP1, JV4-1, JV41, MADH1, MADR1
SMAD2	SMAD family member 2	JV18, JV18-1, MADH2, MADR2, MGC22139, MGC34440, hMAD-2, hSMAD2
SMAD3	SMAD family member 3	DKFZp586N0721, DKFZp686J10186, HSPC193, HsT17436, JV15-

		2, MADH3, MGC60396
SMAD4	SMAD family member 4	DPC4, JIP, MADH4
SOX9	SRY (sex determining region Y)-box 9	CMD1, CMPD1, SRA1
STATH	Statherin	STR
TFIP11	Tuftelin interacting protein 11	FLJ22086, NTR1, TIP39, bK445C9.6, hNtr1
TGFB1	Transforming growth factor, beta 1	CED, DPD1, TGFB, TGFbeta
TGFB2	Transforming growth factor, beta 2	MGC116892, TGF-beta2
TGFB3	Transforming growth factor, beta 3	ARVD, FLJ16571, TGF-beta3
TGFBR1	Transforming growth factor, beta receptor 1	AAT5, ACVRLK4, ALK-5, ALK5, LDS1A, LDS2A, SKR4, TGFR-1
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	AAT3, FAA3, LDS1B, LDS2B, MFS2, RIIC, TAAD2, TGFR-2, TGFbeta-RII
TNF	Tumor necrosis factor (TNF superfamily, member 2)	DIF, TNF-alpha, TNFA, TNFSF2
TUFT1	Tuftelin 1	-
TWIST1	Twist homolog 1 (Drosophila)	ACS3, BPES2, BPES3, SCS, TWIST, bHLHa38
VCAM1	Vascular cell adhesion molecule 1	CD106, DKFZp779G2333, INCAM-100, MGC99561
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor	NR1I1
VEGFA	Vascular endothelial growth factor A	MGC70609, MVCD1, VEGF, VEGF-A, VPF
VEGFB	Vascular endothelial growth factor B	VEGFL, VRF
B2M	Beta-2-microglobulin	-
HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT, HPRT
RPL13A	Ribosomal protein L13a	-
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, MGC88685
ACTB	Actin, beta	PS1TP5BP1

8.3 Specificity of immunocytochemical and histological stainings

Immunocytochemical staining against collagen type I and against collagen type II and toluidine blue staining were verified with positive control slides of rabbit trachea. The antibody against collagen type I specifically recognized the outer fibrous layer of the cartilage ring (see Figure A2-A). The antibody against collagen type II recognized specifically the inner hyaline part of the cartilage ring (see Figure A2-B). Toluidine blue stained GAG (glycosaminoglycan) pink or purple, while other components remained blue (see Figure A2-C). For alcian blue, positive control slide was not included.

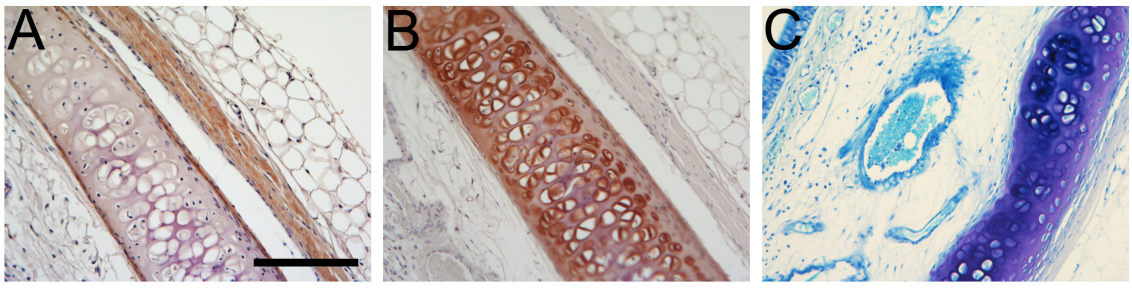

Figure A2 - 5µm positive control slides of rabbit trachea. A: Anti-collagen type I. B: A: Anti-collagen type II. C: Toluidine blue staining. Scale bar 200 µm.

8.4 Estimating missing values for q-PCR data analysis

As a result of pipetting erroneously twice to one well and skipping another, two wells on one 96 well PCR array plate were rendered invalid. The sample affected was the second cell line (HFSC 5/09), two week time point, medium A in Table 2 in the text. The wells affected on the array plate were two of the reference genes. The impact of this error is not as drastic as it may seem, though. This is due to the fact that the RNA samples from the expansion medium cultured aggregates were poor in quality altogether and could not have been used as calibrating samples even without this pipetting error. The qPCR run was not run again due to the price of the array plates and due to the relative unimportance of the error. Still, it was decided that a numerical value was needed for these reference genes to get more comparable data sets. Frankly, numerical values for these two reference gene wells were calculated by using the respective values of another cell line as a comparison. In the table below (Table A2) the C_t values of the reference genes (wells H1 to H5) of the two week time point of two cell lines are presented. The erroneous values are marked with *, and the new calculated values are shown beside the erroneous values. Calculations were done as follows:

First, the relations between reference genes' C_t values in the complete gene set of HFSC 5/09 were calculated. Relations between wells H4 and H1, H4 and H2, H4 and H3 as well as relations between wells H5 and H1, H5 and H2, H4 and H3 were calculated. Next, these relations were used to calculate the missing values for the wells H4 and H5 in the cell line HFSC 6/09.

For example, the relation of H4 and H1 in HFSC 5/09 is 21,3035 / 22,0571 = 0,965834. Using this relation, H4 of the cell line HFSC 6/09 will get a value of 21,6646 * 0,965834 = 20,924407. After calculating the same value twice more using the relations between H4 and H2 or H3, the average of the three numbers obtained was calculated to be 21.

Table A2 - Raw data obtained from q-RT-PCR arrays. Presented in the table are the C_t -values of the five housekeeping genes (see table A1 for details) of two different cell lines (5/09 and 6/09). The missing values of H4 and H5 for the latter cell line were calculated as explained in the text.

HFSC 5/09 Two week time point, Medium A		HFSC 6/09 Two week time point, Medium A		
Well	C_t	Well	C_t	Calculated C_t
H1	22,0571	H1	21,6646	
H2	27,8606	H2	27,2182	
H3	22,1458	H3	22,4157	
H4	21,3035	H4	36,3465 *	**21,09995**
H5	21,5101	H5	22,2163 *	**21,30457**

8.5 Lacking data from one q-RT-PCR plate

The power cord of the laptop to which the qPCR machine was connected to was unplugged and the laptops battery ran out, causing the qPCR machine to abort the current run at cycle 28, a mere 12 cycles short of the intended 40. Hence, only genes with C_t values lower than 28 could be used from this particular sample. The sample affected was the cell line HFSC 5/09, 2 week time point, medium D in Table 1. The sample was not rerun due to the price of the plates, the relative unimportance of one sample and lack of enough remaining RNA from the current sample.

8.6 Technical difficulties with RNA isolation

One major problem accompanied the RNA isolation with the RNeasy® Mini Kit (Qiagen) throughout the study: Despite containing guanidinium thiocyanate, the lysis buffer failed to dissolve the cell aggregates completely, even when assisted by vigorous pipetting up and down. As a result, the RNA samples may not be representative of the whole aggregate, but mainly the surface-most cells. In addition, the aggregates used for cytological stainings may differ in structure from those used for total RNA extraction. This problem could be solved by using additional aggregate disruption and homogenization methods, such as Qiagen's TissueRuptor®, a rotor-stator homogenizer with disposable probes. Due to poor lysis, the amount of 320 ng input RNA for reverse transcription could not be achieved with all samples. These exceptions included all samples cultured in expansion medium and six other samples, totalling 12 samples out of 36. One sample (2Ba, first cell line, 28 days, expansion medium) was discarded altogether due to extremely poor RNA yield. Another sample (2Ad, first cell line, 14 days, chondrogenic medium D) only provided results of the first 28 PCR cycles due to power cut during PCR-cycling. Despite multiple attempts to obtain descriptions of the primers used in the array, the manufacturer did not provide any additional information. One can only assume the efficiencies of all the primer pairs are equal enough.