THE EFFECTS OF HYPOXIC CONDITIONS ON THE CHONDROGENIC DIFFERENTIATION OF ADIPOSE STEM CELLS

MASTER'S THESIS
Anne Mäntymaa
Institute of Medical Technology
University of Tampere
January 2010

ACKNOWLEDGEMENTS

This thesis was carried out at the Regea Institute for Regenerative Medicine, University of Tampere.

First, I would like to acknowledge my supervisor Katja Mäenpää for her support, excellent guidance, advice, and patience during the project and especially during the writing part. I was very lucky to have you as my supervisor. I would also like to thank Susanna Miettinen for her good advice during the project. Special thanks belong to Hilkka Mäkinen with her help in the lab, and all Adult Stem Cell -group members in Regea for being helpful during the experimental part of my thesis project.

Finally, I would like to express my gratitude to my family, and friends for their support and encouragement during this process.

Tampere, January 2010

Anne Mäntymaa

MASTER'S THESIS

Place:	UNIVERSITY OF TAMPERE Faculty of Medicine Institute of Medical Technology, IMT
Author:	MÄNTYMAA, ANNE MAARIT
Title:	The Effects of hypoxic conditions on the chondrogenic differentiation of adipose stem cells
Pages:	64 pp.
Supervisors:	Professor Riitta Suuronen; Katja Mäenpää, M.Sc.; Susanna Miettinen, Ph.D.
Reviewers:	Professor Markku Kulomaa; Katja Mäenpää, M.Sc.
Date:	January 2010

Abstract

Background and aims: Poor self-repairing is a characteristic of articular cartilage damages. Treatments are under investigation, but none has yet proven to be an ideal treatment solution. Tissue engineering offers the possibility to permanent repair or regeneration of cartilage damages. Adult stem cells can be differentiated into mesenchymal cell lineages, such as chondrocytes. High cell density, three-dimensional culture conditions and proper culture medium are known to enhance chondrogenic differentiation. Low oxygen tension is related to normal cartilage development. Therefore, it has been an interesting option when considering the factors enhancing chodrogenic differentiation. The aim of this study was to examine the effects of hypoxic conditions on chondrogenic differentiation of human derived adipose stem cells (ASCs) and to compare the effects of different culture media on differentiation.

Methods: Human ASCs were differentiated in high cell density, in chondrogenic medium with or without transforming growth factor β1 (TGF-β1), either in hypoxic or atmospheric oxygen conditions. The relative expression of the chondorogenic markers and a hypoxic cell survival marker was determined using qRT-PCR method. Cartilaginous matrix formation was evaluated by histological analysis. The degree of differentiation was compared with cells cultured in control conditions.

Results: The expression levels of typical chondrogenic genes generally increased in differentiation conditions. However, a clear difference between hypoxic and atmospheric oxygen conditions was not observed in this study. Type II collagen expression was the highest in hypoxic control conditions, as was the case for hypoxia inducible factor 1α (HIF-1α) expression. Type X collagen expression was high in the presence of TGF-β1. Cartilage-like matrix formation was demonstrated with sulphated-glycosaminoglycan (sulphated-GAG) analysis and histochemical staining. Stronger accumulation of sulphated-GAGs was observed in atmospheric oxygen conditions than in hypoxic conditions.

Conclusions: Low oxygen tension did not enhance the chondrogenic differentiation of ASCs over atmospheric oxygen conditions. However, hypoxic control conditions supported the differentiation based on the highest relative gene expression of know chondrogenic markers. Chondrogenic differentiation was evaluated to be better in atmospheric oxygen conditions than in hypoxic conditions based on sulfated-GAG –staining methods. The effects of hypoxic conditions on chondrogenic differentiation must be further studied.

PRO GRADU -TUTKIELMA

Paikka: TAMPEREEN YLIOPISTO
Lääketieteellinen tiedekunta
Lääketieteellisen teknologian instituutti
Tekijä: MÄNTYMAA, ANNE MAARIT
Otsikko: Hypoksia-olosuhteiden vaikutus rasvan kantasolujen rustoerilaistumiseen
Sivumäärä: 64 s.
Ohjaajat: Professori Riitta Suuronen, FM Katja Mäenpää ja FT Susanna Miettinen
Tarkastajat: Professori Markku Kulomaa ja FM Katja Mäenpää
Aika: Tammikuu 2010

Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Nivelrustovauriot ovat yleisiä. Rustokudoksen uusiutumiskyky on heikko, eikä tehokasta hoitomenetelmää ole vielä kehitetty. Kudosteknologia tarjoaa kuitenkin mahdollisuuden toimiviin hoitomuotoihin ja aikuisen rasvakudoksen kantasoluja voidaan erilaistaa mesenkymaalisten solutyyppien suuntaan, kuten rustosolujen suuntaan. Tiedetään, että korkea solutiheys, mikromassakasvatus ja oikeanlainen kasvatusmedium edistävät rustoerilaistumista. Ruston normaaliin kehitykseen liittyvän niukkahappisen ympäristön vaikutus kantasolujen rustoerilaistukseen on ollut viimeaikoina enenevässä määrin kiinnostuksen kohteena. Tutkimuksen tavoitteena oli tutkia hypoksiaolosuhteiden vaikutusta rasvan kantasolujen rustoerilaistumiseen ja vertailla kasvatusmediumien vaikutusta erilaistumisessa.

Tutkimusmenetelmät: Ihmisen rasvakudoksen kantasoluja erilaistettiin korkeassa solutiheydessä kolmiulotteisena mikromassakasvatuksena kahden rustomediumin avulla, joiden erona oli se, että toinen sisälsi transformaalista kasvutekijä β1:tä (TGF-β1). Erilaistus suoritettiin sekä hypoksia olosuhteissa, että normaalihappipitoisuudessa. Erilaistusta seurattiin rustoerilaistuksessa ilmentyvien markkereiden suhteellisen geenieksprression avulla käyttäen qRT-PCR menetelmää. Rustomatriksin muodostusta arvioitiin histologisten ja immunohistokemiallisten värjäysten avulla. Erilaistumisastetta vertailtiin kontrolliolosuhteissa kasvatettuihin mikromassoihin.

Tutkimustulokset: Rustokudokselle tyypillisten geenien ekspressiotasot nousivat yleisesti ottaen rustoerilaistusolosuhteissa, mutta selvää eroa normaali- ja niukkahappisen ympäristön vaikutuksella ei ollut. Rustokudokselle tyypillisen tyypin II kollageenin ilmentymistasot nousivat eniten hypoksia olosuhteissa kontrollimediumissa, samoin kuin hypoksiamarkkerina toimivan transkriptiotekijän ekspressiotasot. Tyypin X kollageeni ilmeni voimakkaasti TGF-β1:n läsnäollessa. Rustomatriksin kaltaista muodostumista osoitettiin histologisten värjäysten ja sulfatoitujen glykosaminoglygaanien värjäyksen avulla. Normaalihappipitoisuudessa värjäytyminen oli hieman voimakkaampaa kuin hypoksiaolosuhteissa.

Johtopäätökset: Matala happipitoisuus ei parantanut rasvan kantasolujen rustoerilaistumista verrattuna normaalihappipitoisuuteen. Matala happipitoisuus ja kontrollikasvatusmedium kuitenkin saivat aikaan suhteellisesti korkeimmat rustoerilaistusmarkkereiden ilmentymistasot. Sulfatoitujen glykosaminoglykaanien värjäytymisen perusteella rustoerilaistuksen arveltiin olevan tehokkaampaa normaalihappipitoisuudessa. Hypoksiaolosuhteiden merkitystä rustoerilaistukseen on vielä syytä tutkia enemmän.

CONTENTS

ABBREVIATIONS

ACT	autologous chondrocyte transplantation
AGG	aggrecan
ASC	adipose stem cell
BMP-6	bone morphogenetic protein 6
CH	control medium and hypoxia (5%)
CO	control medium and atmospheric oxygen (21%)
COLI	type I Collagen
COLII	type II Collagen
COLX	type X Collagen
COMP	cartilage oligomeric matrix protein
DH	differentiation medium and hypoxia (5%)
DO	differentiation medium and atmospheric oxygen (21%)
D+H	differentiation medium + TGF-β1 and hypoxia (5%)
D+O	differentiation medium + TGF-β1 and atmospheric oxygen (21%)
ECM	extracellular matrix
ESC	embryonic stem cell
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GF	growth factor
HIF-1α	hypoxia inducible factor 1α
IGF	insulin-like growth factor
MSC	mesenchymal stem cell
O_2	oxygen
qRT-PCR	quantitative real-time reverse transcriptase polymerase chain reaction
RT	room temperature
ROS	reactive oxygen species
SOX-9	SRY (sex determining region Y)-box 9
TE	tissue engineering
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor
3D	three-dimensional

1. INTRODUCTION

Cartilage degeneration due to osteoarthritis or trauma resulting in cartilage loss is a major healthcare problem affecting a wide range of people. Treatment of articular cartilage damages is challenging due to the specialized architecture and limited repair capacity of the tissue. To date, there exists no functional and permanent treatment protocol capable of restoring damaged cartilage to its normal phenotype. Various methods in treating cartilage damages have been tried, but none of them has yet been a superior alternative for treatment and better solutions are examined. Treatment possibilities fall into four categories: 1) stimulation of bone marrow to form a repair tissue, 2) autologous osteochondral transplantation, 3) cultured autologous chondrocytes, and 4) autologous or synthetic matrices with and without autologous chondrocytes and growth factors. (Tare et al., 2005) Autologous chondrocyte transplantation has been the first cell-based approach for cartilage repair that has been approved by the U.S. Food and Drug Administration (FDA) for clinical use. Autologous chondrocyte transplantation has good clinical outcomes, but the harvesting procedure of cartilage from undamaged sites of the joint has been associated with progressive degenerative changes in the joint. (Wang et al., 2005)

An alternative to the use of autologous chondrocytes for cartilage repair has been considered to be adult stem cells (Wang et al., 2005). Adult stem cells can be found in many tissues including adipose tissue. Benefits of using adipose stem cells (ASCs) are the amount of adipose tissue and easy accessibility compared to bone marrow derived mesenchymal stem cells (MSCs). Recent studies have shown that ASCs offer great promise for cell-based therapies because of their ability to differentiate towards bone, cartilage, and adipose tissue. One significant advantage of ASCs is the possibility to use patient's own (autologous) cells, which is a major benefit when considering the immunological hurdles of allo- or xenogenous cells. Using autologous cells, hostile immunoreactions are avoidable. (Malladi et al., 2006)

The chondrogenic differentiation of MSCs involves biosynthesis of glycosaminoglycans (GAGs) and deposition of an integrated extracellular matrix (ECM). This process is influenced by a number of factors, including a three-dimensional (3D) culture conditions, and the presence of

appropriate growth factors. (Barry, 2001) An increasing amount of evidence suggests that low oxygen concentration has a significant role in cell differentiation (Giaccia et al., 2004). Recent findings have shown that hypoxic conditions have a critical function in cartilage differentiation and endochondral bone development (Schipani, 2005).

In this study, the chondrogenic differentiation of adipose stem cells was examined *in vitro*. Chondrogenic differentiation was induced with hypoxic conditions, 3D high cell density culture conditions and chondrogenic medium with and without transforming growth factor-β1 (TGF-β1). The aim is to develop chondrogenic differentiation process of ASCs more effective in order to produce tissue engineered cartilage product.

2. REVIEW OF THE LITERATURE

2.1. Cartilage tissue

Cartilage is one form of dense connective tissue. It is an avascular, aneural, and alymphatic tissue, which composes of sparsely distributed chondrocytes embedded within a dense ECM. (McPherson et al., 2000) ECM is a functional element of cartilage, consisting more than 95% of its volume. It is composed of collagen fibers, and abundant ground substance rich in proteoglycan. Chondrocytes are essential in producing and maintaining the matrix. (Ross, 2006) The extracellular matrix of cartilage is solid and firm but somewhat pliable. This accounts for its resilience. (McPherson et al., 2000; Ross, 2006) Cartilage exists as hyaline, elastic or fibrocartilage, depending on molecular composition of their ECM and different anatomical requirements (Ross, 2006).

2.1.1. Hyaline cartilage

Hyaline refers to the glassy nature of hyaline cartilage. It is characterized by matrix rich in type II collagen fibers, proteoglycans, and adhesive glycoproteins. Chondrocytes are located within lacunae in the ECM. Chondrocytes present in clusters called isogenous groups represent recently divided cells. They produce matrix material, which surrounds divided chondrocytes, and they become dispersed. Hyaline cartilage provides a low-friction surface, participates in lubrication in synovial joints, and distributes applied forces to the underlying bone. (Ross, 2006) Collagens confer tensile strength, whereas hydrated GAGs resist compression (Gibson et al., 2008). Cartilage matrix is highly hydrated, and 60-80% of the net weight of hyaline cartilage is intercellular water (Ross, 2006).

2.1.1.1 Collagen molecules

The ECM of hyaline cartilage consists of three major classes of molecules, collagens, proteoglycans, and adhesive glycoproteins. Type II collagen is the major matrix protein constituting the bulk of the fibril. Type IX collagen facilitates fibril interaction with the matrix proteoglycan molecules, and type XI collagen regulates the fibril size. (Ross, 2006) Type X

collagen organizes the collagen fibrils into a 3D lattice (Ross, 2006), and is a marker of hypertrophic cartilage (Schipani, 2005). In addition, type VI collagen is found mainly at the periphery of chondrocytes. It helps in attaching chondrocytes to the matrix framework. Types II, VI, IX, X and XI are referred to as cartilage-specific collagen molecules, since they are found in significant amounts only in the cartilage matrix. (Ross, 2006)

2.1.1.2 Proteoglycans and glycoproteins

The ground substance of hyaline cartilage contains GAGs, such as hyaluronan, chondroitin sulfate, and keratin sulfate. Chondroitin and keratin sulfates are linked to a core protein to form a proteoglycan monomer. The most important proteoglycan in hyaline cartilage is aggrecan. (Roughley et al., 2006; Ross, 2006) Due to the presence of the sulfate groups, aggrecan molecules have a large negative charge and an affinity for water molecules. Hyaluronan molecules are associated with aggrecan molecules, which are bound to the hyaluronan through link proteins. Together they form large proteoglycan aggregates. Water is bound tightly to these aggregates, and this imparts resilience to the cartilage. Some of the water is loosely bound, and therefore allows diffusion of small metabolites to and from chondrocytes. (Ross, 2006) Aggregates are bound to the collagen matrix fibrils by electrostatic interactions and adhesive glycoproteins (Fig. 1). The unique biochemical properties of hyaline cartilage are due to the entrapment of highly charged proteoglycan aggregates within the matrix of collagen fibrils. Other proteoglycans of cartilage matrix, such as decorin, biglycan, and fibromodulin do not form aggregates but interact with GAGs and help in stabilizing the matrix. (Ross, 2006; Hedbom et al., 1992) These are often referred to as small proteoglycans. Cartilage oligomeric matrix protein (COMP) is an example of cartilage ECM protein that appears without GAGs. COMP is shown to express at a specific time during chondrogenesis, distinct from type II collagen and other cartilage matrix constituents. (Hedbom et al., 1992) Adhesive glycoproteins influence interactions between the chondrocytes and the ECM molecules, and are utilized as markers of cartilage turnover and degeneration. Examples of such molecules are cartilage annexin V, tenascin, and fibronectin. (Ross, 2006)

2.1.1.3 Hyaline cartilage as a model for the developing skeleton

Hyaline cartilage is the precursor of bones in early stages of fetal development. Bones develop by the process of endochondral ossification. During the developmental process, most of the cartilage is replaced by bone. (Ross, 2006) Round proliferative chondrocytes (Fig. 2) produce type II collagen and form a columnar layer. Then they differentiate into post-mitotic

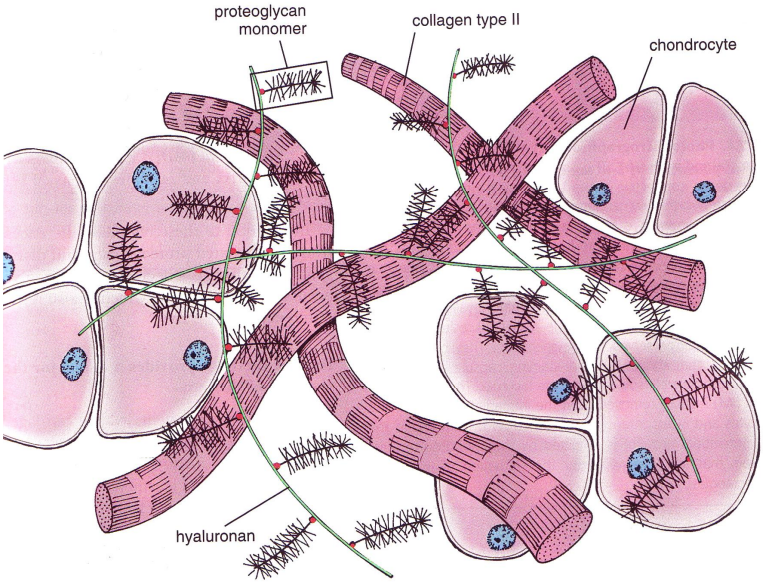

Figure 1. Overview of the cartilage matrix composition. Figure from Ross (2006).

hypertrophic cells. Type X collagen expression is representative for hypertrophic chondrocytes. This differentiation process is followed by mineralization of the surrounding matrix, death of hypertrophic chondrocytes, and blood vessel invasion. Finally, cartilage is replaced with trabecular bone. (Schipani, 2005) The remaining cartilage at the end of bones serves as a growth site for bones. These are called the epiphyseal growth plate. This site of epiphyseal disk remains functional cartilage as long as the bone grows in length. In the adult, cartilage exists on the articular surfaces of joints (articular cartilage), within the rib cage (costal cartilages), as the skeletal unit in the trachea, bronchi, larynx, and nose. (Ross, 2006)

2.1.2. Fibrocartilage

Fibrocartilage constitutes of dense regular connective tissue and hyaline cartilage. It is characterized by abundant of type I collagen fibers in addition to the matrix material of hyaline cartilage. Chondrocytes are similar in appearance to those of hyaline cartilage, but there is less cartilage matrix material associated with them. Fibrocartilage typically exists in intervertebral disks, the symphysis pubis, articular disks of the sternoclavicular and the temporomandibular joints, menisci of the knee joint, the triangular fibrocartilage complex of the wrist, and certain places where tendons attach to bones. Fibrocartilage provides resistance to both compression and shearing forces in these sites, and serves like a shock absorber. (Ross, 2006)

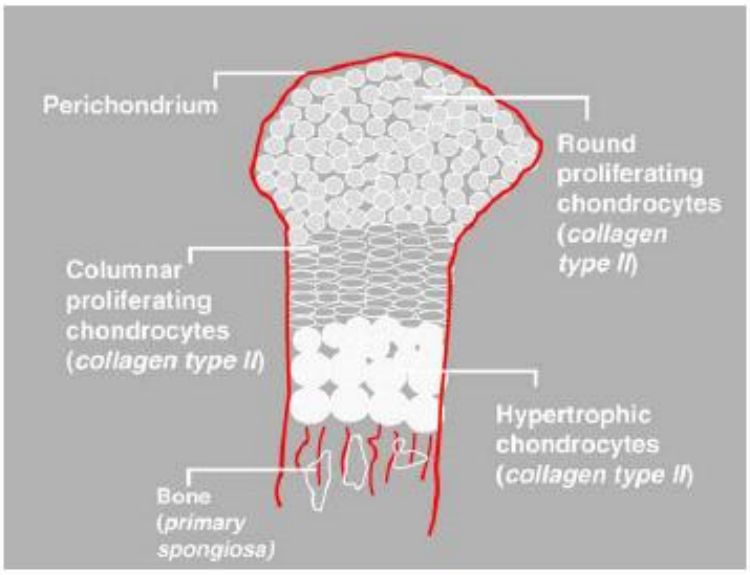

Figure 2. Schematic representation of fetal growth plate, and endochondral ossification. Vascularized areas are shown in red. Figure from Schipani (2005).

2.1.3. Elastic cartilage

Elastic cartilage is distinguished by the presence of elastic fibers and elastic lamellae in addition to the matrix material of hyaline cartilage. The elastic components of this tissue type give the cartilage elastic properties in addition to the resilience and pliability that are characteristic of hyaline cartilage. Elastic cartilage is present in the external ear, the walls of the external acoustic meatus, the auditory tube, and the epiglottis of the larynx. The matrix of elastic cartilage does not calcify during the aging process, as happens with hyaline cartilage. (Ross, 2006)

2.2. Cartilage damages and the challenge of treatment

2.2.1. Cartilages damages

Hyaline cartilage is the type of cartilage that is the most susceptible to stress (Zuscik et al., 2008). Hence regenerative techniques for hyaline cartilage defects that result from traumatic injury or degenerative joint diseases would have the largest impact on patients. Aging population and obesity is estimated to increase the number of osteoarthritis cases in the following years. In addition, cartilage damage followed by sports injuries can often lead in premature cartilage degeneration. (Chung&Burdick, 2008) Traumatic cartilage injuries are susceptible to occur under sudden and high loads while the hydrated ECM has not enough time for controlled deformation and load transmission (Bucwalter, 2002).

Compared to other connective tissues, cartilage turnover rate is slow. On the other hand, cartilage can tolerate considerable amounts of intense and repetitive stress. (Ross, 2006) However, when damaged, articular cartilage exhibits little or no capacity for repair even in the most minor injuries (Buckwalter, 1998). The poor repair capability of articular cartilage could be explained by the lack of blood supply, the immobility of the chondrocytes, the limited ability of mature chondrocytes to proliferate, and a lack of source of undifferentiated cells that can promote repair (Ghivizzani et al., 2000; Ross, 2006). Damage or injury of articular cartilage often leads to progressive degeneration of the joint, as the homeostasis of the tissue is imbalanced. Therefore, cartilage defects need treatment since the imbalanced homeostasis of the tissue eventually results on development of osteoarthritis. (Buckwalter, 1998)

2.2.2. The challenge of treating cartilage damages

In the past decades, the implementation of artificial matrices, growth factors, perichondrium, periosteum, transplanted chondrocytes, and MSCs have been shown to be capable of stimulating formation of cartilaginous tissue in osteochondral and chondral defects in synovial joint (Bucwalter, 2002; Messner&Gillquist, 1996).

Articular cartilage transplantation enables cartilage repair, when intact normal articular cartilage is transplanted in the defect. Periosteal and perichondrial grafts have potential benefit of introduction of progenitor cells with an organic matrix. In addition, they can protect the graft or host cells from excessive loading. (Bucwalter, 2002)

When subchondral bone is penetrated, fibrocartilaginous repair tissue forms on the articular surfaces of synovial joints. In areas with full-thickness loss or advanced degeneration of articular cartilage, penetration of the exposed subchondral bone disrupts subchondral blood vessels. This leads to formation of a fibrin clot that fills the defect and commonly covers the exposed surface. Platelets produce growth factors in fibrin clot in order to enhance the repair process. Undifferentiated MSCs migrate into the defect, proliferate, and differentiate into cells resembling chondrocytes morphologically. During 6 to 8 weeks they form fibrocartilaginous tissue in the chondral portion of the defect. Methods used in penetrating subchondral bone to stimulate formation of new cartilaginous surface include arthroscopic drilling, abrasion of the articular surface and microfracture technique. (Bucwalter, 2002)

Many growth factors have been shown to have an impact on chondrocyte metabolism and chondrogenesis. Osteochondral injuries and exposure of bone due to loss of articular cartilage

release growth factors that affect the formation of cartilage repair tissue. Thus, treating chondral defects locally with growth factors have the potential to stimulate restoration of an articular surface. Delivering growth factors or cell transplants usually requires an artificial matrix. In addition, artificial matrices may stimulate ingrowth of host cell, matrix formation, and binding of new cells and matrix to host tissue. (Bucwalter, 2002)

Transplantation of cells grown *in vitro* is another method to introduce a new cell population into chondral and osteochondral defects. Chondrocytes and undifferentiated MSCs placed in defect sites survive and produce a new cartilage matrix. MSCs and endothelial cells produce many of the used growth factors in repair. (Bucwalter, 2002) One of the latest and most promising cell-based treatment, autologous chondrocyte transplantation (ACT) is based on chondrocytes. ACT involves small biopsies of cartilage from the patient in a minimally invasive manner, isolating chondrocytes from the donor tissue, and expanding the cells *in vitro*. Subsequently, cells are delivered to the cartilage damage site under a periosteum flap to synthesize new cartilage tissue. (Chung&Burdick, 2008)

These preceding treatments have relieved pain in some extent, and improved joint function, but all have disadvantages that can hinder their long-term clinical application (Marlovits et al., 2006). The repaired tissue has a different structure than native cartilage. In addition, cartilage produced from existing treatments is often composed of type I collagen, which is biochemically and biomechanically inferior to hyaline cartilage. Other disadvantages of these treatments include donor side morbidity, complicated surgical procedures, risks of infection, and graft rejection. (Chung&Burdick, 2008) In order to develop an ideal replacement tissue, combining stem cells with natural or artificial scaffolds could be a solution (De Ugarte et al., 2003).

2.3. Cartilage tissue engineering

Tissue engineering (TE) is defined as an interdisciplinary field that connects the principles and methods of engineering and life sciences toward the development of biological substitutes to restore, maintain, or improve tissue function (Langer&Vacanti, 1993). It has the potential to solve problems related to tissue damage and tissue loss due to disease, cancer or trauma (Reddi, 2000). TE could induce tissue regeneration since the events of embryonic skeletal tissue development and morphogenesis are recapitulated (Caplan et al., 1997; Reddi, 2000). TE traditionally involves the use of cells, and biomaterial scaffolds as matrix for cell growth and

differentiation. In addition, signaling factors like growth factors or mechanical stimuli are often used in TE applications. Cells, scaffolds and signaling factors constitute TE solutions alone or in combination (Fig. 3) (Chung&Burdick, 2008).

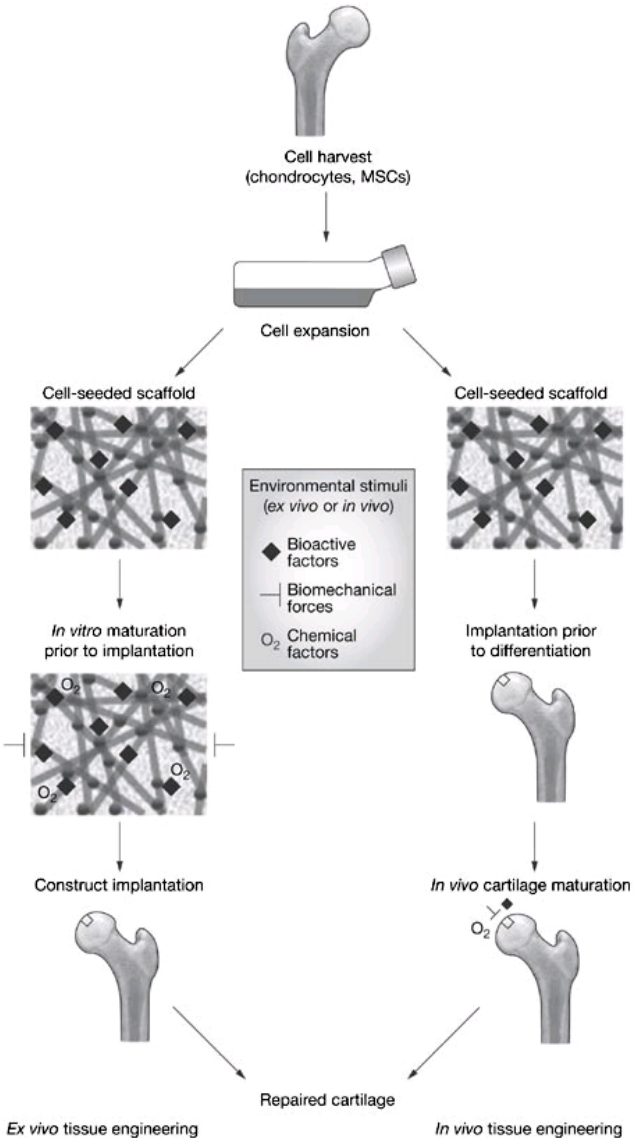

Figure 3. The Process of cartilage tissue engineering is divided to two basic approaches, *ex vivo* tissue engineering and *in vivo* tissue engineering. In *ex vivo* approach, the tissue is generated entirely *in vitro* with full functionality before being transplanted. In *in vivo* approach, the construct is implanted with or without partial *in vitro* cultivation, and allowed to mature *in vivo* for repair and regeneration. Figure from Chen et al. (2006)

Cell source is one of the major challenges for cartilage TE. It is also challenging to obtain sufficient number of cells to fill a clinically relevant defect. The goal is to discover an ideal cell source that 1) could be easily isolated, 2) is capable of expansion, 3) is immunocompatible, and 4) could express and synthesize cartilage-specific molecules like type II collagen and aggrecan. (Chung&Burdick, 2008; De Ugarte et al., 2003) Chondrocytes, fibroblasts, stem cells, and

genetically modified cells have been examined for cartilage repair, but an optimal cell source for cartilage tissue engineering remains to be identified. Recent interest has focused on stem cells, which have multi-lineage differentiation potential and can be obtained from embryo, fetus or adult tissues such as bone marrow and adipose tissue. (Chung&Burdick, 2008)

The use of autologous cells minimizes the need for immunosuppressive therapy after implantation. Chondrocytes may not be the best source of cells for tissue regeneration, because the mature chondrocytes do not generally renew themselves. This provides little potential for further growth. (Vats et al., 2002) In contrast, stem cells are able to divide and renew themselves several times, and they are capable of differentiating into specialized cells (Conrad&Huss, 2005).

2.3.1. Stem cells

Stem cells are defined to have an ability to undergo self-renewal and the ability to differentiate into one or more cell types, depending on their developmental potential. As the understanding of stem cells has grown, the definition of a stem cell has become increasingly complex. Others have argued that the definition must include the ability of stem cells to reconstitute the relevant tissue functionality when transplanted into a suitable host. (Verfaillie, 2002) This criterion is especially relevant when discussing stem cells in the context of regenerative medicine (Rizzino, 2007).

Stem cells are classified according to their origin and their differentiation capacity. Stem cells can be of embryonic, germinal, fetal, or adult origin. According to their differentiation capacity, stem cells can be classified as totipotent, pluripotent, multipotent, and unipotent. Stem cells derived from fertilized oocyte are totipotent until the fourth day of gestation. Totipotent stem cells are capable of differentiating into any cell type that comprises the organism. Stem cells derived from the inner cell mass of the blastocyst are pluripotent. These embryonic stem cells (ESCs) have more limited differentiation capacity, and they give rise to ectoderm, mesoderm, endoderm and germ cells. Adult stem cells are multipotent, and have even more limited differentiation potential. They can give rise to multiple cell lineages. MSCs and ASCs, for example, are multipotent. Unipotent cells have the most limited differentiation capacity, and can give rise to only one or few differentiated cell type. (Shamblott et al., 2000, Rao and Mattson 2001; Choumerianou et al., 2008)

ESCs have the ability to differentiate into all somatic cell types and are capable of many doublings (Chung&Burdick, 2008). However, adult stem cells are preferred to tissue engineering

because they can be used as autologous cells and are thus immunocompatible. Furthermore, adult stem cells have no ethical concerns related to their use. (De Ugarte et al., 2003) Adult stem cells have the capacity to differentiate into multiple cell lineages in appropriate conditions. Sources of adult stem cells investigated for cartilage repair include bone-marrow, adipose tissue, muscle, synovium and periosteum, among others. (Chung&Burdick, 2008) Many authors have found stem cell frequency to be influenced by factors such as age, gender, presence of osteoporosis, and prior exposure to high dose chemotherapy or radiation (Oreffo et al., 1998; Strem et al., 2005). The ideal source of stem cells is one that 1) is easily harvested for autologous transplantation, 2) is abundant, 3) has high proliferation rates for *ex vivo* expansion, 4) has a multilineage differentiation capacity, and 5) has a high yield of cells (Strem et al., 2005; De Ugarte et al., 2003).

2.4. Adipose tissue as a stem cell source

Adipose tissue is abundant, expendable, and easily harvested tissue, which makes it an ideal stem cell source (De Ugarte et al., 2003). White adipose tissue is capable to expand and shrink throughout the life of an adult. This dynamic capacity is mediated by the presence of vascular and non-vascular cells referred to as stem and progenitor cells that have unique regenerative capacity (Strem et al., 2005). The development of adult obesity indicates the presence of adipocyte progenitors or stem cells in adipose tissue depots throughout life (Cornelius et al., 1994). Adipose tissue has been identified as a source of fibroblast-like population of stem cells that can be isolated under local anesthesia with minimal discomfort to the patient (Zuk et al., 2001).

Similar to bone marrow, adipose tissue is a mesodermally-derived organ that contains a stromal population, including microvascular endothelial cells, smooth muscle cells and stem cells (Zuk et al., 2001). Cells can be enzymatically digested out of adipose tissue and separated from the adipocytes with lower mass by centrifugation. A more homogeneous cell population emerges in culture when cultured in conditions supportive of MSC growth. This cell population shares many of the characteristics of its counterpart in bone marrow including extensive proliferative potential and the ability to undergo a multilineage differentiation. (Zuk et al., 2002; De Ugarte et al., 2003) The yield of ASCs is better than that of bone marrow MSCs (Strem et al., 2005), and the volume of human bone marrow accessible under local anesthesia is limited (Bacigalupo et al., 1992). In addition, a harvest of adipose tissue, under local anesthesia, can be easily obtained

excessively more than bone marrow. Besides, stem cell yield is higher compared to that present in bone marrow aspirate. (Aust et al., 2003; Strem et al., 2005)

ASCs have quite similar differentiation capacity, and cell surface phenotype to bone marrow MSCs (Zuk et al., 2002). Multipotent cells identified in adipose tissue have the capacity to differentiate to cells of adipogenic, chondrogenic, myogenic, and osteogenic (Zuk et al., 2002) lineages (Fig.4) when cultured with the appropriate lineage specific stimuli (Gomillion&Burg, 2006). Non-mesenchymal lineage differentiation has been investigated, and some evidence of the transdifferentiation ability of ASCs is confirmed (Strem et al., 2005; Planat-Benard et al., 2004).

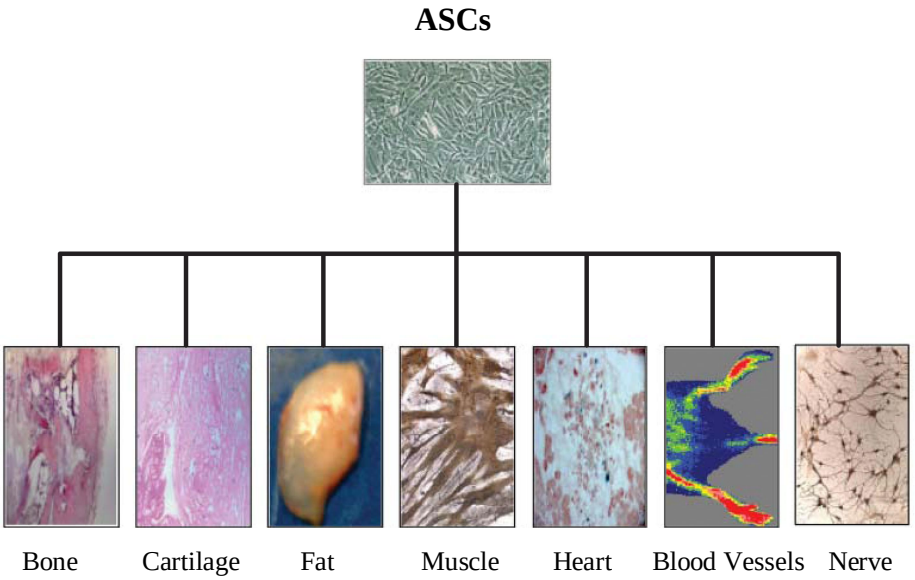

Figure 4. ASCs have the capacity to undergo adipogenesis, chondrogenesis, osteogenesis, and myogenesis. Some evidence of neurogenesis, cardiac myogenesis, and angiogenesis is also presented. Modified from Strem et al. (2005).

2.5. The process of chondrogenesis

The process of chondrogenesis begins with the aggregation and condensation of loose mesenchyme. Factors such as TGF-β1 are known to play critical roles in the condensation process. (Barna&Niswander, 2007) The condensing mesenchyme expresses ECM and cell adhesion molecules during chondrogenesis. Expressed genes include the IIa splice form of type II collagen [Col2a1(IIa)], N-cadherin (Ncad), N-cam (Ncam1), tenascin C (Tnc), and an important transcription factor SRY (sex determining region Y)-box 9 (Sox9) (Zuscik et al., 2008). Sox9 belongs to the Sox family of transcription factors, and is the primary determinant

during the early stages of chondrogenesis. In addition, other Sox family transcription factors have many roles during chondrogenesis and chondrocyte differentiation. (Akiyama et al., 2002) Sox proteins act by binding to a specific consensus motif in the minor groove of DNA and induce DNA bending (DeLise et al., 2000). During the chondrocyte differentiation from the mesenchyme, the cells produce an ECM rich in aggrecan and the IIb splice form of type II collagen [Col2a1(IIb)] (Zuscik et al., 2008). It has been shown that Sox9, L-Sox5 (a new long form of Sox5) and Sox6 form a complex to cooperatively activate Col2a1 gene expression (DeLise et al., 2000). The cells proliferate rapidly after early chondrocyte differentiation. Proliferating cells enlarge the cartilage templates that perform individual skeletal elements. (Zuscik et al., 2008) Further differentiation and hypertrophy leads to the expression of type X collagen and decrease in the expression of type II collagen (DeLise et al., 2000). Hypertrophic differentiation is initiated, when cells near the center of each growing skeletal element withdraw from the cell cycle (Fig. 5). Chondrocyte enlargement, terminal differentiation, mineralization, and ultimately apoptosis are followed by hypertrophic differentiation (Zuscik et al., 2008).

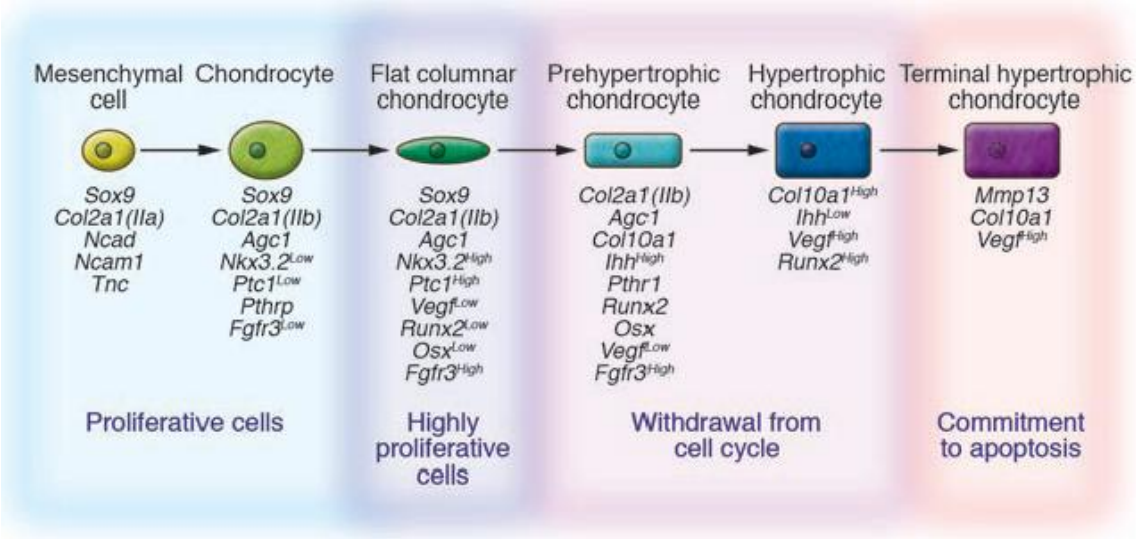

Figure 5. The process of chondrogenesis and chondrocyte differentiation presented in stages. Important markers expressed at each stage of chondrocyte differentiation are listed below each stage. Superscripts after markers indicate the magnitude of gene expression. Figure from Zuscik et al. (2008)

2.5.1. Chondrocyte differentiation and dedifferentiation

Differentiated chondrocytes are round by morphology and produce cartilage specific ECM molecules. *In vitro* monolayer expansion of chondrocytes, causes dedifferentiation. This is characterized by decreased proteoglycan synthesis and decreased type II collagen expression. In contrast, type I collagen expression is increased during dedifferentiation. Various methods have been employed to redifferentiate chondrocytes. (Chung&Burdick, 2008) These include the use of

3D scaffolds, bioreactors (Freed et al., 1998), reduced oxygen tension (Kurz et al., 2004), and the addition of growth factors like TGF-β1, fibroblast growth factor (FGF), and insulin-like growth factor (IGF) (Mandl et al., 2002). Redifferentiation can also be influenced by surface chemistry. Culturing -substrate-material with low adhesion properties supports a chondrocytic phenotype. Low adhesion properties have affected on cells so that they exhibit a round morphology and minimal expression of the fibronectin receptor, α5α1 integrin that interacts with the ECM and mediate various intracellular signals. (Woodfield et al., 2006)

2.6. Adipose stem cells and chondrogenic differentiation

Stem cells isolated from adipose tissue can be isolated and cultured *in vitro* for an extended period with stable expansion and low levels of senescence (Chung&Burdick, 2008). These stem cells are shown to be capable of displaying the biochemical markers associated with mature chondrocytes in the presence of TGF-β, ascorbate, and dexamethasone. (Awad et al., 2003; Erickson et al., 2002; Wickham et al., 2003; Zuk et al., 2001; Zuk et al., 2002) Three-dimensional culture conditions enhance chondrocyte differentiation of ASCs. Differentiation has been achieved in high cell density micromass cultures, and in various scaffolds including alginate, agarose, and collagen-based scaffolds. (Chung&Burdick, 2008; Erickson et al., 2002) After one to two weeks *in vitro,* the ASCs secrete the ECM proteins of cartilage including type II collagen, type VI collagen and aggrecan (Erickson et al., 2002). During *in vitro* culture, differentiated adipose stem cells have been shown to exhibit an increase in equilibrium compressive and shear moduli with accumulation of sulfated-GAGs (Awad et al., 2004).

2.7. Chondrogenic differentiation techniques and stimulating factors in vitro

Chondrogenic differentiation can be enhanced *in vitro* with 3D culture conditions, growth factors, mechanical factors and physical stimuli such as hypoxic conditions.

2.7.1. Micromass technique

Chondrocytes maintain their phenotype in 3D culture when the cell density is high and dedifferentiate in a monolayer culture (Annabi et al., 2003). Ahrens et al. (1977) have developed a micromass technique for chondrogenic differentiation that utilizes a cell suspension of 2×10^7 cells/ml. Among others, Malladi et al. (2007) have modified this micromass technique in which

harvested cells are resuspended in growth medium at 1 x 10^7 cells/mL concentration. Cells are allowed to adhere to each other and to culture dish substratum as they are placed in ten-microliter droplets in culture dishes. Subsequently, chondrogenic medium is carefully added around cell aggregates formed from the droplets of high cell density.

2.7.2. Growth factors and additives

Growth factors and other additives are used in culture medium *in vitro* or in scaffolds *in vivo* in order to control and stimulate cellular differentiation and tissue formation (Chung&Burdick, 2008). Members of the TGF-β family have been shown to play an important role in cartilage development. They have been shown to induce chondrogenesis in ESCs (Hwang et al., 2006), and adult MSCs (Seyedin et al., 1988; Iwasaki et al., 1993), to increase cartilage ECM synthesis (Kim et al., 2003), and to enhance proliferation of chondrocytes (Rosier et al., 1989; Vivien et al., 1990). The members of the TGF-β family act by signalling through specific serine/treonine kinase receptors and their nuclear effectors, Smad proteins (Roelen&Dijke, 2003). Several experiments have shown that TGF-β isoforms differ in their influences on various cell types. TGF-β1 is responsible for initial cell-cell interactions between condensing progenitor cells, as described by Tuli et al. (2003). They showed that MSC condensation is initiated by TGF-β1 within a pellet culture, and is mediated via N-cadherin. This TGF-β1 –mediated condensation is suggested to be a critical step for the progression of chondrogenesis, which is similar to developmental chondrogenesis *in vivo*. TGF-β2 mediates hypertrophic differentiation, and TGF-β3 has said to have a stronger positive effect on bone marrow MSCs differentiation than TGF-β1. (Gooch et al., 2001; Roberts et al., 1990) It is demonstrated in a few studies that under 3D culture conditions in defined media with added TGF-β1, human ASCs express the genes and proteins of the major cartilage matrix macromolecules (Erickson et al., 2002; Wickham et al., 2003; Awad et al., 2004: Leddy et al.,2004).

In addition to TGF-β1, other growth factors have been used in micromass cultures. For instance IGF-1 acts as anabolic factor to increase the production of proteoglycans and type II collagen (Fukumoto et al., 2003; Gooch et al., 2001). The use of FGF-2 and bone morphogenetic protein-6 (BMP-6), also affects the chondrogenesis of ASCs (Chung&Burdick, 2008). FGF-2 is shown to increase cell proliferation and enhance chondrogenesis (Chiou et al., 2006). In addition, BMP-6 is known to upregulate the expression of aggrecan and type II collagen in alginate culture of ASCs (Estes et al., 2006). Among growth factors, other soluble factors such as ascorbate, dexamethasone, and insulin have been explored independently and synergistically for their

effects on cartilage tissue engineering. Growth factors differ in their influences on various cell types, meaning that they may have different effects for example on ASCs and bone marrow MSCs. (Chung&Burdick, 2008)

2.7.3. Mechanical and physical factors

An additional approach to enhance the differentiation is the introduction of mechanical signals through loading regimes or through the use of bioreactors. Loading can be for instance hydrostatic or dynamic compression. Cartilage depends on mechanical forces to maintain its healthy function. Therefore, this approach has been studied to alter cellular differentiation and tissue production. (Chung&Burdick, 2008) The requirement of mechanical stimulus is particularly the case in articular cartilage. Mechanical stimuli sensed by chondrocytes are referred to as mechano-electrochemical events. These regulate the metabolic activity of chondrocytes and the maintenance of the ECM in coordination with other environmental, hormonal, and genetic factors. Compressive loading, hydrodynamic or osmotic pressure, fluid flow, ion flow, and electrical current can be considered as mechano-electrochemical events sensed by chodrocytes. Static loading of cartilage explants in culture is known to reduce chondrocyte metabolism and the expression of aggrecan and collagen. In contrast, dynamic loading seems to have a net anabolic effect. (Zuscik et al., 2008)

Micro-environmental factors, such as oxygen concentration play a role in the regulation of MSC differentiation. Hypoxia alters essential and physiologically important intracellular pathways. Low oxygen levels have been recognized as a critical stimulus for chondrocyte development. (Danet et al., 2003)

2.8. Hypoxic conditions

2.8.1. Oxygen concentration in cartilage tissue

Chondrocytes exist in avascular tissue. They are influenced by hypoxic microenvironment during chondrocyte maturation and endochondral ossification. (Malladi et al., 2007) Articular cartilage has low oxygen (O_2) levels, with estimates of between 1 and 6% depending on the site of the tissue. Oxygen concentration is around 6% at the surface of cartilage, and 2-3% deeper in the tissue (Fig. 6). Compared to other tissues, it is important to notice that, for chondrocytes, 5% O_2 level is normoxic. In order to subject the cells to true hypoxic levels, reduction to lower O_2

tensions is required. Cartilage is relatively thick. Therefore, O_2, nutrients and waste products have to diffuse over long distances. (Gibson et al., 2008)

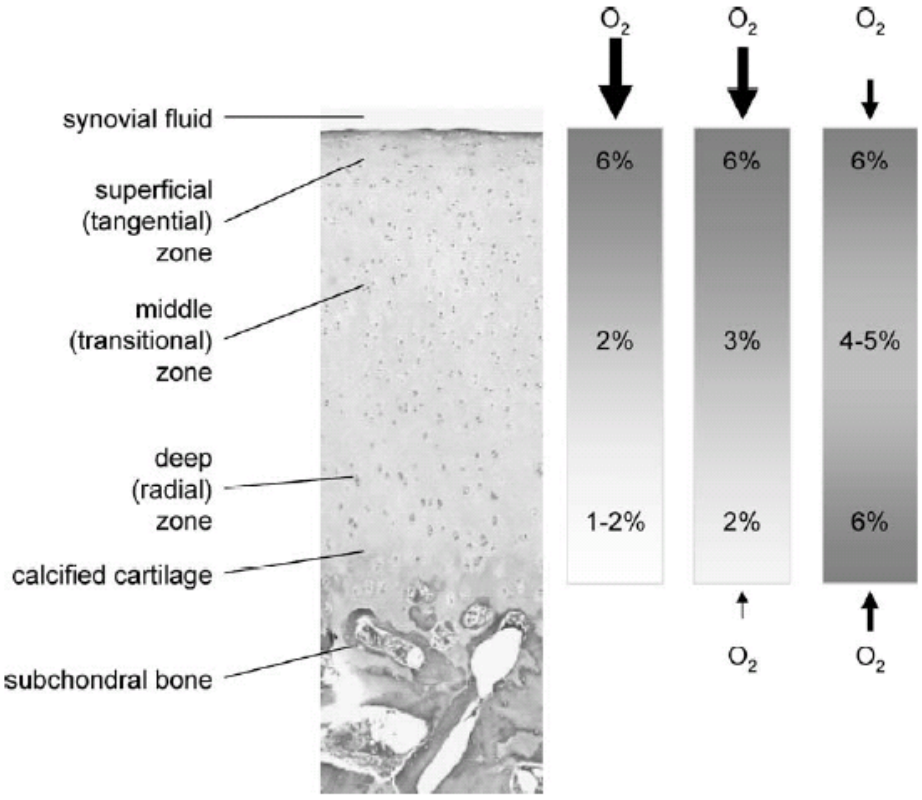

Figure 6. The zones of articular cartilage and its oxygen supply in different depths of the tissue. Figure from Gibson et al. (2008)

Chondrocytes obtain most of their energy from glycolysis (Lee&Urban, 1997), which leads to low O_2 consumption. The consumption rate is only 2-5% of those for other cells (Heywood et al., 2006). Therefore, prolonged culturing at atmospheric O_2 levels can be considered abnormal and may alter normal metabolism and gene expression of chondrocytes (Gibson et al., 2008; Schneider et al., 2007). Hyperoxia is also damaging for them (Ysart&Mason, 1994). In spite of all, chondrocytes do require oxygen because cellular ATP levels, glycolysis and matrix production fall during anoxia (Lee&Urban, 1997). Atmospheric O_2 level is inappropriate and harmful for a cell originating from a tissue of much lower tension. Thus chondrocytes survive better at 5% O_2. (Schneider et al., 2007)

2.8.2. Chondrocyte response to low oxygen concentration

Cells under hypoxic conditions alter their expression of genes involved in cell cycle, differentiation, and apoptosis. The transcription factor hypoxia inducible factor-1α (HIF-1α) is a survival factor that is induced in hypoxic environments in MSCs and chondrocytes. (Zuscik et

al., 2008; Schipani et al., 2001) It has a significant role in adaptive resposes to hypoxia (Schipani, 2005). HIF-1α controls genes associated with cell proliferation, cell metabolism, and angiogenesis, like vascular endothelial growth factor (VEGF) (Semenza, 2000). HIF-1α is associated in differentiation process as it inhibits cell proliferation but increases ECM production (Zuscik et al., 2008; Schipani et al., 2001).

HIF-1 composes of 2 isoforms, HIF-1α and HIF-1β. It is a heterodimeric protein. Both isoforms are constitutively expressed in cells. (Schipani, 2005) HIF-1β is stable in normoxic conditions, but HIF-1α is highly unstable in atmospheric oxygen conditions due to hydroxylation on specific proline residues by prolyl-hydroxylase (Schipani, 2005; Jaakkola et al., 2001; Min et al., 2002). Under hypoxic conditions, oxygen sensitive prolyl-hydroxylase activity is reduced, and HIF-1α translocates into the nucleus. There, it binds to the second part of a heterodimer, HIF-1β and binding proteins. This complex binds to specific hypoxic responsive elements, and causes the initiation of transcription of specific genes. (Chan et al., 2002; Kallio et al., 1998; Leo et al., 2004; Bishop et al., 2004) Massive apoptosis of chodrocytes in hypoxic areas is followed by the deletion of the gene encoding HIF-1α. In 3D micromass cultures of ASCs, targeted deletion of HIF-1α leads to reduced chondrogenic potential. This indicates that hypoxia-induced upregulation of HIF-1α supports chondrogenic differentiation. (Zuscik et al., 2008) The lack of HIF-1α *in vitro* has inhibiting influence on the anaerobic glycolysis and ECM production of chondrocytes (Pfander et al., 2003). Hypoxia is suggested to be an important stress factor that enhances the chondrogenic potential of mesenchymal cell populations during the development of normal tissues that have limited blood supply. In addition, hypoxia may enhance the chondrogenic potential of MSCs under pathologic conditions, e.g., following disruption of vascularity after injury. (Zuscik et al., 2008)

Another member of HIF family, HIF-2α shares 80% sequence homology to HIF-1α. Like HIF-1α, it also interacts with HIF-1β. (Prabhakar et al., 2009) Hypoxia promotes the phenotype of human articular chondrocytes through HIF-2α –mediated Sox9 pathway, and thus induces the expression of the main matrix genes. HIF-2α is known to promote both Sox9-dependent and –independent factors important for cartilage homeostasis. Therefore, it could be possible to manipulate HIF-2α, instead of Sox9. This may enhance the chondrocyte phenotype and, hence, cartilage repair. (Lafont et al., 2008)

2.8.3. Hypoxia in chondrogenic differentiation

Due to low oxygen concentration of normal cartilage tissue, hypoxic conditions are an interesting approach in examining chondrogenic differentiation of MSCs, including ASCs. In the previous studies, MSCs are shown more chondrogenic at low O_2. (Guilak et al., 2006) Xu et al. (2007) have shown that mouse ASC early chondrogenesis was greater when ASCs were expanded in 2% oxygen concentration, rather than in 21% concentration. ASCs expanded in lower oxygen concentration proliferated at a faster rate. The differentiation was performed in 21% oxygen concentration in their experiment. In contrast to enhanced chondrogenic capacity, inhibited osteogenic capacity was observed in the same experiment. (Xu et al., 2007) Wang et al. (2005) published data indicating that 5% oxygen tension and chondrogenic conditions can significantly increase the synthesis of total protein, collagen, and sulfated-GAGs of human ASCs, but inhibit cell proliferation.

HIF-1α is demonstrated to be a critical regulator of chondrogenic differentiation under hypoxic conditions (Xu et al., 2007; Malladi et al., 2007). Vascular endothelial growth factor (VEGF) is one of the known downstream targets of HIF-1α (Malladi et al., 2007). According to their studies, Malladi et al. (2007) provided some evidence of HIF-1α signaling through VEGF. Since the micromass is a heterogeneous structure with a normoxic periphery and a relatively hypoxic interior, analysis of gene expression is inconsistent (Malladi et al., 2007). Malladi et al. therefore used HIF-1α-deleted ASCs in monolayer to study the gene expression profile. They found out that Sox9, was significantly decreased when HIF-1α was deleted. In addition, aggrecan and type II collagen were also significantly downregulated. These data suggested that HIF-1α may directly regulate the expression of Sox9, aggrecan, type II collagen, and VEGF in ASCs. Although it is unknown whether Sox9 is transcriptionally regulated by the members of HIF-1 family, Malladi et al. study suggests that Sox9 is tightly associated with hypoxia responsiveness in these cells. (Malladi et al., 2007)

It has been shown that under long-term hypoxic stress, chondrogenic differentiation is significantly decreased. This may be due to over-production of reactive oxygen species (ROS) under lower oxygen conditions. (Malladi et al., 2006) Oxygen and ROS play a crucial role in controlling homeostasis and facilitate the turnover and remodeling of the ECM in the physiology and pathology of cartilage differentiation (Carlo&Loeser, 2003; Henrotin et al., 2005). ROS production may lead to exceeding the antioxidant capacities of the cells. This results in

“oxidative stress”, which leads to structural and functional cartilage damage, like cell death and matrix degradation (Henrotin et al., 2005).

3. AIMS OF THE RESEARCH

The aim of the research was to examine the effects of hypoxic conditions on chondrogenic differentiation of human derived ASCs. The effects of hypoxia on the differentiation were evaluated in different culture media. The aim is to develop more effective chondrogenic differentiation technique for ASCs in order to produce a tissue engineered cartilage product.

4. METHODS

4.1. Culture conditions and chondrogenic differentiation of adipose stem cells

ASCs used in this research were isolated from humans who have given informed consent. ASCs from four patients were used. Human adipose tissue was received from surgical operations of the Tampere University Hospital and private clinic Terveystalo. The study was carried out under approval of the ethical committee of Pirkanmaa hospital district (R03058).

Chondrogenic differentiation was compared between hypoxic conditions (5% O_2) and conditions with atmospheric oxygen concentration (21% O_2). Differentiation was induced in two differentiation media and compared to that in control media (Table 1).

Table 1. Growth conditions ant their abbreviations used in differentiation protocol.

21% Oxygen	**O**	**5% Oxygen (hypoxia)**	**H**
Control medium	CO	Control medium	CH
Differentiation medium	DO	Differentiation medium	DH
Differentiation medium + TGF-β1	D+O	Differentiation medium + TGF-β1	D+H

Chondrogenic differentiation was induced using the micromass culture technique modified from Ahrens et al. (1977). High cell density (1 x 10^7 cells/ml) was induced with droplets of 2 x 10 µl adipose stem cell suspension. ASCs of passage 5 and 6 were used. Two high cell density droplets were plated into the center of each well and allowed to attach for 3 h in the incubator (5% CO_2, 21% O_2). Control medium and differentiation media were gently added so as not to detach the plated micromasses. Cultures were maintained in the incubator for one week and media were changed a day after plating and then every third day. The micromasses were cultured either in control medium (DMEM/F12; Gibco Invitrogen, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamin) or in differentiation medium with and without TGF-β1 (10 ng/ml). Differentiation medium comprised of DMEM/F12, supplemented with 1xITS+1, (10 µg/ml insulin, 5,5 µg/ml human transferrin, 5ng/ml sodium-selenite, 0,5 µg/ml bovine serum

albumine, 4,7 µg/ml linoleic acid, Sigma), 0,2% antibiotic/antimycotic (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amfoterisin, Lonza), 1% L-glutamin, 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 55 µg/ml sodium pyruvate (Lonza, Biowhittaker) and 23 µg/ml L-proline (Sigma, St. Louis, USA).

Cultures were transferred to Cell IQ machine (Chip-Man Technologies Ltd.) seven days after plating. In Cell IQ, the control well plate was maintained in traditional incubator conditions (5% CO_2 and 21% O_2) and the hypoxic well plate was maintained in 5% CO_2 and 5% O_2 for one week. Medium was changed every other day. After a week Cell IQ period, micromasses were harvested for total RNA isolation, sulfated-GAG and histological analysis.

4.2. Histology

4.2.1. Preparation of histological sections

The samples were fixed overnight in 4% paraformaldehyde in PBS, and dehydrated through a graded series of ethanol, cleared in xylene in Tissue Embedding Console System (Tissue-Tek® VIP, Miles Diagnostic division, Mishawaka, Indiana, USA). Finally, the samples were embedded in paraffin and sectioned at 5 µm thickness with the microtome. Samples were deparaffinized in xylene and hydrated in a degrading ethanol series before staining.

4.2.2. Alcian blue and Safranin O staining of histological sections

The presence of sulfated-GAGs in micromass sections was detected with Alcian blue and Safranin O staining. Rabbit larynx and trachea samples were used as the positive controls along staining. Alcian blue staining was performed at pH 1 for 30 min. Tap water was used to wash the stain. Samples were rinsed in distilled water before counter stain in Nuclear Fast Red solution (Biocare Medical, Concord, California, USA) for 5 minutes. Weigert´s iron hematoxylin was used as a counter stain in Safranin O staining. Sections were treated with 0,001% Fast Green and 1% acetic acid before the staining. The stained sections were dehydrated through an ascending ethanol series and finished in xylene. Finally, the sections were mounted with entellane.

4.3. Immunohistochemistry

Micromasses of one individual were stained immunohistochemically for type I and type II collagen, the ECM proteins in fibro- and hyaline cartilage, respectively. Rabbit larynx and trachea samples were used as the positive controls along staining.

After deparaffinized (see 4.2.1), samples were predigested with 0,2% trypsin in 0,1% $CaCl_2$ for 30 minutes at 37˚C and then with 0,25 U/ml chondroitinase ABC (Sigma) and 1,45 U/ml hyaluronidase (Sigma) for 30 minutes at 37˚C. PBS wash was performed between and after enzyme digestions, and after every step in the procedure. Quencing of endogenous peroxidase activity was done in 3% hydrogen peroxide for 30 minutes at room temperature (RT). Normal horse serum 10% was used for blocking. Primary antibody treatment over night was performed with mouse monoclonal Collagen type I (Sigma, St.Louis USA) and mouse monoclonal collagen type II (II-II6B3 AB, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City).

On the next day, secondary antibody treatment was performed using biotinylated anti-mouse antibody, made in horse (Vector). Avidin-biotin –complex ABC (Vector) was added before diaminobenzene (DAB, DAB Substrate Kit Zymed). Reaction was stopped with 0,5 M Tris at pH 7,4 and water. Mayer's hematoxylene was used as counter stain. After staining, the sections were dehydrated through an ascending ethanol series and finished in xylene and then mounted with entellane.

4.4. Sulfated glycosaminoglycan analysis

The total amount of sulfated-GAGs in micromasses were analysed with Blyscan^{TM} Sulfated Glycosaminoglycan Assay (Newtownabbey, Ireland) according to the manufacturer´s instruction.

Micromasses were stored at -20°C in control medium after the differentiation. Micromasses were washed with PBS and digested over night with 0,25U/ml papain (Sigma) at 60°C. Papain lysate was then used in sulfated-GAG assay and DNA analysis. Blyscan dye reagent was added on papain lysate. Dye is bound to sulfated-GAGs, and the sulfated-GAG-dye complex precipitates out of solution. The samples were centrifuged, and unbound dye solution was removed by

inverting and draining the sample tubes. Dissociation reagent was added to release the bound dye into solution. Spectrofotometer (656 nm) was used to measure the intensity of bound cationic dye (methylmethylene blue) to sulfated parts of the GAG molecules. Sulfated-GAG amount was then compared to the overall amount of DNA in the samples. DNA quantification was performed using Hoechst 33258 (Bio-Rad) stain and the amount of fluorescence signal was measured with Victor (PerkinElmer) multiplate reader (excitation 360 nm and emission 460 nm).

4.5. Total RNA isolation, cDNA synthesis and qRT-PCR analysis

Total RNA was isolated from micromasses after 2 weeks of differentiation using Eurozol® -reagent (EuroClone™, Pero, Italy) according to the manufacturer's instruction. The wells of micromass samples were rinsed with PBS and 2-3 wells of micromasses were pooled together in 1ml Eurozol® -reagent to ensure an adequate yield of total RNA. The samples were stored at -70°C for 2 days – 3 weeks before RNA isolation. All the samples were incubated in Eurozol® at room temperature (RT) for 5 minutes before freezing. 100 µl chloroform was added to 1 ml Eurozol/sample –suspension and samples were shaken vigorously by hands. Samples were incubated 2-3 minutes RT and centrifuged at 12 000 rcf (4°C) for 15 minutes. The upper phase including RNA, was mixed with 500 µl isopropanol. Samples were incubated for 10 minutes at RT, and RNA was pelleted by centrifugation at 12 000 rcf (4°C) for 10 minutes. Subsequently, RNA pellets were washed in 75% ethanol and centrifuged at 7500 rcf (4°C) for 15 minutes. The RNA pellets were dissolved in 30 µl distilled water and the extracted RNA was stored at -70ºC. Nanodrop (Thermo Fisher Scientific, Wilmington, USA) was used to measure total RNA yield by optical density at 260 nm and to assure sample purity from the ratio of A260/A280.

A High-Capasity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, California, USA) was used to prepare cDNA from total RNA according to the manufacturer's instructions, Table 2.

The Protocol used in reverse transcription (GeneAmp PCR System 2400, Perkin Elmer, Connecticut, USA) was as follows:

Initiation	25ºC, 10 min
Reverse transcription reaction	37ºC, 2 h
	85ºC, 5 seconds
Hold	4ºC, ∞

The cDNA product was stored at -20ºC.

Table 2. The composition of master mix in reverse transcription.

Reagent	**Volume per reaction (µl)**
10 x reverse transcriptase buffer	6
25 x dNTP mixture	2.5
10 x Random Primers	6
50 U/µl Multiscribe reverse transcriptase	3
Distiller water	12.5
Total volume of master mix	**30**
5 µg total RNA diluted in distilled H_2O	30
Total volume of reaction	**60**

Differences in gene expressions of micromasses grown in control medium and differentiation media, as well as hypoxic and normal oxygen conditions, were analyzed on the basis of mRNA levels by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) method. Applied Biosystems Primer Express® software version 3.0 was used to design the primers. These primer sequences and accession numbers are presented in Table 4. Reactions were carried out in duplicates in MicroAmp optical 96-cell reaction plates (Applied Biosystems, Singapore). Reaction mixture components are presented in Table 3.

Table 3. The qRT-PCR master mix and sample addition.

Reagent	**Volume (µl)**
2 x SYBR® Green Master mix including SYBR® Green 1 dye, Amplitaq Gold® DNA polymerase, dNTPs with dUTP, Passive reference 1, and buffer components (Applied Biosystems, Warrington, UK)	7.5
10 µM Forward primer	0.5
10 µM reverse primer	0.5
Distilled H2O	5.5
cDNA samples 50 ng in dH2O	2
Reaction total volume	**16**

The Protocol used in qRT-PCR reaction (ABI Prism® 7300 Sequence Detection System, Applied Biosystems, Singapore):

Enzyme activation 95ºC, 10 min

45 x cycles of:

Denaturation	95ºC, 15 seconds
Anneal and extend	60ºC, 1 min
Hold	4ºC, ∞”

The gene expression data were analyzed with ABI Prism® 7300 Sequence Detection Systems version 1.4 software (Applied Biosystems). Melting curve analysis was used to check the quality of the PCR products. The results were normalized against RPLP0 (human acidic ribosomal phosphoprotein P0) expression according to mathematical model described by Pfaffl (2001). Normalization was performed using a cycle threshold (CP) value obtained from the exponential area of amplification curves for each sample and a slope value of a linear standard curve. Duplicate sample value averages of RPLP0 were used for calculations. The equation is presented in Figure 7.

$$Ratio = \frac{(E_{target})^{\Delta CP_{target}\,(control-sample)}}{(E_{ref})^{\Delta CP_{ref}\,(contol-sample)}}$$

Figure 7. The equation for normalizing the gene expression results against RPLP0 expression. $E = 10^{(-1/slope)}$.

4.5.1. Primers used in qRT-PCR reaction

Aggrecan (AGG), cartilage oligomeric matrix protein (COMP), type II collagen (COLII) and SRY (sex determining region Y)-box 9 (SOX9) were chosen as the indicators of chondrogenic differentiation based on known cartilage ECM proteins and chondrogenic differentiation transcription factors. Type I collagen (COLI) was analyzed to detect a possible osteogenic or fibrocartilage differentiation. Type X collagen (COLX) was examined as a marker of chondrocyte hypertrophic differentiation. Hypoxia inducible factor 1α (HIF1α) was analyzed to examine the effects of hypoxic conditions to the cell response and expression. The primers (Table 4) were supplied by Oligomer Oy, Helsinki, Finland.

Table 4. The primer sequences.

Gene	Accession number	Primer sequence	Product size
AGG	NM_001135	Forward 5´-TCG AGG ACA GCG AGG CC-3´	85
		Reverse 5´-TCG AGG GTG TAG CGT GTA GAG A-3´	
COMP	NM_000095	Forward 5´-GCCTGGCTGTGGGTTACACT-3´	73
		Reverse 5´-TCCGTGACCGTGTTCACATG-3´	
HIF1α	NM_001530	Forward 5´-CATGGAAGGTATTGCACTGCA-3´	65
		Reverse 5´-CTGAGGTTGGTTACTGTTGGTATCA-3´	
SOX9	NM_000346	Forward 5´-AAAGGCAACTCGTACCCAAATTT-3´	75
		Reverse 5´-TGATTGGCCACAAGTGGGTAA-3´	
COLI	NM_00088	Forward 5´-CCA GAA GAA CTG GTA CAT CAG CAA-3´	95
		Reverse 5´-CGC CAT ACT CGA ACT GGA ATC-3´	
COLII	NM_001844	Forward 5´-GAG ACA GCA TGA CGC CGA G-3´	67
		Reverse 5´-GCG GAT GCT CTC AAT CTG GT-3´	
COLX	NM_000493	Forward 5´-CACGCAGAATCCATCTGAGAATAT-3´	92
		Reverse 5´-GTTCAGCGTAAAACACTCCATGAA-3´	

5. RESULTS

Sample abbreviations and explanations used in results and discussion chapters are presented in Table 1.

5.1. Cell monitoring during differentiation

All ASC cultures were monitored during growth period with light microscope (Fig.8). In addition, micromasses (Fig.9) were monitored with Cell IQ machine.

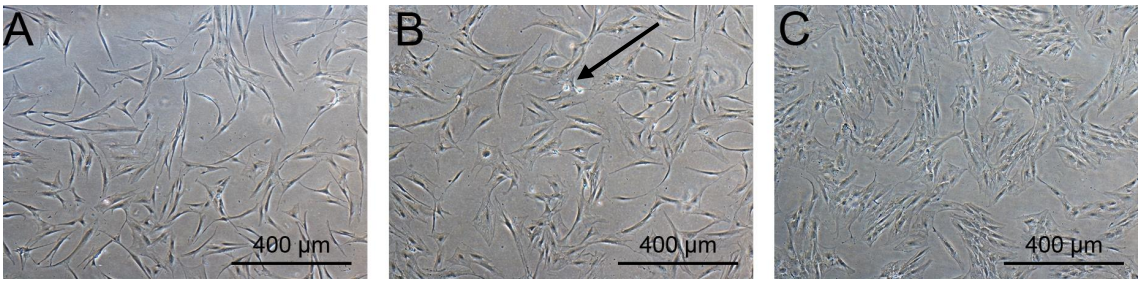

Figure 8. Monolayer cultures of ASCs during expansion. ASCs at passage 1 (A), at passage 2 (B), and at passage 4 (C) are from different individuals. Dividing ASC (B) is pointed with arrow.

A day after plating, the micromasses were big and translucent (Fig.9A, B, C). By the third day, the micromasses had condensed from that of the beginning. Control micromasses (Fig.9D, G) were smaller than those differentiated in chondrogenic media (Fig.9E, F, H, I) and were attached more firmly to the well substratum. The size of micromasses differentiated without TGF-β1 was approximated to be larger than those differentiated with TGF-β1. During the differentiation process, the control micromasses attached more firmly to the bottom of the wells and finally were difficult to detach, while chondrogenic micromasses were rather floating in the medium. Micromasses without TGF-β1 were evaluated to attach a bit more firmly to the wells than those with TGF-β1. The surfaces of differentiated micromasses are smooth in microscopic images, but in control micromasses there are more cells attached to the micromass surface forming a “star-like” structure (Fig.9G). In control wells, there were more cells attached around to the well than in chondrogenic wells. Cells spread less from the chondrogenic micromasses that were cultured without TGF-β1 than those cultured with TGF-β1.

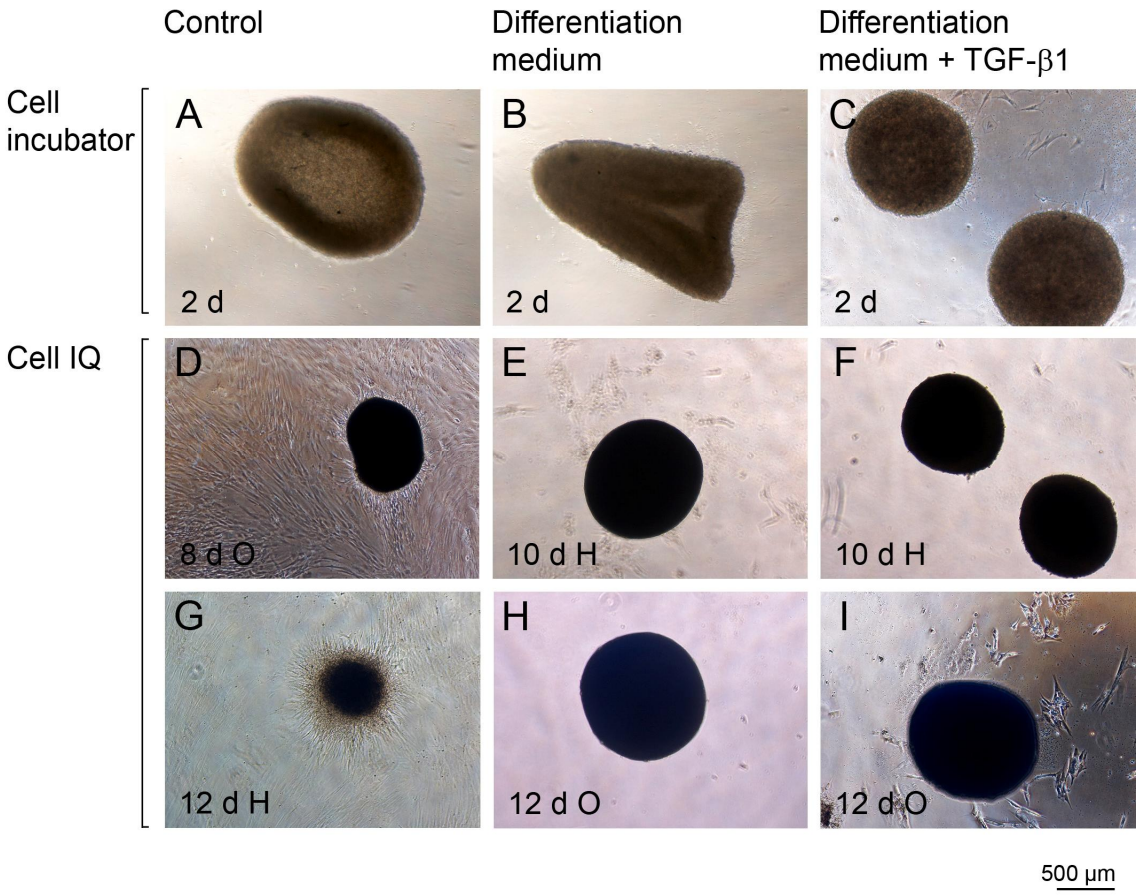

Figure 9. The progression of chondrogenic differentiation in micromass culture. Light microscope images of micromasses (A-C) are taken a day after micromass plating. The micromasses were grown 8-10 days (D-F) and 12 days (G-I) either in normal oxygen concentration (O) or in hypoxic conditions (H). It can be seen that after 8-12 days, the control micromasses were smaller than chondrogenic micromasses.

5.2. Histology

5.2.1. Alcian blue and Safranin O staining

Control samples did not stain with Alcian blue (Fig.10) and Safranin O (Fig.11) for sulfated-GAGs. Alcian blue staining was detected in all micromass samples cultured in differentiation media. A bit stronger staining was observed in atmospheric oxygen samples (Fig.10B, C) than in hypoxic conditions (Fig.10E, F). Samples without TGF-β1 in atmospheric oxygen stained more intensively (Fig.10B), than those with TGF-β1 (Fig.10C). In hypoxic conditions, the situation was opposite; samples differentiated with TGF-β1 stained stronger (Fig.10F), than those without TGF-β1 (Fig.10E).

Round longitudinally orienting zone was observed under the surface of micromasses in atmospheric oxygen with and without TGF-β1 (Fig.10G, I, J) in Alcian blue staining. It was clearly detectable in lower magnification pictures G and I. The zone in the presence of TGF-β1 (Fig.10G) had a blue appearance, while the zone in the micromass without TGF-β1 had white appearance (Fig.10I).

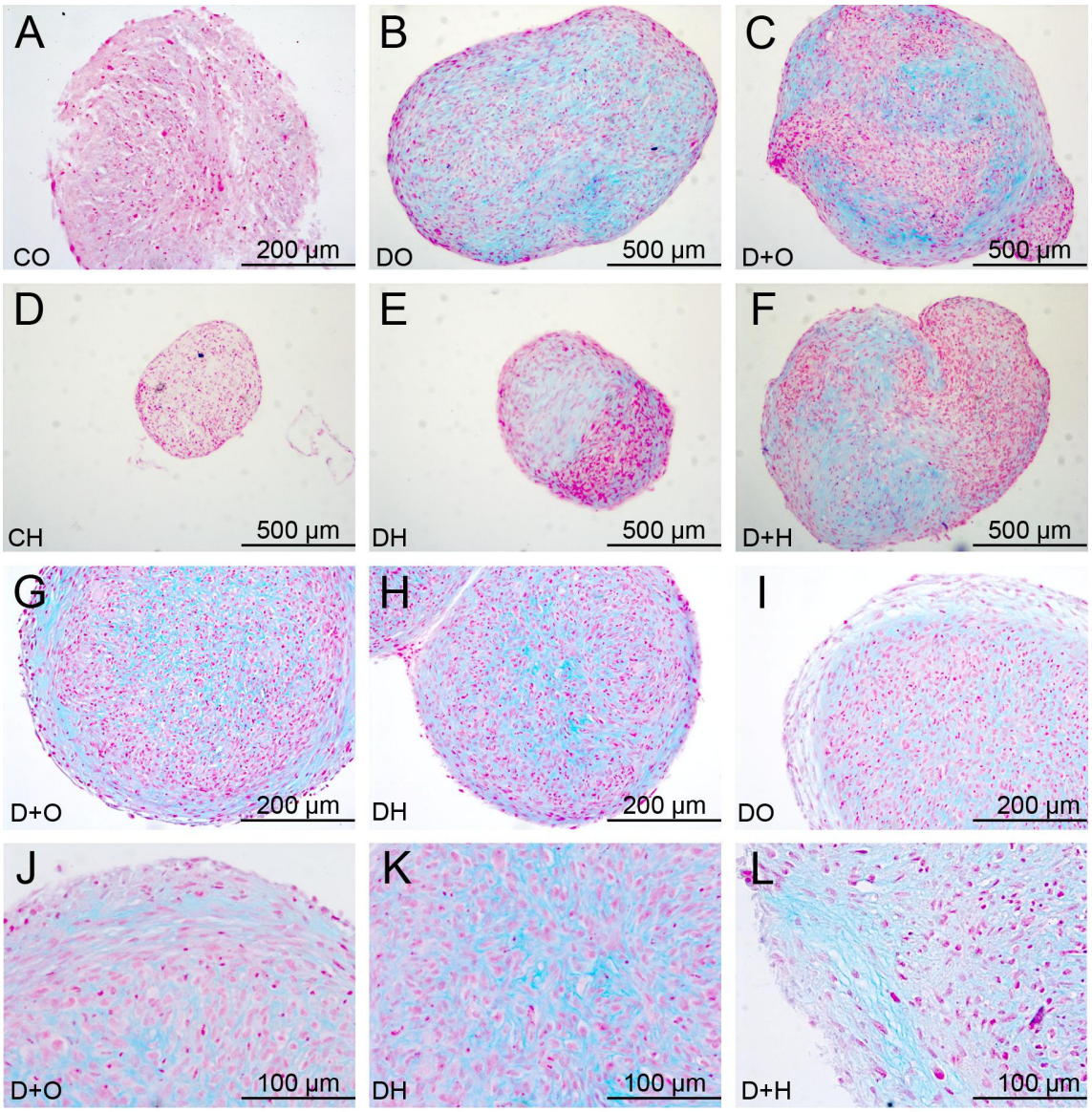

Figure 10. Alcian blue stained micromasses from control (A and D), chondrogenic (B, E, H, I and K) and chondrogenic + TGF-β (C, F, G, J and L) samples.

Positive staining for sulfated-GAGs with Safranin O was detected in micromass samples cultured in DO and D+O conditions (Fig.11B, C, D). Compared to the control sample, a slight positive staining was detected in DH conditions (Fig.11E). In D+H sample, the staining was more intense than in DH condition (Fig.11F).

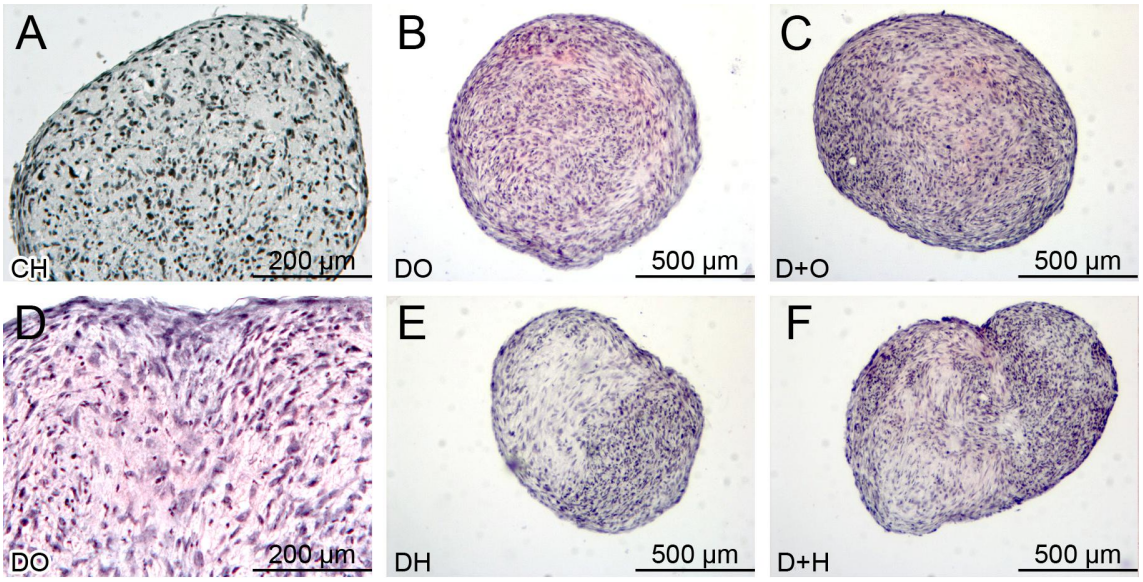

Figure 11. Safranin O stained micromasses from control (A), chondrogenic (B, D and E) and chondrogenic + TGF-β1 (C and F) samples.

When comparing Alcian blue and Safranin O staining in the sections sliced from the same micromass, positive staining could be distinguished in the same areas of the micromasses (Fig.12), where cells are sparsely spread. Stronger positive staining was observed for micromasses differentiated in hypoxic conditions with Alcian blue, than Safranin O.

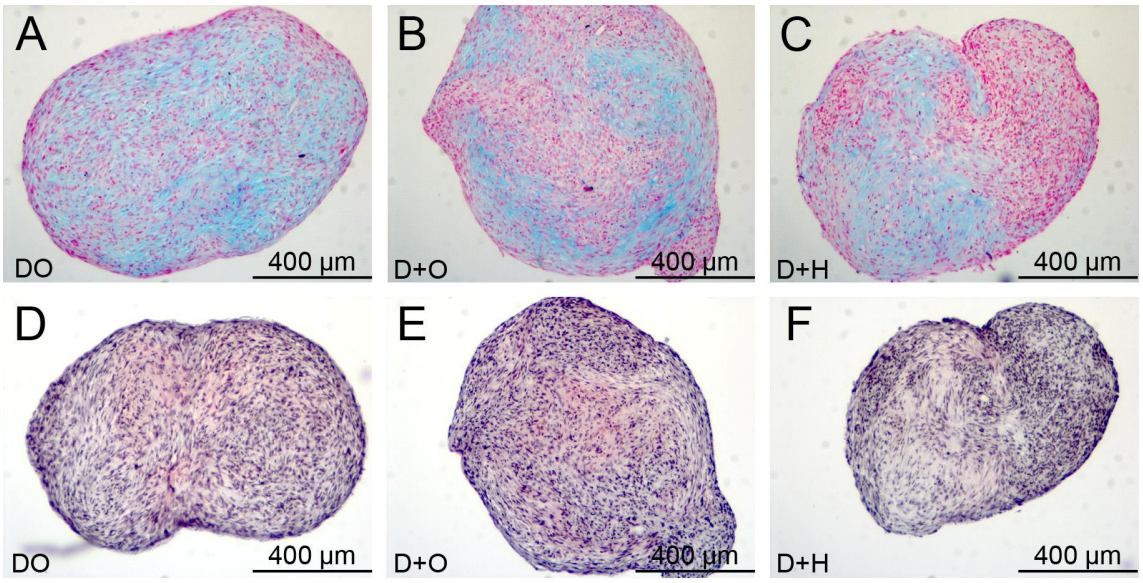

Figure 12. Comparison of Alcian blue (A-C) and Safranin O (D-F) stained differentiation micromass samples. Note that the sections are from different depths of the micromass and are not identical.

5.3. Immunohistochemistry

The histological sections of micromasses were stained for type I and II collagen using immunohistochemistry. Positive staining was not detected for type I or type II collagen in control samples. Light positive staining for type I collagen was detected in micromass samples cultured in differentiation medium (Fig.13B, C, H, I). The strongest staining was observed in D+O conditions (Fig.13G). Sample differentiated in D+H (Fig.13I) also stained, but the level of staining was milder than for the corresponding DH sample without TGF-β1.

Positive staining for type II collagen was detected neither in the controls nor in the differentiated samples, except for the extremely light positive staining for the sample differentiated in chondrogenic medium in atmospheric oxygen conditions (Fig.13H).

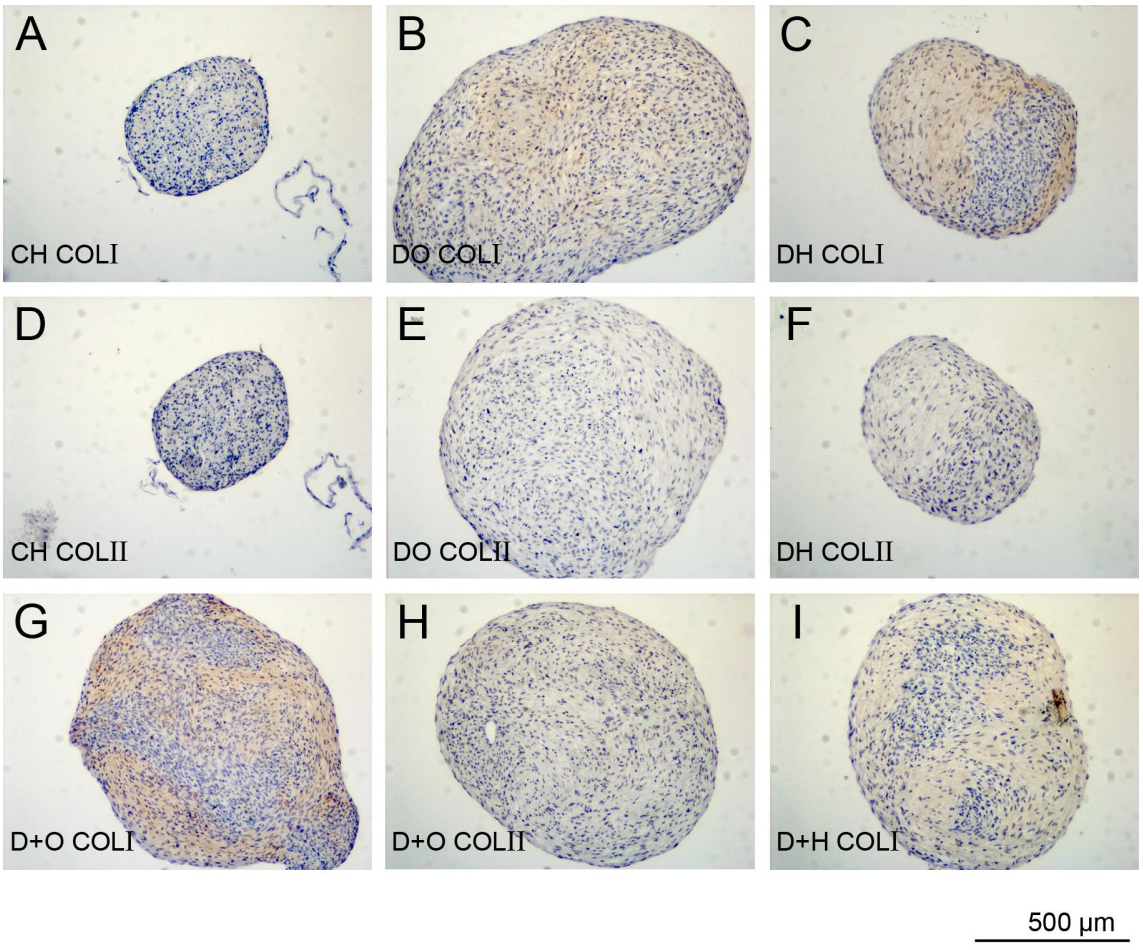

Figure 13. Immunohistochemically stained micromasses for type I and II collagen (COLI and COLII) from control (A and D), chondrogenic (B, E, C, and F) and chondrogenic + TGF-β (G, H and I) samples.

Alcian blue, Safranin O and immunohistochemically stained sections from the same micromass were compared. Positive staining was detected approximately in the same areas of the micromass sections, where cells are sparsely located (Fig.14).

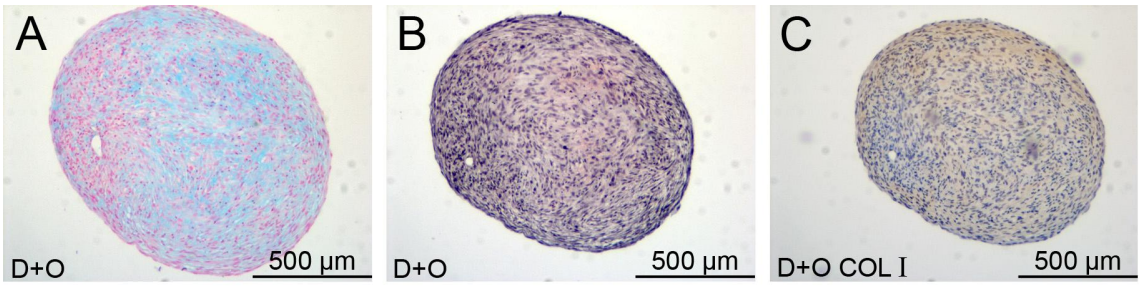

Figure 14. Comparison of Alcian blue (A) and Safranin O (B) and immunohistochemically (C) stained sections of the same micromass from D+O condition.

5.4. Sulphated glycosaminoglycan analysis

The accumulation of sulfated-GAGs in micromasses were analysed with Blyscan™ Sulfated Glycosaminoglycan Assay. D+O samples showed the highest relative amount of sulfated-GAGs (Fig.15). In atmospheric conditions, samples differentiated with TGF-β1 showed higher amount of sulfated-GAGs, than those without TGF-β1. Control samples cultured in hypoxic conditions had higher accumulation of sulfated-GAGs than those cultured in normal oxygen. Accumulation of sulfated-GAGs varied less in different hypoxic samples, and the medians of each condition were close to each other. Individual variations were observed in the accumulation of sulfated-GAGs.

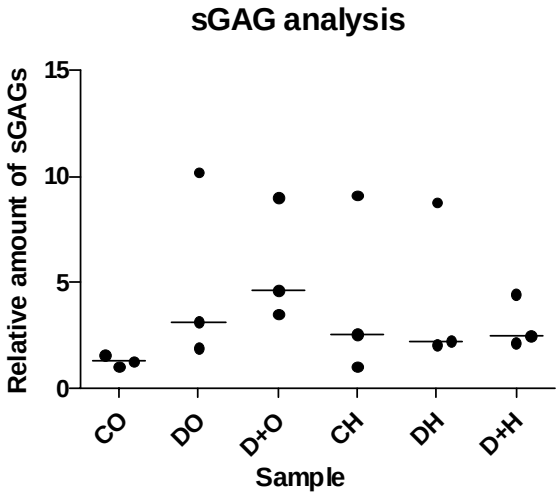

Figure 15. Relative amount of sulfated-GAGs. Horizontal lines represent the median.

5.5. Quantitative real-time reverse transcriptase PCR

qRT-PCR was used to investigate the relative changes in the gene expressions of aggrecan, type I, II and X collagens, SOX-9, HIF-1α and COMP. Aggrecan, type II collagen, SOX-9 and COMP are cartilage specific genes and type X collagen is the marker for hypertrophic chondrocytes. Type I collagen was investigated to detect a possible fibrocartilage or osteogenic differentiation and HIF-1α to detect the responsiveness of the cells to hypoxic conditions.

Expression of aggrecan (Fig.16) in all differentiated samples was higher than in control samples. The expression was highest in hypoxic conditions in the presence of TGF-β1. The expression of AGG of samples differentiated in DO and D+O conditions, was approximately the same. The expression of these samples was about 4-5 folds greater than in control samples. Control sample cultured in hypoxic conditions, expressed even lower amounts of AGG than normal oxygen samples. Hypoxic samples without TGF-β1 expressed less AGG than those differentiated in DO.

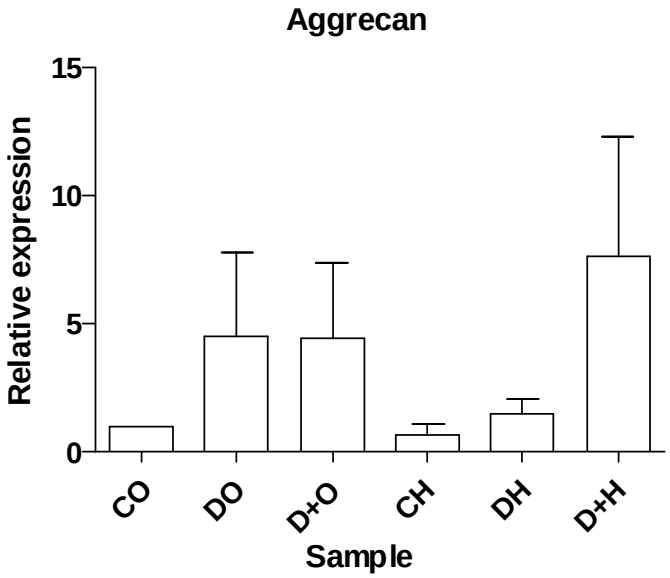

Figure 16. The relative expression of aggrecan (n=3).

Type X collagen (Fig. 17) expression was manifold in differentiated samples in the presence of TGF-β1. Expression of COLX in atmospheric oxygen differentiated samples was high, and the sample in D+O conditions had the highest expression level of all samples. Expression followed a similar pattern in hypoxic conditions, but was lower than in corresponding atmospheric conditions.

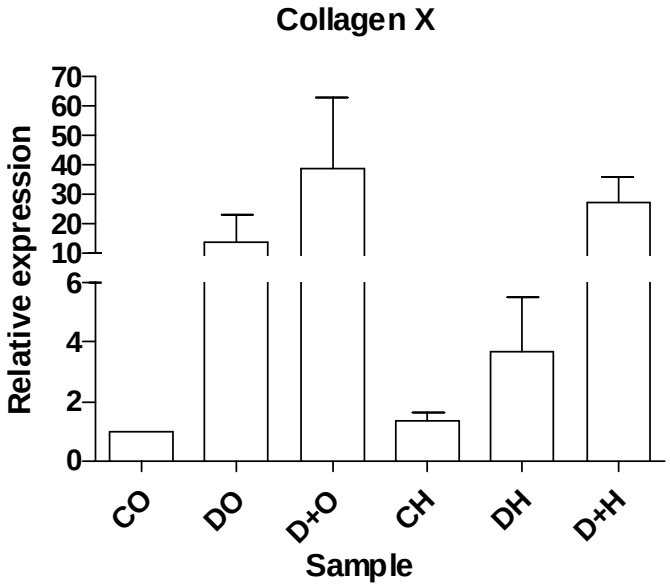

Figure 17. The relative expression of type X collagen (n=4).

Type I collagen expression (Fig. 18) in differentiation media in both oxygen concentrations was about twofold compared to control samples. Expression was similar in differentiation medium with or without TGF-β1. In addition, the expression was similar in atmospheric oxygen and hypoxic conditions. The qRT-PCR results for type I collagen are consistent with immunohistochemistry results, which show the higher expression of type I collagen in samples differentiated in chondrogenic media, than for control samples.

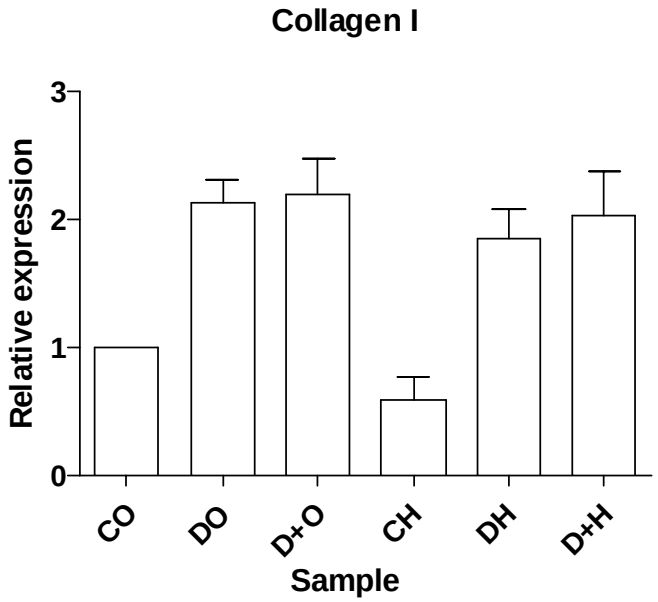

Figure 18. The relative expression of type I collagen (n=4).

Expression level of type II collagen (Fig. 19) was highest in control samples cultured in hypoxic conditions. The expression of control samples cultured in atmospheric oxygen and in DO conditions were almost similar. A bit higher expression was observed in samples differentiated with TGF-β1 in atmospheric oxygen. Approximately four times higher expression was observed in hypoxic control samples, than in control samples with normal oxygen concentration. Samples differentiated in DH and D+H conditions expressed type II collagen almost similarly.

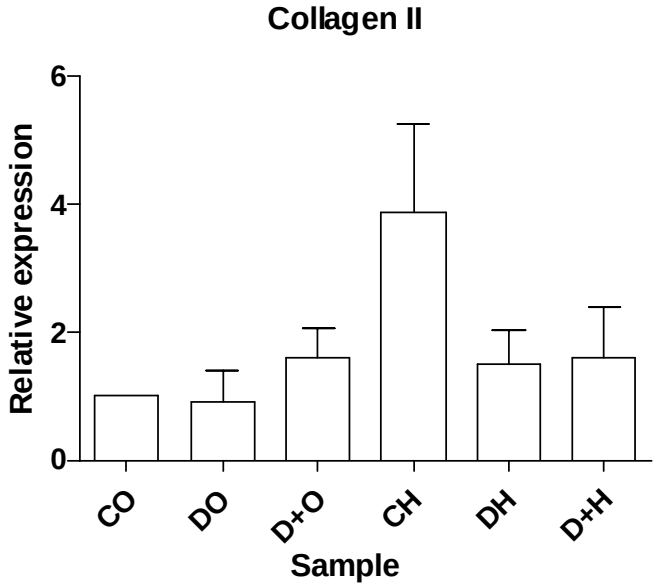

Figure 19. The relative expression of type II collagen (n=4).

HIF-1α expression (Fig. 20) was highest in hypoxic conditions in micromasses cultured in control medium and relatively low in micromasses cultured in differentiation media. The expression of control sample in atmospheric oxygen was higher than that of all differentiated samples. Hypoxic control samples had 1.5-2 fold higher expression levels than normal oxygen control samples. Expression levels were observed to be similar for all differentiated samples.

SOX9 expression (Fig.21) was highest in control micromasses cultured in hypoxic conditions. In atmospheric oxygen conditions, the expression of SOX9 was highest in DO samples. In hypoxic conditions, the expression was highest in control samples, and lowest in samples differentiated without TGF-β1. Samples differentiated without TGF-β1 had approximately two-fold higher expression in atmospheric oxygen, than in hypoxic conditions. In contrast, samples differentiated in the presence of TGF-β1 had lower expression in normal oxygen, than in hypoxic conditions.

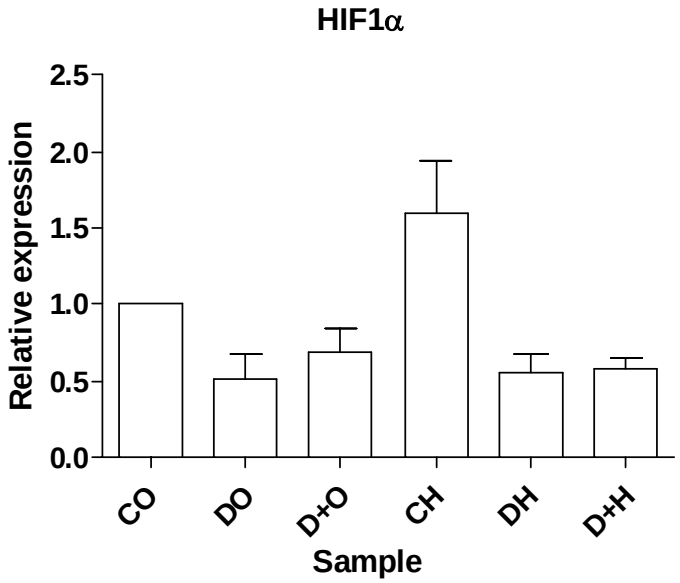

Figure 20. The relative expression of HIF-1α (n=4).

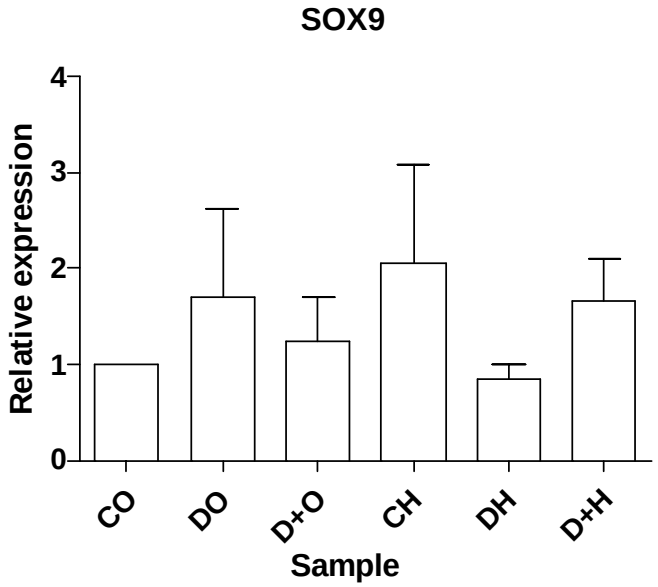

Figure 21. The relative expression of SOX9 (n=4).

Expression of COMP (Fig. 22) was strongest for the samples differentiated with TGF-β1 in both oxygen concentrations. The expression in samples differentiated in DO conditions, was only a bit higher than that of atmospheric oxygen control samples, and higher than the corresponding DH samples. The expression of COMP in control samples cultured in hypoxic conditions was higher than in the corresponding normal oxygen control samples.

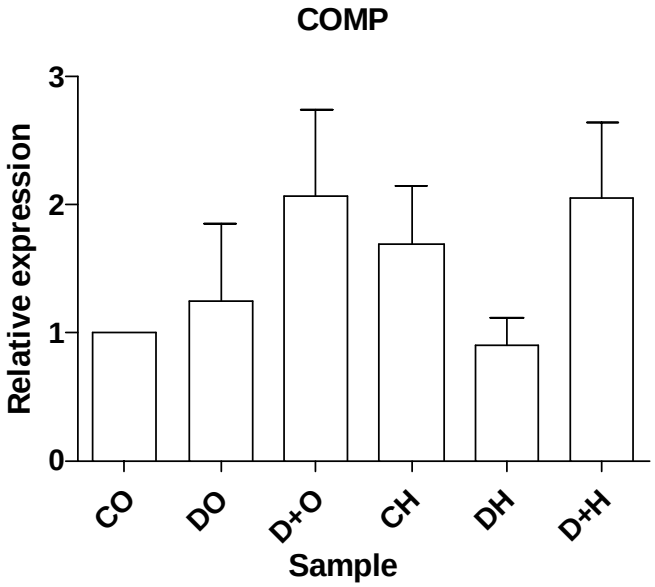

Figure 22. The relative expression of COMP (n=4).

6. DISCUSSION

The chondrocytic growth plate is hypoxic during fetal development. An increasing amount of evidence strongly suggests that low oxygen tension has an important role also in cell differentiation, and recent findings have demonstrated that hypoxia has a critical function in cartilage differentiation. (Schipani, 2005; Giaccia et al., 2004; Chen et al., 1999) In this experiment, the effects of hypoxic conditions on chondrogenic differentiation of ASCs were evaluated using two differentiation media and a control medium. In general, no clear advantage of hypoxic conditions over atmospheric oxygen conditions was detected in this study.

A caution should be taken when comparing the results of this study with other studies, since they may have been performed in different conditions, with different cells and animals of cell origin, varying oxygen levels, and time periods used in cell expansion and differentiation. Similar study with the exact same parameters has not been reported. Thus, the results of this research were compared with those having the same experimental elements or goals with the present study.

6.1. Micromass monitoring during differentiation

Micromasses condensed soon after plating. The process of mesenchymal cell condensation is crucial for chondrogenesis, and the process is directed by cell-cell and cell-matrix interactions as well as secreted factors interacting with their receptors. The establishment of cell-cell interactions is probably involved in triggering signal transduction pathways that initiate chondrogenic differentiation. (DeLise et al., 2000)

Micromass size increased during the differentiation due to ECM molecule production and secretion from cells inside the micromasses. Size difference between control and differentiated micromasses (Fig.9) indicates a higher ECM production of differentiated micromasses. Barry et al. (2001) reported similar size increase than in this study, in their bone marrow MSC pellet culture differentiation experiment in the presence of TGF-β1, -β2, or -β3. Without added TGF-βs, the control pellet cultures were considerably smaller and showed no evidence of matrix

accumulation or chondrogenesis. The results of this study were similar to the findings of Barry et al. (2001), because differentiated micromasses in the presence of TGF-β1 were detected with bigger size, and thus higher ECM synthesis than the control micromasses. However, TGF-β1 alone had no such effect compared to differentiated micromasses without TGF-β1. Conversely, micromasses differentiated without TGF-β1 were observed to be even larger. Micromasses grown in control medium were smaller than differentiated ones. Malladi et al. (2006) found out in their mice ASC chondrogenic differentiation study under hypoxic (2% O_2) conditions that the sizes of the hypoxic micromasses were remarkably larger than those differentiated in atmospheric oxygen conditions. In this study, it was visually inspected (data not shown), that hypoxic micromasses were smaller than those differentiated in atmospheric oxygen concentration. Pilgaard et al. (2009) studied hypoxic expansion of human ASCs in order to enhance the chondrogenesis. They found out that cells expanded in lower oxygen levels appeared smaller in morphology than those at higher oxygen concentrations.

In control conditions, the cells spread more than in differentiation conditions. Control micromasses were grown in the presence of FBS, which includes adhesion molecules such as fibronectin. In contrast, differentiation medium contained ITS, which is chemically defined medium that does not include adhesion molecules (Sigma-Aldrich, 2009). This was believed to reduce both the cell spreading, and micromass attachment on the wells. Woodfield et al. (2006) described that redifferentiation into chondrogenic phenotype can be influenced by surface chemistry. Substrate material with low adhesion properties supports a chondrogenic phenotype, where cells exhibit a round morphology and minimal expression of adhesion molecules such as integrins. Based on this, the medium was appropriate since the differentiation micromasses did not adhere to the bottom of the wells. Pilgaard et al. (2009) noticed more detached cells and cells with condensed nuclei indicating apoptosis at the lowest oxygen level (1%) in monolayer cultures. The visual observation of the micromasses and cells during this experiment also revealed that the micromasses and spread cells detached more easily in hypoxic conditions.

6.2. Sulphated glycosaminoglycans

All differentiated samples showed Alcian blue staining for sulfated-GAGs. Control micromasses were not stained in either condition. Huang et al. (2004) reported positive Alcian bue staining for sulfated-GAGs in ASC micromass culture as early as 24 hours after micromass plating and TGF-β1 induction. Safranin O staining for sulfated-GAGs was not so intense for all differentiated

samples; in hypoxic conditions the staining was only slightly detectable. Micromasses differentiated in hypoxic conditions stained stronger in Alcian blue method when compared to Safranin O staining. Kiviranta et al. (1985) suggested that Safranin O staining for sulfated-GAGs could be more specific than Alcian Blue staining.

Hye-Joung & Gun-Il (2009) compared the ASC and bone marrow MSC chodrogenic differentiation, and found out that ASCs have lower chondrogenic potentials than bone marrow MSCs. However, they reported that chondrogenesis comparable to bone marrow MSCs can be induced from ASCs using a greater dose combination of growth factors. In their study TGF-β2 and IGF raised the GAG level. Their results from Safranin-O staining generally paralleled those from GAG analyses. On the other hand, Awad et al. (2003) reported that the combination of TGF-β1 and ITS+ (medium with insulin, transferrin, and selenious acid) stimulated cell growth and synthesis of proteins and proteoglycans by human ASCs in alginate bead cultures.

Contrary to Alcian blue and Safranin O stainings, sulfated-GAG assay pointed that there were sulfated-GAGs present in hypoxic control samples. However, the results were quite similar compared to Alcian blue stainings, when viewing the differentiated samples. In addition, there was variation between individuals in sulfated-GAG analysis and gene expression results. Pilgaard et al. (2009) reported about distinct inter-individual variability. Thus, to obtain more comprehensive results, larger sample size is recommended, but individual variability still has to be taken into account.

Pilgaard et al. (2009) found out that even though hypoxic incubation did not have any significant effect on the accumulation of sulfated-GAGs compared to normal oxygen levels, the Alcian blue staining revealed the highest ECM proteoglycan synthesis in cultures grown under atmospheric and 15% oxygen conditions. The same observation was done in this study, when the sulfated-GAG accumulation and Alcian blue staining of the micromasses were detected. There was variation between the sulfated-GAG analysis and staining results in the same manner as in Pilgaard et al. (2009) study.

Malladi et al. (2006) reported a significant decrease in sulfated-GAG accumulation in the hypoxic micromasses compared with those differentiated in atmospheric conditions. In this study, such a significant difference in sulfated-GAG content was not detected, but the amount of

sulfated-GAGs was similarly lower in hypoxic conditions than in atmospheric oxygen concentration.

Round longitudinally orienting zone can be seen under the surface of some micromasses (Fig.10G, I, J) in Alcian blue staining. This zone was detected commonly in atmospheric oxygen samples. The zone in the presence of TGF-β1 (Fig.10G) had a dense blue appearance, while the zone in the micromass without TGF-β1 had looser white appearance (Fig.10I). Barry et al. (2001) reported that in general, cells close to the periphery of the pellet culture differentiate first. They showed that differentiation proceeded in this fashion until cells in the center of the pellets were differentiated. Differentiation was controlled with type II collagen immunostaining. They also reported that in some cases, possibly when supply of nutrients was limited, a type II collagen-rich peripheral zone was formed around an undifferentiated central zone. In these cases, the outermost cells of the pellet were the last to differentiate. In this study, it may indicate that the micromasses with this zone have differentiated more in the periphery of the micromasses than in the center. On the other hand, in some micromasses, less viable cell areas can be detected in the center of the micromasses, indicating possible cell death or yet undifferentiated state of the cells (Fig.10C, E, F). Apoptosis can be due to restricted diffusion of nutrients and oxygen toward the center of the micromass (Malladi et al., 2007).

6.3. Gene expressions and immunohistochemistry in different culture conditions

Aggrecan expression was the highest in the presence of TGF-β1 under hypoxic conditions, suggesting that hypoxic conditions in addition to TGF-β1 enhance aggrecan synthesis. Pilgaard et al. (2009) showed the expression of aggrecan and type II collagen to decrease with declining oxygen levels. Similar results were detected in this study, a decrease in aggrecan and type II collagen in the differentitiation samples in hypoxic conditions compared with the corresponding samples in atmospheric oxygen concentration. TGF-β1 has been shown to increase the synthesis of the major cartilage matrix components aggrecan and type II collagen in human articular chondrocytes (Recklies et al., 1998). Contrary to this expectation, in this study, the expression of type II collagen was not increased in the presence of TGF-β1 in hypoxic conditions if compared to hypoxic control sample. However, if only differentiated micromasses are compared, the presence of TGF-β1 shows a slightly increased expression of type II collagen.

The expression of type X collagen was highest in the presence of TGF-β1 in both oxygen concentrations. Estes et al. (2006) have confirmed increased expression of type X collagen induced by TGF-βs. This should be studied more in order to find out if there really is a correlation between the expression of type X collagen and TGF-β1. Type X collagen is predominantly associated with hypertrophic cartilage. It has been reported to exist in normal articular cartilage at the undermost zone, and is also expressed by articular chondrocytes in culture. (Barry et al., 2001) Pilgaard et al. (2009) showed type X collagen expression to drop drastically towards low oxygen concentrations. Such a significant reduction in the relative expression levels were not observed in this study but the expression was higher in corresponding differentiation samples under atmospheric conditions.

Type I collagen expression was shown to be similar in all differentiated samples, suggesting that in this experiment oxygen concentration and the presence of TGF-β1 had no differing effects on the expression. However, type I collagen did shown increased expression compared to control samples in both oxygen concentrations. Pilgaard et al. (2009) showed decreased type I collagen expression with reduced oxygen levels. In this study, immunohistochemical staining for type I collagen was positive, while staining for type II collagen was negative.

HIF-1α is a critical regulator for chondrogenic differentiation under hypoxic conditions. In this study, the expression of HIF-1α seems to have a similar expression pattern to type II collagen. However, it has to be taken into account, that differentiation micromasses were highly condensed and therefore not fully diluted in the harvesting phase, while control micromasses were successfully broken. This might have an effect on the gene expression results of other studied genes as well.

Altough type II collagen expression was the highest in hypoxic control samples, immunohistochemistry did not provide staining (Fig.13D and Fig.19). More sensitive results are obtainable with qRT-PCR than with immunohistochemistry methods. On the other hand, the immunohistochemistry was performed by using only one individual samples, and the cells of the control micromass of that individual seemed unviable. Hye-Joung & Gun-Il (2009) found out that for ASC micromass differentiation, type II collagen expression was upregulated with the addition of IGF and TGF-β2. Barry et al. (2001) immunostained pellets treated with TGF-β1, -β2, or -β3, and reported interestingly that differentiation to a chondrocytic phenotype was least when TGF-β1 was used. In addition, due to different cell type (MSCs) used in their experiments,

these results of Barry et al. (2001) are not comparable to results of the present study. However, their findings lead to a consideration if some other growth factors should have been added to obtain enhanced type II collagen synthesis in this study. On the other hand, type II collagen expression in hypoxic conditions has been shown to be connected to HIF-1α expression. Thus, the conditions beneficial for HIF-1α expression could also enhance type II collagen expression.

Sox9 has been shown to express in parallel with type II collagen in cartilage development and fully formed cartilages of mouse embryos. In addition, it has been demonstrated that Sox9 cooperatively activate Col2a1 through direct binding to a consensus sequence in the Col2a1 enhancer region. Gene inactivation experiments in mouse have shown the significant role of Sox9 in MSC chondrogenic differentiation. Gene inactivation demonstrated that without Sox9, cells of the chondrocytic lineage were unable to form precartilage markers, including Col2a1 and aggrecan. (Lefebvre et al., 2001) In this study, Sox9 expression is similar to type II collagen expression, and resembles also the expression of HIF-1α. These genes had the highest expression in hypoxic control samples (Fig.19, Fig.20 and Fig.21) which might indicate positive differentiation effects of hypoxia in control conditions. Malladi et al. (2007) demonstrated, by deleting HIF-1α, decreased expression of Sox9 and type II collagen. It has been demonstrated that Sox9 expression slightly precedes that of type II collagen (DeLise et al., 2000). In contrast, there seems to be no similar pattern between Sox9 and aggrecan expression (Fig.16 and Fig.21). Aggrecan has the lowest expression levels in control samples under hypoxic conditions. Lafont et al. (2008) reported in their experiment utilizing human articular chondrocytes and 1% oxygen, that cartilage ECM genes aggrecan and type II collagen, in addition to chondrocyte regulator transcription factor Sox9 were identified as hypoxia-inducible genes. Pilgaard et al. (2009) studied the chondrogenic inducibility of the human ASCs expanded in a series of varying hypoxic levels. Chongrogenic differentiation was examined in high-density pellet cultures. In their experiment, Sox9, aggrecan, type I, II and X collagen were upregulated and their expression levels showed a declining trend proportional with reduced oxygen concentration.

In this study, COMP expression was the highest in the presence of TGF-β1 in both, atmospheric and hypoxic conditions. It has been demonstrated, that COMP biosynthesis in synovial cells as well as in articular chondrocytes is strongly induced by TGF-β1 (Recklies et al., 1998).

6.4. The stage of differentiation

Type I collagen expression in differentiation conditions, and positive staining in immunohistochemistry indicate rather fibrocartilage-like differentiation than hyalinecartilage-like differentiation. This hypothesis is supported by relatively low type II collagen expression and non-visible staining in immunohistochemistry. The stage of differentiation in the presence of TGF-β1 is interesting, since the expression of type X collagen was significantly higher than without TGF-β1. TGF-β1 is known to be an important factor in chondrogenic differentiation. However, it may cause problems if it has a role in the overexpression of type X collagen, which indicates hypertrophic differentiation of chondrocytes.

6.5. The role of hypoxia in differentiation

In this study, it was explored if hypoxia has a positive effect on the chonrogenic differentiation of ASCs. HIF-1α is known as a critical regulator for chondrogenic differentiation under hypoxic conditions (Xu et al., 2007), and is known to participate in modulation of oxygen consumption by affecting glucose transporters and glycolytic enzymes to maintain oxygen homeostasis (Malladi et al., 2007). Malladi et al. (2007) demonstrated with mouse ASCs that the deletion of HIF-1α decreased expression of Sox9 and type II collagen. The results of this study are consistent with the results of Malladi et al. (2007), since here the relatively low HIF-1α expression of hypoxic differentiation samples indicated relatively low type II collagen and Sox9 expression of the same samples.

Malladi et al. (2006) demonstrated in their study that hypoxic conditions inhibit the *in vitro* chodrogenesis in mice ASCs. In this experiment, hypoxic differentiation conditions did not support the differentiation over atmospheric oxygen conditions, but in control micromasses HIF-1α and type II collagen expression was the highest. To evaluate the effects of hypoxic conditions more reliably, different setups are needed, which include different oxygen concentrations, changes in medium composition and differentiation/proliferation times and conditions (Malladi et al., 2006; Pilgaard et al., 2009). To study different conditions in the same experiment would reveal which factors affect the differentiation the most. Oxygen concentration is a significant factor also during medium exchanges. If this step of the experiment could be performed in low oxygen conditions, the cells would not expose to atmospheric oxygen concentrations at all during the hypoxic period. This could have an effect on the HIF-1α expression. Pilgaard et al.

(2009) used four hypoxic conditions (15, 10, 5 and 1% O_2) in their study. They independently pre-equilibrated the medium to each oxygen level before its application. They also pointed out that the choice of expansion medium should be considered, because the expansion medium has been proven to have an effect on the outcome of cell population and its differentiation potential. It could be also a considerable issue in continuing the experiments of this study.

In this study, ASCs were expanded in atmospheric conditions. In addition, the first week of differentiation period was carried out in 21% oxygen concentration. The second week of differentiation was performed under hypoxic conditions for half of the samples. This proliferation and differentiation set up did not support chondrogenic differentiation under hypoxia more than atmospheric conditions. Chen et al. (2006) have demonstrated that chondrogenesis-specific transcriptional machinery can be induced by hypoxic exposure, but demonstrated that atmospheric oxygen levels better support the full course of chondrogenesis (Chen et al., 2006). Pilgaard et al. (2009) and several other recent investigations reported by them have utilized a hypoxic preconditioning step to improve the function and survival of stem cells prior to differentiation or clinical application.

Wang et al. (2005) reported inhibited proliferation of human ASCs, but increased protein synthesis, total collagen synthesis and GAG synthesis under low oxygen tension (5%). Their findings suggested that oxygen tension has a role in regulating the proliferation and metabolism of human ASCs as they undergo chondrogenesis. In addition, the exogenous control of oxygen tension may provide a means of increasing the overall accumulation of matrix macromolecules in tissue-engineered cartilage. In contrast, Malladi et al. (2005) showed that reduced oxygen tension (2%) significantly decreased chondrogenesis in mice ASCs, while 20% oxygen environment induced chondrogenesis in 3D micromass culture model with added TGF-β1. Xu et al. (2007) reported that mouse ASC expansion in 2% oxygen before 3D micromass differentiation had been selected for chondrogenic progenitors with an enhanced ability to survive and differentiate. In contrast to other studies, Lin et al. (2006) demonstrated that hypoxia reversibly arrests preadipocytes in an undifferentiated state. They further demonstrated that the HIF-1 constitutes an important mechanism for the inhibition of adipogenic differentiation by hypoxia, and suggested that hypoxia in the stem cell niche is critical for the maintenance of the undifferentiated stem or precursor cell phenotype.

As can be seen from these varying results concerning hypoxic experiments, the experimental settings are likely to influence the outcome of the study. Different culture conditions (medium, growth factors, oxygen concentration, culturing periods, and the timing of hypoxic exposure), cell source and individual variations should be studied more carefully and optimized further to obtain better chondrogenic differentiation conditions of ASCs.

7. CONCLUSIONS

Articular cartilage damage does not undergo self-repair, and often leads to an incurable process of osteoarthritis. Tissue engineering provides a method for repairing the degenerated cartilage. Adipose tissue is an abundant and accessible origin of adult stem cells. ASCs are known to undergo chondrogenic differentiation among other tissues of mesenchymal origin, and can be used as autologous cells.

In this research, the chondrogenic differentiation of ASCs was studied in high cell density micromass culture under hypoxic and atmospheric conditions in chondrogenic medium with or without TGF-β1. The chondrogenic differentiation at some extent was confirmed by the the presence of sulfated-GAGs in harvested micromasses. The effects of hypoxic conditions on the differentiation were evaluated based on the expression of HIF-1α and cartilage specific genes.

Hypoxic conditions were not observed to induce chondrogenic differentiation of ASCs more than atmospheric conditions in differentiation conditions. This is thought to be due to differentiation conditions which did not support HIF-1α expression, which again regulates the expression of specific chondrogenic genes Sox9 and type II collagen. On the other hand, hypoxic control conditions supported the differentiation based on highest gene expression of HIF-1α, type II collagen and Sox9. Chondrogenic differentiation thus occurred in differentiation conditions when detection was based on Alcian blue and Safranin O staining. Better chondrogenic differentiation was evaluated in atmospheric oxygen conditions than in hypoxic conditions based on sulfated-GAG –staining methods. In hypoxic conditions in general, TGF-β1 had a positive effect in the expression of aggrecan, type X collagen, Sox9 and COMP. Overall, the differentiation was evaluated to be directed more on hypertrophic phenotype due to relatively high type X collagen expression in differentiation conditions. Chondrogenic differentiation conditions of ASCs must be optimized and developed further to obtain better differentiation in order to develop a stem cell based treatment for cartilage defects. This would include the testing of different culture conditions, such as medium, growth factors, oxygen concentration, culturing periods, and the timing of hypoxic exposure.

REFERENCES

Ahrens PB, Solursh M & Reiter RS. Stage-related capacity for limb chondrogenesis in cell culture. Dev Biol 1977; 60: 69-82.

Akiyama H, Chaboissier MC, Martin JF, Schedl A & De Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 2002; 16: 2813-2828.

Annabi B, Lee YT, Turcotte S, Naud E, Desrosiers RR, Champagne M, Eliopoulos N, Galipeau J & Beliveau R. Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. Stem Cells 2003; 21: 337.

Aust L, Devlin B, Foster SJ, Halvorsen YD, Hicok K, du Laney T, Sen A, Willingmyre GD & Gimble JM. Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy 2003; 6(1): 7-14.

Awad HA, Halvorsen YD, Gimble JM & Guilak F. Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. Tissue Eng 2003; 9(6): 1301-1312.

Awad HA, Wickham MQ, Leddy HA, Gimble JM & Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 2004; 25(16): 3211-3222.

Bacigalupo A, Tong J, Podesta M, Piaggio G, Figari O, Colombo P, Sogno G, Tedone E, Moro F & Van Lint MT. Bone marrow harvest for marrow transplantation: effect of multiple small (2 ml) or large (20 ml) aspirates. Bone Marrow Transplant 1992; 9(6): 467-470.

Barna M & Niswander L. Visualization of cartilage formation: insight into cellular properties of skeletal progenitors and chondrodysplasia syndromes. Dev Cell 2007; 12: 931-941.

Barry F, Boynton RE, Liu B & Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell res 2001; 268(2): 189-200.

Bishop T, Lau K, Epstein A, Kim S, Jiang M & O'Rourke D. Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans. PloS Biol 2004; 2: e289.

Buckwalter JA. Articular cartilage: injuries and potential for healing. J Orthop Sports Phys Ther 1998; 28(4): 192-202.

Caplan AI, Elyaderani M, Mochizuki Y, Wakitani S & Goldberg VM. Principles of cartilage repair and regeneration. Clin Orthop Relat Res 1997; 342: 254-269.

Carlo MD & Loeser RF. Increased oxidative stress with aging reduces chondrocyte survival: correlation with intracellular glutathione levels. Arthritis Rheum 2003; 48, 3419-3430.

Chan DA, Suthphin PD, Denko NC & Giaccia AJ. Role of prolyl hydroxylation in oncogenically stabilized hypoxia-inducible factor-1alpha. J Biol Chem 2002; 277: 40112-40117.

Chen E, Fujinaga M & Giaccia A. Hypoxic microenvironment within an embryo induces apoptosis and is essential for proper morphological development. Teratology 1999; 60: 215-225.

Chen FH, Rousche KT & Tuan RS. Technology Insight: adult stem cells in cartilage regeneration and tissue engineering. Nature Clinical Practice Rheumatology 2006; 2: 373-382.

Chen L, Fink T, Ebbesen P & Zachar V. Hypoxic treatment inhibits insulin-induced chondrogenesis of ATCD5 cells despite upregulation of DEC1. Connect Tissue Res 2006; 47: 119-123.

Chen L, Fink T, Ebbesen P & Zachar V. Optimized chondrogenesis of ATCD5 cells through sequential regulation of oxygen conditions. Tissue Eng 2006; 12: 559-567.

Chiou M, Xu Y & Longaker MT. Mitogenic and chondrogenic effects of fibroblast growth factor-2 in adipose derived mesenchymal cells. Biochem Biophys Res Commun 2006; 343(2): 644-652.

Choumerianou DM, Dimitriou H & Kalmanti M. Stem cells: promises versus limitations. Tissue Eng. Part B Rev 2008; 14: 53-60.

Chung C & Burdick JA. Engineering cartilage tissue. Adv Drug Deliv Rev 2008; 60(2); 243-262.

Conrad C & Huss R. Adult stem cell lines in regenerative medicine and reconstructive surgery. J Surg Res 2005; 124(2): 201-208.

Cornelius P, MacDougald OA & Lane MD. Regulation of adipocyte development. Annu Rev Nutr 1994; 14: 99-129.

Danet GH, Pan Y, Luongo JL, Bonnet DA & Simon MC. Expansion of human SCID-repopulating cells under hypoxic conditions. J Clin Invest 2003; 112: 126-135.

DeLise AM, Fischer L & Tuan RS. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage 2000; 8(5): 309-334.

De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J & Hedrick MH. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 2003; 174(3): 101-109.

Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H & Guilak F. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun 2002; 290(2): 763-769.

Estes BT, Wu AW & Guilak F. Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. Arthritis Rheum 2006; 54(4): 1222-1232.

Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjak-Novakovic G. Chondrogenesis in a cell-polymer-bioreactor system. Exp Cell Res 1998; 240(1): 58-65.

Fukumoto T, Sperling JW, Sanyal A, Fitzsimmons JS, Reinholz GG, Conover CA & O'Driscoll SW. Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis in vitro. Osteoarthritis Cartilage 2003; 11(1): 55-64.

Ghivizzani SC, Oligino TJ, Robbins PD & Evans CH. Cartilage injury and repair. Phys Med Rehabil Clin N Am 2000; 11(2): 289-307.

Giaccia A, Simon M & Johnson R. The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes Dev 2004; 18: 2183-2194.

Gibson JS, Milner PI, White R, Fairfax TP & Wilkins RJ. Oxygen and reactive oxygen species in articular cartilage: modulators of ionic homeostasis. Pflugers Arch 2008; 455(4): 563-573.

Gomillion CT & Burg KJ. Stem cells and adipose tissue engineering. Biomaterials 2006; 27(36): 6052-6063.

Gooch KJ, Kwon JH, Blunk T, Langer R, Freed LE & Vunjak-Novakovic G. Effects of mixing intensity on tissue-engineered cartilage. Biotechnol Bioeng 2001; 72(4): 402-407.

Gooch KJ, Blunk T, Courter DL, Sieminski AL, Bursac PM, Vunjak-Novakovic G & Freed LE. IGF-1 and mechanical environment interact to modulate engineered cartilage development. Biochem Biophys Res Commun 2001; 286(5): 909-915.

Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B & Gimble JM. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol 2006; 206: 229-237.

Hedbom E, Antonsson P, Hjerpe A, Aeschlimann D, Paulsson M, Rosa-Pimentel E, Sommarin Y, Wendel M, Oldberg A & Heinegård D. Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J Biol Chem 1992; 267(9): 6132-6136.

Henrotin Y, Kurz B & Aigner T. Oxygen and reactive oxygen species in cartilage degradation: friends or foes? Osteoarthritis Cartilage 2005; 13, 643-654.

Heywood HK, Bader DL & Lee DA. Rate of oxygen consumption by isolated articular chondrocytes is sensitive to medium glucose concentration. J Cell Physiol 2006; 206: 402-410.

Huang JI, Zuk PA, Jones NF, Zhu M, Lorenz HP, Hendrick MH & Benhaim P. Chondrogenic potential of multipotential cells from human adipose tissue. Plastic and Reconstructive Surgery 2004; 113(2): 585-594.

Hwang NS, Kim MS, Sampattavanich S, Baek JH, Zhang Z & Elisseeff J. Effects of three-dimensional culture and growth factors on the chondrogenic differentiation of murine embryonic stem cells. Stem Cells 2006; 24(2): 284-291.

Hye-Joung K & Gun-Il I. Chondrogenic differentiation of adipose tissue-derived mesenchymal stem cells: Greater doses of growth factor are necessary. Journal of Orthopaedic Research 2009; 27(5): 612-619. (Hye-Joung & Gun-Il, 2009)

Iwasaki M, Nakata K, Nakahara H, Nakase T, Kimura T, Kimata K, Caplan AI & Ono K. Transforming growth factor-beta 1 stimulates chondrogenesis and inhibits osteogenesis in high density culture of periosteum-derived cells. Endocrinology 1993; 132(4): 1603-1608.

Jaakkola P, Mole D, Tian Y, Wilson M, Gielbert J & Gakell S. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 2001; 292: 468-472.

Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makimo Y & Tanaka H. Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. EMBO J 1998; 17(22): 6573-6586.

Kim SE, Park JH, Cho YW, Chung H, Jeong SY, Lee EB & Kwon IC. Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: implications for cartilage tissue engineering. J Control Release 2003; 91(3): 365-374.

Kiviranta I, Jurvelin T, Tammi M, Säämänen AM & Helminen HJ. Microspectrophotometric quantitation of glycosaminoglycans in articular cartilage sections stained with Safranin O. Histochemistry 1985; 82(3): 249-255.

Kurz B, Domm C, Jin M, Sellckau R & Schunke M. Tissue engineering of articular cartilage under the influence of collagen I/III membranes and low oxygen tension. Tissue Eng 2004; 10(7-8): 1277-1286.

Lafont JE, Talma S, Hopfgarten C & Murphy CL. Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and –independent pathways. J Biol Chem 2008; 283(8): 4778-4786.

Langer R & Vacanti JP. Tissue engineering. Science 1993; 260(5110): 920-926.

Leddy HA, Awad HA & Guilak F. Molecular diffusion in tissue-engineered cartilage constructs: Effects of time and culture conditions. J Biomed Mater Res 2004; 70B: 397-406.

Lee RB & Urban JPG. Evidence for a negative Pasteur effect in articular cartilage. Biochem J 1997; 321: 95-102.

Lefebvre V, Behringer RR & De Crombrugghe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis and cartilage 2001; 9: S69-S75.

Leo C, Giaccia A & Denko N. The hypoxic tumor microenvironment and gene expression. Semin Radiat Oncol 2004; 14: 207-214.

Lin Q, Lee YJ & Yun Z. Differentiation arrest by hypoxia. J Biol Chem 2006; 281(41): 30678-30683.

Malladi P, Xu Y, Chiou M, Giaccia AJ & Longaker MT. Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. Am J Physiol Cell Physiol 2006; 290(4): C1139-1146.

Malladi P, Xu Y, Chiou M, Giaccia AJ & Longaker MT. Hypoxia inducible factor-1alpha deficiency affects chondrogenesis of adipose-derived adult stromal cells. Tissue Eng 2007; 13(6): 1159-1171.

Mandl EW, van der Veen SW, Verhaar JA & van Osch GJ. Serum-free medium supplemented with high-concentration FGF2 for cell expansion culture of human ear chondrocytes promotes redifferentiation capacity. Tissue Eng 2002; 8(4): 573-580.

Marlovits S, Zeller P, Singer P, Resinger C & Vecsei V. Cartilage repair: generations of autologous chondrocyte transplantation. Eur J Radiol 2006; 57(1): 24-31.

McPherson JM & Tubo R. Articular Cartilage Injury, In:Lanza RP, Langer R, Vacanti J (eds), Principles of Tissue Engineering. Academic Press, San Diego, 2000, pp. 697-709.

Messner K & Gillquist J. Cartilage repair: A critical review. Acata Orthop Scand 1996; 67: 523-529.

Min JH, Yang H, Ivan M, Gentler F, Kealin Jr WG & Pavletich NP. Structure of an HIF-1alpha-pVHL complex: hydroxyproline recognition in signaling. Science 2002; 296: 1886-1889.

Oreffo RO, Bord S & Triffitt JT. Skeletal progenitor cells and ageing human populations. Clin Sci (Lond) 1998; 94(5): 549-555.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29(9): e45.

Pfander D, Cramer T, Schipani E & Johnson RS. HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes. J Cell Sci 2003; 116(9): 1819-1826.

Pilgaard L, Lund P, Duroux H, Taylor J, Emmersen J, Fink T, Ragoussis J & Zachar V. Transcriptional signature of human adipose tissue-derived stem cells (hASCs) preconditioned for chondrogenesis in hypoxic conditions. Exp Cell res 2009; 315: 1937-1952.

Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L & Casteilla L. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004; 109: 656-663.

Prabhakar NR, Kumar GK & Nanduri J. Intermittent hypoxia-mediated plasticity of acute O_2 sensing requires altered red-Ox regulation by HIF-1 and HIF-2. Ann N Y Acad Sci 2009; 1177: 162-168.

Rao MS & Mattson MP. Stem cells and ageing: expanding the possibilities. Mech Ageing Dev 2001; 122: 713-734.

Recklies AD, Baillargeon L & White C. Regulation of cartilage oligomeric matrix protein synthesis in human synovial cells and articular chondrocytes. Arthritis & rheumatism 1998; 41(6): 997-1006.

Reddi AH. Morphogenesis and Tissue Engineering, In: Lanza RP, Langer R & Vacanti J (eds), Principles of Tissue Engineering. Academic Press, San Diego, 2000, pp. 81-91.

Rizzino A. A challenge for regenerative medicine: proper genetic programming, not cellular mimicry. Dev Dyn 2007; 236(12): 3199-3207.

Roberts AB, Kondaiah P, Watanabe S, Good P, Danielpour D, Roche NS, Rebbert ML, Dawid IB & Sporn MB. Mesoderm induction in Xenopus laevis distinguishes between the various TGF-beta isoforms. Growth Factors 1990; 3(4): 277-286.

Roelen RAJ & Dijke P. Controlling mesenchymal stem cell differentiation by TGFβ family members. J Orthop Sci 2003; 8: 740-748.

Rosier RN, O'Keefe RJ, Crabb ID & Puzas JE. Transforming growth factor beta: an autocrine regulator of chondrocytes. Connect Tissue Res 1989; 20(1-4): 295-301.

Ross MH, Kaye GI & Pawlina W. Histology A Text and Atlas, Lippincott Williams & Wilkins, Baltimore, Philadelphia, 2003, pp. p.875. 164-206

Roughley PJ, Melching LI, Heathfield TF, Pearce RH & Mort JS. The structure and deagradation of aggrecan in human intervertebral disc. Eur Spine J 2006; 15(3): S326-332.

Schipani E, Ryan HE, Didrickson S, kobayashi T, Knight M & Johnson RS. Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev 2001; 15(21): 2865-2876.

Schipani E. Hypoxia and HIF-1 alpha in chondrogenesis. Semin Cell Dev Biol 2005; 16(4-5): 539-546.

Schneider N, Mouithys-Mickalad A, Lejeune JP, Duyckaerts C, Sluse F, Deby-Dupont G & Serteyn D. Oxygen consumption of equine articular chondrocytes: influence of applied oxygen tension and glucose concentration during culture. Cell Biol Int 2007; 31(9): 878-886.

Semenza GL. HIF-1 and human disease: one highly involved factor. Genes Dev 2000; 14(16): 1983-1991.

Seyedin SM, Rosen DM & Segarini PR. Modulation of chondroblast phenotype by transforming growth factor-beta. Pathol Immunopathol Res 1988; 7(1-2): 38-42.

Shamblott MJ, Edwards BE & Gearhart JD. Pluripotent stem cells, In: Lanza RP, Langer R & Vacanti J (eds), Principles of tissue engineering. Academic press, San Diego, 2000, pp. 369-381.

Sigma-Aldrich® website, available in http://www.sigmaaldrich.com/finland.html (21.11.2009)

Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, Fraser JK, Hedrick MH. Multipotential differentiation of adipose tissue-derived stem cells. Keio J Med 2005; 54(3): 132-141.

Tare RS, Howard D, Pound JC, Roach HI & Oreffo RO. Tissue engineering strategies for cartilage generation - micromass and three dimensional cultures using human chondrocytes and a continuous cell line. Biochem Biophys Res Commun 2005; 333(2): 609-621.

Tuli R, Tuli S, Nandi S, Huang X, Mannet PA, Hozack WJ, Danielson KG, Hall DJ & Tuan RS. Transforming growth factor-beta-mediated chondrogenesis of Human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J Biol Chem 2003; 278(42): 41227-41236.

Vats A, Tolley NS, Polak JM & Buttery LD. Stem cells: sources and applications. Clin otolaryngol Allied Sci 2002; 27(4): 227-232.

Verfaillie CM. Adult stem cells: assessing the case for pluripotency. Trends Cell Biol 2002; 12(11): 502-508.

Vivien D, Galera P, Lebrun E, Loyau G & Pujol JP. Differential effects of transforming growth factor-beta and epidermal growth factor on the cell cycle of cultured rabbit articular chondrocytes. J Cell Physiol 1990; 143(3): 534-545.

Wang DW, Fermor B, Gimble JM, Awad HA & Guilak F. Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. J Cell Physiol 2005; 204(1): 184-191.

Wickham MQ, Erickson GR, Gimble JM, Vail TP & Guilak F. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. Clin Orthop Relat Res 2003; 412: 196-212.

Woodfield TB, Miot S, Martin I, van Blitterswijk CA & Riesle J. The regulation of expanded human nasal chondrocyte redifferentiation capacity by substrate composition and gas plasma surface modification. Biomaterials 2006; 27(7): 1043-1053.

Xu Y, Malladi P, Chiou M, Bekerman E, Giaccia AJ & Longaker MT. In vitro expansion of adipose-derived adult stromal cells in hypoxia enhances early chondrogenesis. Tissue Eng 2007; 13(12): 2981-2993.

Ysart GE & Mason RM. Responses of articular cartilage explant cultures to different oxygen tensions. Biochim Biophys Acta 1994; 1221: 15-20.

Zuk PA, Zhu M, Mizuno H, Huang H, Futrell JW, Katz AJ, Benhaim P, Lorenz HP & Hendrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001; 7(2): 211-228.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P & Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002; 13(12): 4279-4295.

Zuscik MJ, Hilton MJ, Zhang X, Chen Z & O'Keefe RJ. Regulation of chondrogenesis and chondrocyte differentiation by stress. J Clin Invest 2008; 118(2): 429-438.