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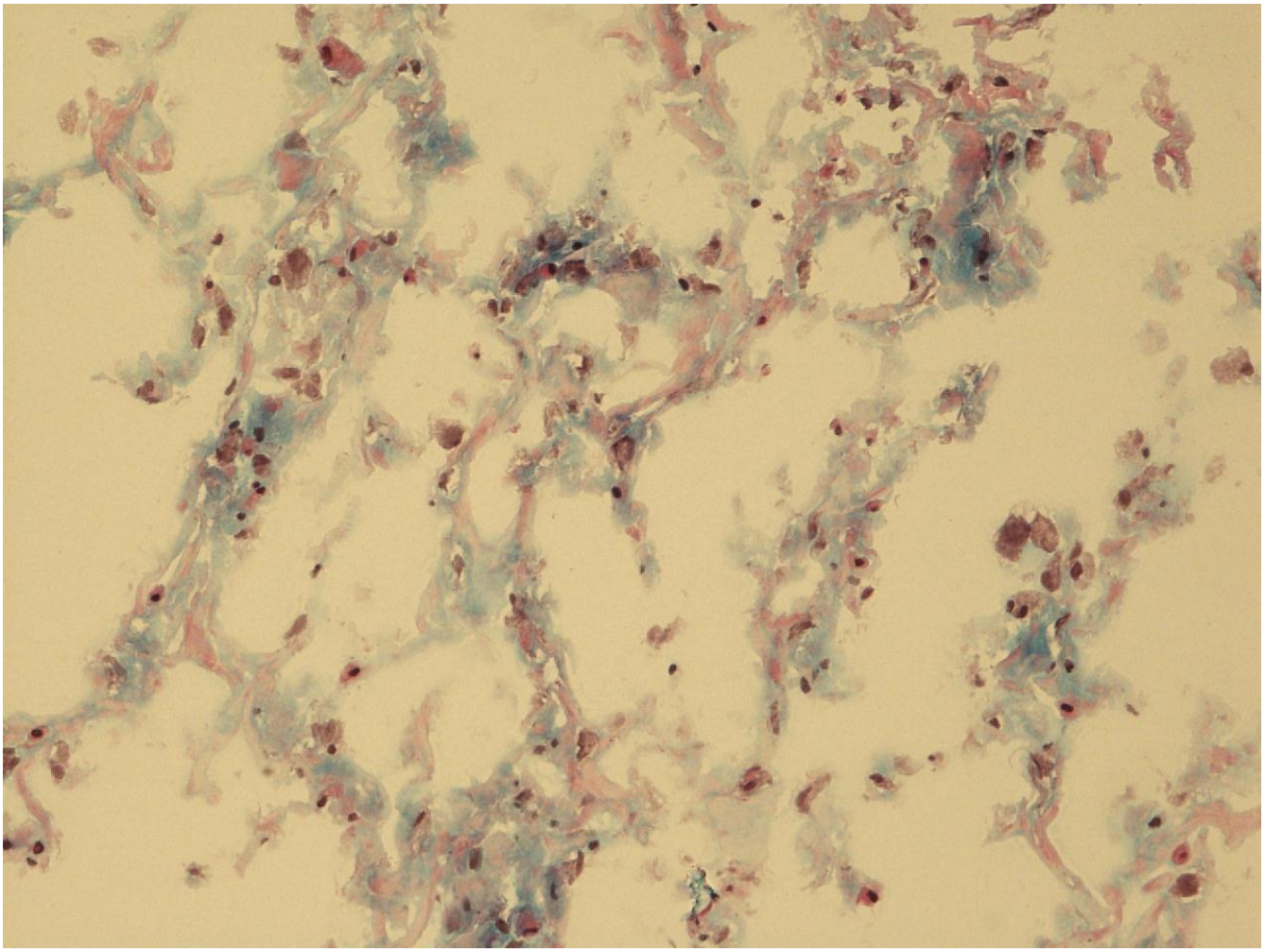

Effects of different pH and oxygen levels on proliferation and chondrogenic differentiation of human mesenchymal stem cells cultured in hydrogels

Master of Science thesis in Biotechnology

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Cover image: Alcian Blue van Gieson staining of human mesenchymal stem cells cultured in the hydrogel HydroMatrix™ at 10% O_2.

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Abstract

Low back pain is a very common health problem and 70-85% of the world's population suffers from it at some point in their life. One possible cause for this pain is intervertebral disc (IVD) degeneration. Current treatments for IVD degeneration include surgical treatments which are very invasive. Thus there is a need for a strategy which is minimally invasive and for this cell therapy could be a solution. The IVD degeneration often starts in the center of the IVD, in the nucleus pulposus (NP), where chondrocyte-like cells reside. As human mesenchymal stem cells (hMSC) can undergo chondrogenic differentiation, these cells are a good candidate for the use in cell therapy to treat IVD degeneration. Transplantation of these cells together with a hydrogel into a degenerated IVD is thought to arrest the degeneration as hMSC could undergo chondrogenic differention while the hydrogel acts as a temporary matrix. The environment inside a degenerated IVD is hypoxic and acidic and thus the effects of these factors on the hMSC are important to study. The aim of this study was to evaluate the effects of different pH and oxygen levels on the proliferation and chondrogenic differentiation of human bone marrow-derived MSC cultured in the hydrogels HydroMatrix™ and PuraMatrix™. In the oxygen experiment, cells were cultured in the hydrogels for 14 days at either 10% O_2 or 21% O_2 (standard cell culturing condition). In the pH experiment, cells were cultured in a hydrogel for 6 days either at 10% O_2 or 21% O_2 and the pH levels tested were pH 7.4 (standard cell culturing condition), 7.1, 6.8 and 6.5 representing pH levels in healthy and degenerated IVDs.

FACS was used to characterize the hMSC population and it could be concluded that the vast majority of the cells were in fact MSC. The results from the oxygen experiment showed gene and protein expressions of SOX9 and gene expression of collagen IIA1 in MSC cultured in HydroMatrix™ at both oxygen levels indicating chondrogenic differentiation. In addition, sulfated glycosaminoglycans were present in samples of hMSC cultured in HydroMatrix™ at 10% O_2 indicating that a decreased oxygen level of 10% O_2 could induce chondrogenic differentiation of hMSC. Cells were also shown to proliferate in HydroMatrix™ at both oxygen levels as cells positive for PCNA were observed in IHC studies. In the PuraMatrix™ samples, *SOX9* and *collagen IIA1* gene expressions were observed in some samples showing indications of chondrogenic differentiation. Deviating trends of *HIF-1α* gene expression were observed which suggested that 10% O_2 might not be low enough to induce the HIF-1α gene.

In the pH experiment, the results showed that the presence of viable hMSC decreased with decreasing pH, indicating that a decreased pH affects the survival of hMSC by resulting in a lower cell viability. However, some viable cells could be observed when cultured in HydroMatrix™ at pH 6.8 and 10% O_2. This indicated that some hMSC could survive in the pH found in mildly degenerated IVDs at a decreased oxygen level of 10% O_2. When comparing the results of culturing hMSC at different pH at 10% O_2 with 21% O_2, no clear difference in the presence of viable or dead cells could be observed however, quantification was not performed. Thus it cannot be said whether a combination of decreased pH and an oxygen level of 10% has a stronger negative effect on the survival of hMSC than a decreased pH alone.

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Introduction

Low back pain is a very common problem and 70-85% of the world's population suffers from it at some point in their life (Andersson, 1999). Hoy et al. performed a study in which the global burdens of different musculoskeletal conditions were estimated. Low back pain received the highest number when considering the number of years the person lived with the disability (Hoy et al., 2014). Therefore it is of outmost importance to do research in this area and develop new treatments for this. This pain can be caused by the degeneration of one or several intervertebral discs (IVDs) (Luoma et al., 2000) and a possible treatment for this condition could be to transplant human mesenchymal stem cells (hMSC) into the degenerated disc tissue. The idea is that when the hMSC have been transplanted into the degenerated disc, they will differentiate into chondrocyte-like cells, which are found in the center of the disc, to arrest the degeneration (Yang et al., 2009). In parallel to this injection, a hydrogel could be injected to act as a temporary matrix for the cells as it resembles the ECM in many cartilaginous tissues (Drury and Mooney, 2003). To study the effect of a decreased pH and oxygen level is of relevance as the environment in the center of the IVD is hypoxic due to the avascular nature of the tissue (Ishihara and Urban, 1999). In a degenerated IVD, the diffusion of nutrients including oxygen and glucose is decreased resulting in a decreased level of oxygen. A consequence of this is a high concentration of lactic acid resulting in a lower pH. The pH in a normal IVD varies between 7.0-7.2 while in mildly and severely degenerated discs the pH is estimated to be 6.8 and 6.5 respectively (Li et al., 2012) (Razaq et al., 2003). Therefore it is of importance to study these effects on the mesenchymal stem cells as this is the environment that the cells will be exposed to after transplantation.

Aims

This study aimed to investigate the effects of different pH and oxygen levels on proliferation and chondrogenic differentiation of hMSC. The cells were cultured in the hydrogels HydroMatrix™ and PuraMatrix™ as these are potential clinical scaffolds for the hMSC to grow in after their transplantation into a degenerated IVD.

Limitations

This project was limited to testing the two hydrogels HydroMatrix™ and PuraMatrix™ and the cell type that was used was hMSC derived from the iliac crest. Cells from three different patients were studied and only one time point was investigated in the hydrogel experiments. No *in vivo* studies were performed as the project is limited to *in vitro* studies.

Theory

In this section, the theory needed to understand the aim and relevance of this project is presented.

Anatomy of the intervertebral disc

Intervertebral discs (IVDs) are avascular fibrocartilaginous structures located in the spine and 23 IVDs are situated in the human spine (Roberts et al., 2006) (Bibby et al., 2001) (Shankar et al., 2009). As can be seen in the sagittal view of **Figure 1**, each IVD is positioned between two vertebrae with hyaline cartilage endplates separating the IVD from the vertebral bodies (Bibby et al., 2001). The IVD provides flexibility and support to the spine while the cartilage endplates assist in resisting load (Bibby et al., 2001) (Shankar et al., 2009). The IVD is comprised of two parts; the nucleus pulposus (NP) and the annulus fibrosus (AF) (**Figure 1**). The NP, which is found in the center of the disc, is very different from the AF that is surrounding the NP. The NP is a soft and gelatinous fibrocartilaginous structure that is highly hydrated while the AF is hard and less hydrated, consisting of a collagen network organized as lamellae around the NP. The extracellular matrix (ECM) in the NP contains collagens, mainly type II collagen, and proteoglycans including aggrecan. The proteoglycans have a water retention capacity and can thereby facilitate the hydration of the nucleus. By this hydration, there is an osmotic pressure in the NP (Urban and Roberts, 2003) and the NP can thereby resist load and deformation (Ciapetti et al., 2012). The proteoglycans also govern the flow of fluid in and out of the IVD, where negative charges in the proteoglycans assist in attracting fluid back into the IVD after deformation or loading (Urban et al., 1979) (Massey et al., 2012). Another difference between the NP and AF is the collagen content. The highest collagen content is in the AF and it gradually decreases towards the center of the NP (Bibby et al., 2001) (Roberts et al., 2006) (Urban and Roberts, 1995) (Shankar et al., 2009).

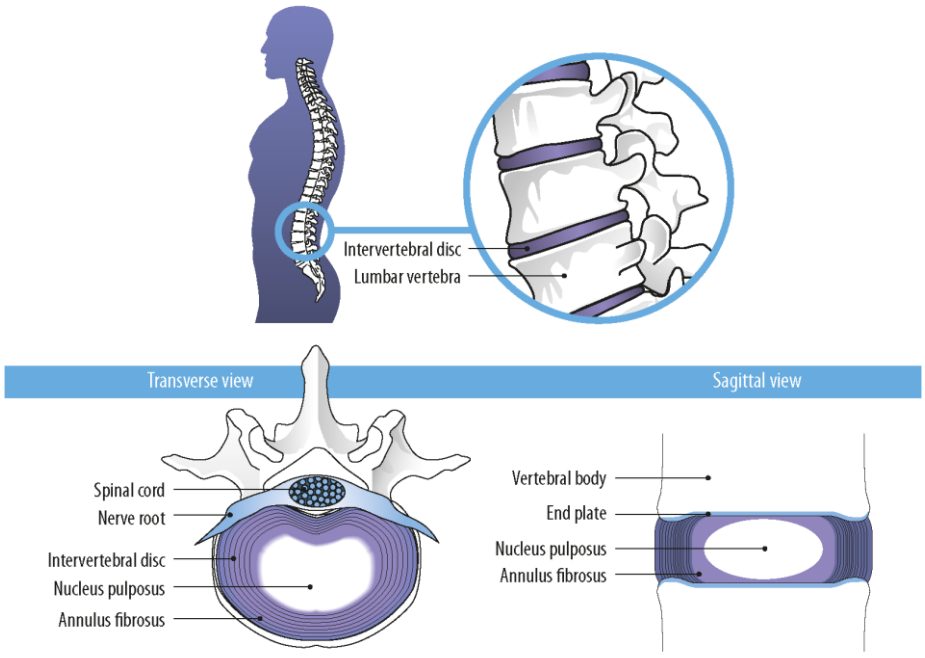

Figure 1. Illustration of the position and composition of intervertebral discs in the spine of a human adult.

The composition of the NP and AF is not the only factor setting the two regions apart; the cells residing in the regions are also different. In an adult human IVD, the cells present in the NP are chondrocyte-like cells which have an oval morphology and mainly synthesize type II collagen. In the AF on the other hand, cells have an elongated morphology resembling fibroblasts (Bibby et al., 2001) (Roberts et al., 2006) (Ciapetti et al., 2012). The cells in the IVD do not only synthesize the ECM in the different regions but they also secrete matrix metalloproteinases (MMPs) which degrade the ECM. In addition, tissue inhibitors for metalloproteinases (TIMPs) which inhibit the MMPs are also secreted by the cells (Roberts et al., 2000) (Bibby et al., 2001). Thus the cells are important for the turnover of ECM. A disruption of the balance between synthesis and degradation of the ECM, where more ECM is degraded than is synthesized, is considered to have a role in IVD degeneration (Cawston et al., 1999) (Weiler et al., 2002) (Shankar et al., 2009) (Urban and Roberts, 1995). There is however a degree of regeneration of degenerated human IVD as cells isolated from degenerated AF and NP have been shown to express stem cells markers (Risbud et al., 2007). This regeneration indicates that stem cell niches exist in the IVD. These niches are microenvironments in which the stem cells reside. In the article by Henriksson et al. cells in the AF region were observed to express stem cell markers also suggesting that stem cell niches exist in the IVD (Henriksson et al., 2009a).

Degeneration of intervertebral disc

IVD degeneration typically begins in the NP as the disc matrix composition changes within the NP with increasing age. For instance the amount of collagens and proteoglycans present in the NP decrease leading to dehydration of the disc (Roberts et al., 2006) (Urban and Roberts, 2003) (Adams and Roughley, 2006) (Singh et al., 2009). This dehydration results in a decreased size of the NP as well as a decrease in osmotic pressure resulting in an increased transfer of compressive stress to the AF region (Adams et al., 1996). Also in the beginning of IVD degeneration, the level of enzymes regulating the matrix synthesis including MMPs is changed. When the disc cells secrete high levels of collagenases including MMP-1, more collagen is degraded resulting in a change in tissue composition and a loss of tissue integrity (Weiler et al., 2002) (Le Maitre et al., 2007) (Le Maitre et al., 2006). This will for instance result in a less organized morphology of the lamellae and clefts and tears will form. Another feature for degenerated IVDs is that the cells start to form more clusters. There is also an increase in cell necrosis and a decrease in proteoglycan content leading to dehydration. This ultimately leads to a decrease in the height of the disc (Urban and Roberts, 1995) (Urban and Roberts, 2003) (Roberts et al., 2006).

The occurrence of disc degeneration in humans increase with age however, there are also other influential factors including repetitive high loading to the spine and genetic factors. With increasing age, the disc matrix composition changes resulting in dehydration of the disc (Roberts et al., 2006) (Urban and Roberts, 2003) (Adams and Roughley, 2006) (Singh et al., 2009). In addition, the nutrient supply to the NP and the removal of metabolic waste products decrease with increasing age. This is because the endplates become less permeable and thereby the diffusion of nutrients including oxygen and glucose from this direction is limited (Shankar et al., 2009). As more lactic acid is formed at low oxygen concentrations, a reduced

nutrient supply results in a decreased pH in the NP (Urban et al., 2004). This decrease in pH is harmful for the cells and thus the number of viable cells in the NP also decrease with increasing age (Buckwalter, 1995). Another factor that is important to consider in IVD degeneration is repetitive high loading to the spine (Urban and Roberts, 1995) (Adams et al., 2000). A repetitive high loading to the spine appears to be injurious to the IVDs as cleft and tears can be formed and the adult IVD has a limited healing potential (Adams et al., 2000). In a study conducted by Kelsey et al. the repetitive twisting and high loading to the spine was evaluated (Kelsey et al., 1984). The authors concluded that a high and repetitive loading to the spine increases the risk of low back pain due to damage to the IVD. Physical load leading to IVD damage is a factor that accelerates the degeneration of the IVD (Battie et al., 2009).

Genetic factors are also important for IVD health (Adams and Roughley, 2006) (Adams et al., 2000) (Shankar et al., 2009). Genes affecting the degeneration of the IVD include those associated with type IX collagen, the proteoglycan aggrecan and MMP-3. These genetic factors affect the skeletal tissue and the collagen framework in the IVD (Adams and Roughley, 2006) (Adams et al., 2000) (Annunen et al., 1999). Polymorphism in these genes is associated with IVD degeneration (Annunen et al., 1999) (Kawaguchi et al., 1999) (Takahashi et al., 2001).

Current treatments for degenerated disc

Current strategies for treating degenerated discs include physiotherapy and the use of pain killers (Urban and Roberts, 2003). There are also surgical approaches including spinal fusion and artificial/total disc replacement (Schizas et al., 2010) (Urban and Roberts, 2003). In spinal fusion, vertebral bodies are joined together using bone tissue that can either be derived from the patient (autograft) or from a donor (allograft). The fusion is intended to stabilize the spine and thus relieve the pain caused by motion (Schizas et al., 2010). In artificial/total disc replacement, a degenerated disc is replaced with an artificial disc. The artificial disc can be composed of different materials including polyethylene and titanium. The replacement of the degenerated disc with an artificial disc is intended to relieve the pain by restoring the mechanics of the spine where the spine can be aligned. However, the long-term effect of a disc replacement including wear of the artificial disc needs to be more thoroughly studied (Schizas et al., 2010). These surgical treatments are very invasive and therefore there is a need for a treatment which is minimally invasive. One such strategy could be using cell therapy. In this approach, mesenchymal stem cells (MSC) can be injected into the degenerated disc together with a cell carrier such as a hydrogel. The idea is that MSC could then differentiate into chondrocyte-like cells. But for such a therapy to be beneficial, it would be necessary to select patients that have a sufficient nutrient supply to the degenerated IVD. Otherwise the benefits of this approach will be lost (Urban et al., 2004).

Cell therapy for degenerated disc

There are different types of stem cells which can be used in cell therapy strategies. These include human embryonic stem cells (hESC) and human MSC (hMSC) (Gerecht-Nir and Itskovitz-Eldor, 2004) (Drazin et al., 2012). hESC are pluripotent while the hMSC are multipotent. However, there are ethical issues associated with using hESC in cell therapy

strategies together with practical problems (Leist et al., 2008) (Gerecht-Nir and Itskovitz-Eldor, 2004). One such practical issue is the agglomeration of embryoid bodies formed during differentiation of hESC. The agglomeration arises when culturing a large number of cells, which is required for cell therapy strategies. Solutions to this problem have been found in small scale cultivations, however not when the process is scaled up (Dang et al., 2002). Another drawback with using hESC is that *in vivo* there is a formation of teratomas which are tumors consisting of tissues from the three germ layers (Prokhorova et al., 2009). The main issues concerning the hESC are however ethical where the question is if the embryo has a moral value and one has to weigh destructive embryo research against the possible benefits (Leist et al., 2008) (McLaren, 2001).

For the purpose of treating degenerated IVDs, an appropriate candidate choice is the usage of bone marrow-derived hMSC. This is because the hMSC have a multilineage differentiation potential but are still easy to isolate and expand *ex vivo* (Harichandan, 2012) (Minguell et al., 2001). To differentiate the hMSC into adipocytes, chondrocytes and osteoblasts *in vitro* is also possible to do by the influence of external factors including the composition of growth medium (Chamberlain et al., 2007). An additional benefit with using hMSC is that the ethical issues regarding the hESC are avoided (Drazin et al., 2012). Transplantation of hMSC has been performed into injured disc of xenogenic porcine (Henriksson et al., 2009b). Henriksson et al. injected the hMSC with and without the hydrogel PuraMatrix™. The authors concluded that the hMSC survived in the disc and immunohistochemistry together with real time PCR-analysis showed an expression of chondrogenic markers indicating a differentiation of the hMSC into disc cells. In addition, the hMSC had a higher potential to differentiate and proliferate in the discs transplanted together with the hydrogel carrier.

Mesenchymal stem cells

MSC are a type of stem cells that originate from the mesoderm and can differentiate into cell types from a mesodermal lineage including adipocytes, chondrocytes, osteoblasts and cell types from other lineages including pancreatic cells and hepatocytes (Harichandan, 2012). Thereby the MSC have a multilineage differentiation potential. The hMSC are found in the bone marrow but they also exist in other tissues including adipose tissue, placenta and umbilical blood (Zannettino et al., 2008) (Minteer, 2012) (Battula et al., 2008) (Zvaifler et al., 2000). In the bone marrow, the hMSC exist in scarce amounts; approximately 1 out of 10000 nucleated cells in the bone marrow is considered to be a MSC (Chamberlain et al., 2007). The procedure of hMSC isolation includes that bone marrow samples containing anticoagulantia are centrifuged in special tubes containing FICOLL and the phase containing the mononuclear cells is collected. Thereafter, the cells are cultured in growth medium in cell culture flasks. The hMSC will adhere to the surface of the culture flasks under approximately 1-2 weeks while the other cells are eliminated during consecutive change of growth medium. In this thesis, frozen bone marrow samples are obtained from the iliac crest of patients. hMSC already in passage are used and isolation is not performed.

In order for cells to be characterized as hMSC, they should have an adherence to plastic and have the ability to differentiate into adipocytes, chondrocytes and osteoblasts *in vitro*

(Dominici et al., 2006) (Chamberlain et al., 2007). In addition, the cells should express certain cells surface proteins and lack the expression of hematopoietic markers. The cultured cells should be positive for cluster of differentiation 73 (CD73), CD90 and CD105 expression and negative for CD11b or CD14, CD34, CD45, CD79α or CD19 and HLA-DR expression (Dominici et al., 2006). The MSC can also have a positive expression of STRO-1 and CD166 (Harichandan and Buhring, 2011) (Jones et al., 2006) (Chamberlain et al., 2007). CD166 is an activated leukocyte cell adhesion molecule (ALCAM) while STRO-1 is a cell surface protein found in MSC from bone-marrow (Dennis et al., 2002) (Dominici et al., 2006). CD73 is an ectonucleotidease and CD90 is a cell surface protein also known as thy-1 while CD105 is a glycoprotein present on the surface of cells, also known as endoglin. The negative markers CD11b, CD14, CD34 and CD45 are markers which hematopoietic cells express. CD14 is a lipopolysaccharide receptor expressed by macrophages and monocytes which also express CD11b. CD79α and CD19 expression indicates a population of B cells while expression of HLA-DR does not occur unless the MSC have been stimulated with interferon- γ (IFN-γ). CD45 is a leukocyte antigen while CD34 expression indicates a population of precursors for hematopoietic or endothelial cells (Dominici et al., 2006) (Pittenger et al., 1999) (Chamberlain et al., 2007). The expression of the specific cell surface markers can be measured using fluorescence-activated cell sorting (FACS) described in the Methods section.

Hydrogels

Hydrogels are three-dimensional polymer networks consisting of water-absorbing polymers that are cross-linked. These can either be natural polymers including collagen, alginate and hyaluronic acid (HA) or synthetic polymers for instance poly vinyl alcohol (PVA) and poly acrylic acid (PAA) (Zavan et al., 2009). As the hydrogels can absorb and retain water and some are biocompatible, it makes them a good candidate material for tissue engineering approaches (Zavan et al., 2009). The hydrogels can be used in various applications in the field of tissue engineering. For instance, they can be used as carriers to deliver different molecules to a specific tissue or as space filling scaffolds. The structure of the hydrogels resembles the ECM of many cartiliginous tissues and therefore the hydrogels can serve as an environment for cells to attach, grow and differentiate in provided the appropriate external factors. Another application is the use of hydrogels as carriers of cells to a specific tissue (Drury and Mooney, 2003).

For the purpose of transplanting cells into a degenerated disc, a hydrogel can be injected in parallel to the cells. To use a hydrogel for this purpose is advantageous as the hydrogel ensures that the transplanted cells are located in the specific tissue along with providing an environment mimicking the ECM to increase the chance of survival of the transplanted cells. In addition, since the hydrogel has similar characteristics as the gelatinous NP, the hydrogel will serve as a temporary healthy NP and restore the disc height until the MSC have differentiated into chondrocyte-like cells and these cells can produce their own matrix (Henriksson et al., 2009b). Transplantation of MSC together with a hydrogel has been performed in degenerated discs in rabbits (Sakai et al., 2003). In the study by Sakai et al. the autologous MSC were first mixed with Atelocollagen® gel and then transplanted into the degenerated disc. The authors concluded that the transplantation of MSC in the hydrogel

slowed the process of disc degeneration by stabilizing the AF structure. In addition, the transplanted MSC showed the potential to differentiate into disc cells after transplantation. hMSC together with the hydrogel PuraMatrix™ have also been transplanted into a xenogeneic porcine model (Henriksson et al., 2009b). The results of this study are discussed in the next section.

PuraMatrix™

One of the hydrogels that are studied in this thesis is PuraMatrix™ (BD Biosciences, New Jersey, USA). PuraMatrix™ is a synthetic peptide hydrogel, consisting of 1% w/v amino acids and 99% water, that forms a three-dimensional structure by self-assembly after exposure to salts. This hydrogel has an acidic pH ranging between 2.0-2.5 that is equilibrated to normal pH after exposure to cell culture medium. It is composed of a peptide with the sequence (arginine-alanine-aspartic acid-alanine)$_4$, which also can be written as $(RADA)_4$, and is thereby composed of alternating hydrophobic and hydrophilic amino acids. This 16 amino acid long peptide is also called RADA16. The PuraMatrix™ hydrogel thus has the sequence AcN-$(RADA)_4$–CNH_2 and due to the alternating hydrophilicity and hydrophobicity, resulting to a high water-solubility, it contains stable β-sheets (Sieminski et al., 2008) (Puramatrix) (Zhang et al., 1995).

The gelation of the hydrogel is initiated by the addition of cations which can cause an interaction between individual β-sheets resulting in formation of filaments which in turn can form fibers (Sieminski et al., 2008) (Zhang et al., 1993). The gelation is not only stabilized by the ionic interactions between side-chains but also by hydrogen bonds in the backbone of the β-sheets (Zhang et al., 1995). The fibers formed are in nanoscopic scale creating an average pore size ranging between 5–200 nm (Zhang, 2006). This three-dimensional network of fibers in the hydrogel makes it similar to the size and structure of ECM in many tissues (Sieminski et al., 2008) (Wang et al., 2012) (Zhang, 2007) (Zhang, 2006). The PuraMatrix™ hydrogel contains arginine-alanine-aspartic acid (RAD) sequences. The RAD sequence resembles arginine-glycine-aspartic acid (RGD) sequences which allows for cell attachment via integrin receptors on the cells (Zhang, 2006) (Zhang et al., 1995). However, if the RAD sequences functions in a similar way as the RGD sequences, which integrin receptors recognize, has not been proven (Holmes et al., 2000) (Zhang et al., 1995). A possible mechanism of an integrin-independent cell attachment to the RAD sequence could be via ionic interactions between components on the surface of cells and the charged side-chains in the peptide hydrogel (Zhang et al., 1995).

A benefit of using PuraMatrix™ in the application of MSC transplantation into degenerated disc is that there are PuraMatrix™ gels which are produced in GMP-grade processes and can be used in clinical settings. From a production perspective, the PuraMatrix™ hydrogel is appropriate as it can be sterilized using UV (Zhang, 2007). This hydrogel is currently being tested in clinical trials as a void filler for dental bone in order to obtain a FDA approval. The idea is that PuraMatrix™ Bone Void Filler is added into voids in the bone and then eventually is substituted with healthy bone after resorption. Clinical investigations of the hydrogel as a possible hemostat are also presently being planned and outlined. The idea is that PuraMatrix™ will arrest the bleeding after application by gelation (PuraMatrix, 2013).

PuraMatrix™ has previously been used in the application of transplanting hMSC into degenerated IVD of xenogeneic porcine (Henriksson et al., 2009b). The hMSC were either co-injected with the hydrogel or with a suspension of cell culture medium. In this study it was concluded that the proliferative and differential potential was higher when the cells were injected with the hydrogel compared to without the hydrogel. This suggests that the hydrogel promotes differentiation of the hMSC into chondrocyte-like cells as well as providing an environment aiding the proliferation of the transplanted cells. In another study performed by Florine et al. hMSC were cultured in PuraMatrix™ in medium containing TGF-β and dexamethasone (Florine et al., 2013). Sulfated GAGs as well as collagen types I and II were studied after 21 days of culture using toluidine blue dye staining and immunohistochemistry. It was observed that the sulfated GAG content increased after 21 days of culture compared to 14 days of culture in hMSC cultured in PuraMatrix™. This indicated chondrogenic differentiation of hMSC in the hydrogel. Chondrogenic differentiation has also been observed in bovine MSC cultured in PuraMatrix™ (Kopesky et al., 2010).

As there is a detailed knowledge regarding the structure and sequence of PuraMatrix™, there is a possibility to tailor-make it for specific applications. However, a detailed understanding of how each amino acid and peptide affect the final structure has not yet been obtained (Zhang, 2007). In the article by Wang et al. PuraMatrix™ was tailor-made through the addition of motifs (Wang et al., 2012) . The authors modified PuraMatrix™ by adding a link N motif to the hydrogel and used it as a carrier for rabbit NP cells. The authors concluded that the new hydrogel promoted NP cell attachment as well as cell migration and matrix synthesis.

HydroMatrix™

The other hydrogel that is studied in this thesis is HydroMatrix™ Cell Culture Scaffold (Sigma-Aldrich, St. Louis, USA). This is a three-dimensional peptide hydrogel which forms a nanofiber scaffold matrix when exposed to salts or to a change in temperature by self-assembly (Sigma-Aldrich, 2014). The composition of this hydrogel is not specified however, similarly to PuraMatrix™, it is synthetic and not derived from animals. This is beneficial as the possible risk of infection or contamination from animal is avoided (Sigma-Aldrich, 2009). Also similar to the PuraMatrix™ hydrogel, this hydrogel has an acidic pH which is estimated to be pH 3 at 25°C (Sigma-Aldrich, 2013).

The HydroMatrix™ hydrogel has previously been used as a scaffold for cell culture (Stoppoloni et al., 2013). In the article by Stoppoloni et al. human primary chondrocytes were cultured in the HydroMatrix™ together with the molecule L-carnitine which is involved in the cells' energy metabolism. The cell proliferation and the production of ECM in the hydrogel were compared to monolayer cultures. The authors found that the L-carnitine promoted cell proliferation and that ECM production increased with increasing L-carnitine concentration mainly in the hydrogel (Stoppoloni et al., 2013).

Growth factors

Growth factors are molecules involved in cell proliferation, cell differentiation, extracellular signaling and ECM turnover. Specific growth factors can promote cellular events including cell survival and cell proliferation (Alberts et al., 2008) (Tolonen et al., 2006). Examples of

growth factors are epidermal growth factor (EGF), platelet-derived growth factors (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β). Growth factors have been observed in both healthy and degenerated IVDs indicating that cells in the IVD can synthesize these growth factors. bFGF and TGF-β have been found in degenerated human IVDs as well as in injured merino AF (Tolonen et al., 2006) (Melrose et al., 2002). In addition, Tolonen et al. found TGF- β1 and TGF- β2 in normal control discs (Tolonen et al., 2006). The authors hypothesized that the growth factors are involved in the cellular remodeling during the process of IVD degeneration. In the article by Melrose et al. bFGF and TGF-β were localized to areas of matrix remodeling which indicates that these growth factors are involved in this process. Due to this localization and the fact that these growth factors are involved in the turnover of ECM, there is an interest of the growth factors being used as therapeutic molecules in IVD degeneration (Masuda, 2008) (Thompson et al., 1991). The growth factors used in this thesis are human recombinant bFGF, also called FGF2, and human recombinant TGF-β1. bFGF is used to maintain the hMSC in their undifferentiated state and to retain their differentiation potential while TGF-β1 is used to promote differentiation of hMSC into chondrocyte-like cells.

bFGF

bFGF is a growth factor which is involved in processes including angiogenesis where it is a pro-angiogenic growth factor that binds to FGF receptors present on target cells to stimulate a response (Presta et al., 2005). This growth factor binds to heparin and heparan sulfate proteoglycans (HSPGs) and has been shown to interact with endothelial cells via this binding (Asada et al., 2009) (Presta et al., 2005). bFGF has been shown to inhibit differentiation of mesenchymal stem cells from rat. In the study by Lai et al. the results suggested that bFGF inhibited MSC differentiation by stimulating *Twist2* and *Spry4* expressions indicating that these two genes are negative regulators of MSC differentiation (Lai et al., 2011). In a study by Tsutsumi et al. bone marrow-derived hMSC were cultured in monolayer and in pellet cultures in the presence of bFGF (Tsutsumi et al., 2001). The authors concluded that the addition of the growth factor enhanced the MSC proliferation in monolayer cultures as well as retained the MSC differentiation potential in the pellet cultures. The chondrogenic differentiation potential was measured by detecting GAG production as well as mRNA levels of markers of chondrogenesis including type II and X collagen. The level of GAGs and expression of the markers were higher in pellet cultures with added growth factor compared to without growth factor. These results indicate that the bFGF retains the MSC differentiation potential (Tsutsumi et al., 2001).

TGF-β

TGF-β is a growth factor that is involved in the turnover of ECM and in many cellular processes including proliferation and differentiation (Shi and Massague, 2003) (Ignotz and Massague, 1986) (Bassols and Massague, 1988). The growth factor exerts its action by binding to two receptor serine/threonine kinases which cross-phosphorylates each other resulting in a phosphorylation cascade causing the activation of Smad proteins. The activation of this pathway inhibits cell proliferation for most cell types including epithelial cells and B lymphocytes. The growth factor is thereby a negative regulator of this process for these cell

types (Huang and Huang, 2005) (Shi and Massague, 2003) (Bassols and Massague, 1988). TGF-β regulates the expression of ECM components including fibronectin, collagen and chondroitin/dermatan sulfate proteoglycans and is therefore important for the turnover of ECM (Ignotz and Massague, 1986) (Bassols and Massague, 1988). Ignotz and Massague found that TGF-β induces a higher expression of ECM proteins in chick embryo fibroblasts (Ignotz and Massague, 1986). In addition, the relative incorporation of fibronectin and collagen in the ECM was changed in the presence of TGF-β which suggests that this growth factor not only increases matrix production but also changes its composition (Ignotz and Massague, 1986).

TGF- β1 has been observed to promote chondrogenesis of rabbit periosteal explants from tibiae (Miura et al., 2002). The explants contain MSC which can differentiate into chondrocytes. Miura et al. suggested that one of the mechanisms by which this growth factor can act to promote chondrogenesis is by promoting chondrogenesis in pre-chondrocytes (undeveloped chondrocytes). In order for this mechanism to take place, the growth factor has to be able to stimulate differentiation of MSC into chondrocytes (Miura et al., 2002). TGF-β has also been observed to promote chondrogenesis of rabbit bone marrow-derived MSC (Johnstone et al., 1998). In the article by Johnstone et al. the addition of the growth factor resulted in a higher expression of chondrogenic markers including type IIA collagen. In addition, the MSC aggregate culture morphology changed into being more similar to cartilage. In a study by Tuli et al. the mechanism by which TGF-β1 can promote chondrogenesis of mesenchymal progenitor cells (MPC) was studied (Tuli et al., 2003). TGF-β is suggested to activate proteins having a role in the process of chondrogenesis including mitogen-activated protein (MAP) kinases. p38, extracellular signal-regulated kinase-1 (ERK1) and *c*-Jun N-terminal kinases (JNK) are subtypes of the MAP kinases which TGF-β is suggested to interact with. The results from the study by Tuli et al. indicate that TGF-β promotes differentiation of MPC into chondrocytes by acting on ERK-1 and p38. The roles of these kinases in the process of chondrogenesis have been studied by Oh et al. (Oh et al., 2000). In mesenchyme derived from chick limb buds, p38 was found to stimulate chondrogenesis while ERK-1 was observed to repress chondrogenesis.

Oxygen level and pH in the intervertebral disc

The IVD is the biggest avascular structure in the human body (Roberts et al., 2006) (Urban et al., 2004) (Grunhagen et al., 2006). Due to the avascular nature of the tissue, the diffusion of nutrients, including oxygen and glucose, from the endplates into the NP is of great importance (Urban et al., 2004) (Bibby et al., 2001). As the AF is closer to the endplates than the NP, gradients of the nutrients arise where the lowest concentrations are found in the NP. Thus the IVD environment is hypoxic with the lowest concentration of oxygen found in the NP (Bibby et al., 2001) (Ishihara and Urban, 1999). How steep the gradients are depend on the rate of diffusion into the IVD and the rate of nutrient consumption (Urban et al., 2004). A consequence of low concentrations of oxygen and glucose in the NP is that there is an increased rate of anaerobic glycolysis which results in a high concentration of lactic acid and consequently there is a low pH in the NP (Urban et al., 2004) (Bibby et al., 2001) (Li et al., 2012) (Razaq et al., 2000). The pH is also affected by proteoglycans consisting of GAGs with

negative charges, including aggrecan, present in the IVD. The negative charges attract cations including hydrogen ions (H^+) which results in a more acidic pH compared to the tissues surrounding the IVD (Razaq et al., 2003).

The pH varies between 7.0-7.2 in healthy IVDs while in degenerated IVDs the pH level is estimated to be 6.5-6.8 (Li et al., 2012) (Razaq et al., 2003). The severity of the degeneration increases with decreasing pH. It is unclear whether a reduced nutrient supply initiate IVD degeneration or if it is the degeneration of the IVD that causes a reduced nutrient supply (Urban et al., 2004) (Bibby et al., 2001). The possible mechanism could be that with age or injury, the endplates become less permeable which impairs the diffusion of nutrients to the NP and also the removal of waste products from the IVD. One of these waste products is lactic acid which accumulates in the IVD resulting in a low pH which can be harmful to the cells. In addition, a reduced nutrient supply can disturb the balance between matrix synthesis and degradation possibly initiating IVD degeneration. (Buckwalter, 1995) (Bibby et al., 2001) (Razaq et al., 2003) (Ishihara and Urban, 1999).

As there is a low oxygen level and low pH in the IVD, it is important to study these effects on the hMSC as this is the environment that the cells will be exposed to after transplantation. The exact oxygen level inside a human IVD has not been measured however it is known that it is a hypoxic environment. Different oxygen levels ranging between 2-3% O_2 have been studied to mimic the *in vivo* situation (Stoyanov et al., 2011) (Risbud et al., 2007) (Baumgartner et al., 2010). The oxygen level that is tested in this thesis is 10% O_2 and is compared to a control cultured at standard cell culturing conditions (21% O_2 and pH 7.4) (Urban et al., 2004). The oxygen level of 10% was chosen to observe if hMSC could even survive at a decreased oxygen level of 10% in the hydrogels. This is because the oxygen tension inside bone marrow has been estimated to range between 1-7% through modeling (Chow et al., 2001). 10% O_2 was considered an appropriate starting value to study as this is approximately half of the standard condition (21%) and above 7%. In combination with a decreased oxygen level, the cells are cultured in the hydrogels in chondrogenic medium with added transforming growth factor (TGF-β) to promote differentiation of the hMSC into chondrocyte-like cells.

The pH levels that are tested in the second part of this thesis are 7.4, 7.1, 6.8 and 6.5. These levels of pH are studied as they represent the pH in standard cell culturing condition, healthy IVD, mildly degenerated IVD and severely degenerated IVD respectively (Li et al., 2012). One setup of these will be cultured at 21% O_2 to only study the effect of a decreased pH and another setup will be cultured in 10% O_2 to study the combined effect of pH and low oxygen level to mimic the environment in the IVD.

Methods

In this section, the principles of the methods used in this thesis are presented.

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) is a technique which can be used for separating specific cells from a cell population. This technique utilizes fluorescently labeled antibodies which bind to specific surface proteins only present on the cells of interest. As can be

observed in **Figure 2**, single cells move through the FACS machine following a liquid stream which will send the cells past a laser beam. The fluorescence emitted by the fluorescently labeled cells will be detected by a detector. Thereafter, a vibrating nozzle produces droplets of the fine stream which can contain one cell. The single cell droplets receive either a positive or negative charge depending on if the cell had emitted fluorescence or not. The droplets then move through an electric field which separates and deflects the droplets of different charge to different containers. Thereby the cells of interest are separated from the rest of the cell population and can be collected for further analysis. Droplets containing many cells are not given a charge and are transferred to a waste container (Alberts et al., 2008). FACS is used in this project to verify that the hMSC used are actually MSC and have not differentiated. Antibodies conjugated to fluorophores are used to bind to specific proteins on the surface of the hMSC.

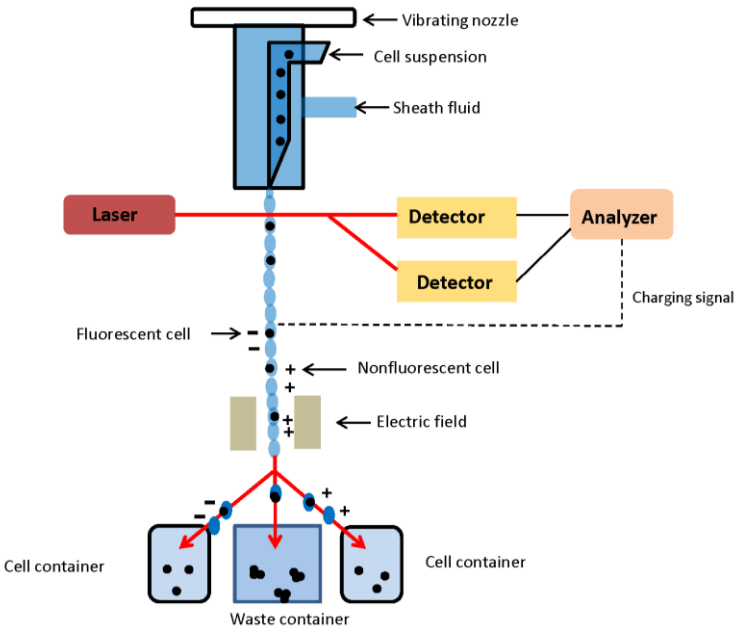

Figure 2. Illustration of fluorescence-activated cell sorting (FACS). Single cells move through the FACS machine following a fine stream that sends the cells past a laser beam. Fluorescence is detected by the detector and a charge is given to the cells depending on if the cell is fluorescent or not. An electric field then deflects and separates the cells into separate containers. (Modified image from Alberts, 2008)

Alcian blue van Gieson staining

To study the histology of the cells and the environment they have produced, Alcian blue van Gieson (ABvG) staining is performed. The main mode of binding of Alcian blue is coulombic attraction as Alcian blue is positively charged which enables the binding to sulfated GAGs which are negatively charged. This results in that proteoglycans in which sulfated GAGs are present are stained blue. Van Gieson stains collagens pink/red and by using the ABvG staining the cell nuclei are visualized brown/black (Quintarelli and Dellovo, 1965).

Immunohistochemistry

The principle of immunohistochemistry (IHC) is the action of antibodies binding to specific antigens. The number of antibodies used for IHC can vary; typically either one or two

antibodies are used. In the first case, one labeled antibody that binds to the antigen is used. The antibody can be fluorescently labeled or conjugated to an enzyme. In the second case, two antibodies are used; a primary antibody that binds to the antigen and a secondary antibody which binds to the primary antibody. The secondary antibody can be fluorescently labeled enabling visualization by fluorescence microscopy or it can be conjugated to an enzyme, such as horseradish peroxidase (HRP), which can catalyze the conversion of a colorless substrate into a colored product. Many secondary antibodies can bind to a single primary antibody yielding an amplification of the signal making it a more sensitive method. To use two antibodies for IHC is therefore the more common case (Buchwalow, 2010).

SOX9

To study the differentiation of hMSC into chondrocytes, an antibody for SRY (sex determining region Y)-box 9 (SOX9) is used in IHC studies as this is a chondrogenic lineage marker. The SOX9 protein has a DNA binding domain and can act as a transcription factor (Lefebvre et al., 1997). In the study by Lefebvre et al., the results indicated that SOX9 is involved in the activation of the gene encoding collagen IIA1 in chondrocytes. High levels of SOX9 expression is observed in undeveloped chondrocytes (pre-chondrocytes) however, the expression decrease as the progenitors differentiate towards fully matured chondrocytes. The SOX9 expression increases again in fully differentiated chondrocytes (Wright et al., 1995) (Ng et al., 1997) (Akiyama et al., 2002). Deletion of the *SOX9* gene has been observed to result in an impaired cartilage and bone formation in limb buds in mouse embryos (Akiyama et al., 2002). The authors concluded that SOX9 is needed in multiple stages in the differentiation process of MSC into chondrocytes. Thus, the prevalence of SOX9 protein is observed to study the differentiation of MSC into chondrocyte-like cells.

PCNA

To study the proliferation of the hMSC cultured in the hydrogels, an antibody for proliferating cell nuclear antigen (PCNA) is used. The PCNA is a 36kDa homotrimer which is found in the nucleus of mammalian cells and is highly expressed in proliferating cells. The cyclic homotrimer slides along double-stranded DNA during DNA replication to connect polymerases to the DNA (Essers et al., 2005). The relative proportion of this protein decreases with decreased cell proliferation and can therefore be used as a proliferation marker (Bravo et al., 1982).

Real-time reverse transcription PCR

Real-time reverse transcription polymerase chain reaction (RT-PCR) is a method to measure gene expression. This method can be a one-step or two-step process depending on whether the reverse transcription is separated from the PCR. The two-step method in which reverse transcription of mRNA is performed in a separate tube than the PCR is used in this thesis. In order to perform PCR, mRNA needs to be converted into complementary DNA (cDNA) by a DNA polymerase which is RNA-dependent. This enzyme is referred to as reverse transcriptase and it is commonly extracted from retroviruses containing RNA including avian myeloblastosis virus (AMV) (Bustin, 2000) (Glick et al., 2009). This enzyme can act as both a RNA-dependent DNA polymerase as well as an exoribonuclease specific for DNA:RNA hybrids. In the presence of oligo-dT and random primers, the reverse transcriptase first

performs reverse transcription of the mRNA into cDNA. The enzyme then degrades the mRNA strands that are annealed to cDNA strands i.e. RNA in DNA:RNA hybrids. This results in single-stranded cDNA. The mixture of oligo-dT and random primers is used to transcribe all the mRNA in the sample to obtain high cDNA amounts (Qiagen, 2009).

The formed cDNA is then amplified exponentially in the real-time PCR (Bustin, 2000). Conventional PCR consists of repeating cycles with three steps; denaturation, renaturation and synthesis. In the first step, the DNA is heated to allow for denaturation and is mixed with DNA polymerase, primers and the four types of deoxyribonucleotides. The temperature is lowered in the second step allowing for the annealing of the primers to the DNA. Finally, the temperature is slightly increased again in the third step to allow for DNA synthesis by the DNA polymerase (Glick et al., 2009). In real-time PCR, a Taq polymerase is used. The Taq polymerase is a thermostable 5'-to 3'- exonuclease which can extend newly synthesized DNA strands (Innis et al., 1995) (Dorak, 2006). In addition, a probe which is a fluorescently labeled oligonucleotide that can bind to DNA is used. The probe is designed to bind to the target gene, specifically between the locations of the forward and reverse primers binding sites. Using the principle of fluorescence resonance energy transfer (FRET), a reporter molecule which is fluorescent is bound to the 5' end of the probe while a quencher is bound to the 3' end of the probe. When the reporter molecule and quencher are close in distance, the fluorescence from the reporter is quenched by the quencher. However, when the Taq polymerase exerts its exonucleolytic action, i.e. cleaves the probe, it will free the reporter molecule which results in a separation between the reporter and quencher ultimately resulting in a fluorescence signal (Nolan et al., 2006) (Innis et al., 1995) (Dorak, 2006). The fluorescence is monitored and the formation of PCR products can be detected and visualized in real-time. The probe that is used in this thesis is a 6-FAM™ dye-labeled TaqMan® minor groove binding probe (Applied Biosystems, Foster City, USA). The 6-FAM™ is an example of a reporter (Dorak, 2006).

The process of real-time PCR can be divided into four phases; the linear phase, the early exponential phase, the exponential phase and the plateau stage. In the first phase, the fluorescence signal from each cycle is lower than the background level resulting in a linear curve. The fluorescence signal eventually attains a threshold value where the signal is significantly higher than the background. This is referred to as the early exponential phase and the cycle where this phase starts is referred to as the threshold cycle (C_T). From the C_T value, one can derive the target DNA amount in the sample as the early exponential phase is reached more quickly in samples with a higher DNA content. Thus a low C_T value correlates to a high target DNA amount. The exponential phase is characterized by the doubling of PCR products with each cycle whereas a limitation of reaction components arises at the plateau stage resulting in unusable data (Glick et al., 2009).

Besides performing PCR on your genes of interest, reference genes (housekeeping genes) are required to be included in the PCR run. These reference genes are used to normalize the data so that comparisons can be made between samples (Bustin et al., 2009). The C_T values of the genes of interest are first subtracted with the C_T value of a reference gene creating a ΔC_T value. Thereafter, the ΔC_T of a sample is subtracted with the ΔC_T of a reference sample

creating a $\Delta\Delta C_T$ value. The reference sample is chosen based on it having an even expression of the genes of interest. Finally, by taking 2 to the power of $-\Delta\Delta C_T$, relative values are obtained and this is set to 1 for the reference sample. The relative values can be compared between samples (Livak and Schmittgen, 2001). In addition, interplate calibrators (IPC) are used in every RT-PCR run to check for series variations to allow for comparison between different runs.

An example image of a graph obtained from a RT-PCR in which a threshold is set is presented in **Figure 3**. In this figure, the normalized fluorescence is plotted against the number of cycles. The figure shows duplicates of different samples run in the RT-PCR and a fluorescence threshold value is set to cross the linear phase of the samples. The C_T-values of each sample is read on the x-axis specifically where the threshold line crosses the sample lines. The two lines below the threshold line are duplicates of no template control (NTC) which do not contain any RNA.

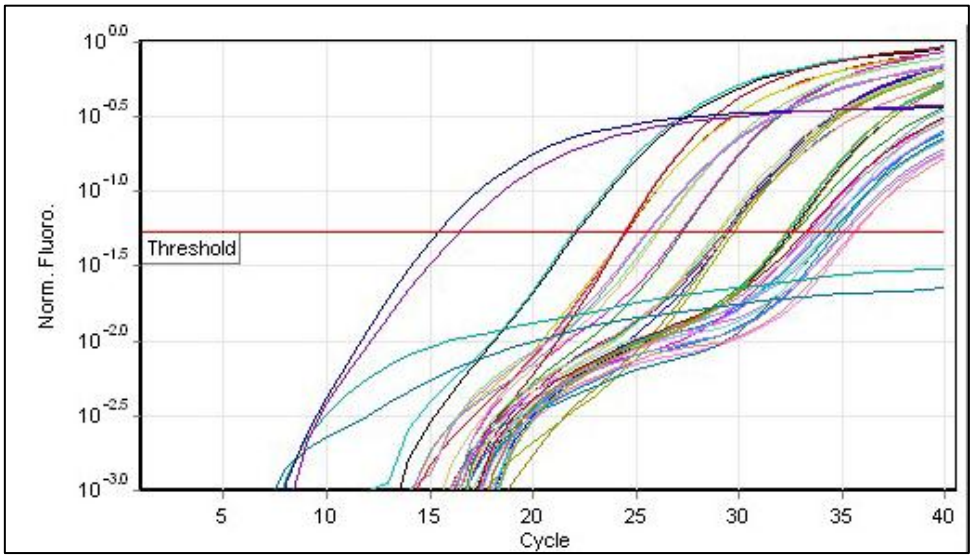

Figure 3. Example graph obtained from a RT-PCR run in which a threshold line is set. Normalized fluorescence is plotted against the number of cycles. Duplicates of each sample were run and the fluorescence threshold value is set to cross the sample lines in the linear phase. The C_T values are read on the x-axis specifically where each sample line crosses the threshold line.

SOX9

The expression of the gene *SOX9* is measured by RT-PCR to study it both on a protein and genetic level. As pre-chondrocytes have a high expression of SOX9, it can be used to study the differentiation of MSC into chondrocyte-like cells.

Collagen IIA1

The expression of the gene encoding collagen IIA1 is used to study the differentiation of hMSC into chondrocyte-like cells. The collagen IIA1 protein is a marker for late chondrogenesis however; it is an early marker for ECM accumulation that occurs in the last stages of chondrogenesis. Collagen IIA1 can be observed in the NP where chondrocyte-like cells synthesize and deposit collagen type II. Thus the presence of this protein is an indication of chondrogenic differentiation of the hMSC (Roberts et al., 2006) (Lefebvre et al., 1997).

HIF-1α

The expression of the gene encoding the protein hypoxia-inducible factor-1α (HIF-1α) is also measured by RT-PCR. Under normoxic conditions, the HIF-1α protein concentration and its mRNA level is lower than the levels found under hypoxia which was considered to be 2% O_2 in the article by Wagegg et al. (Wagegg et al., 2012). In other articles, hypoxic conditions that have been considered have ranged between 2-3% O_2 (Stoyanov et al., 2011) (Baumgartner et al., 2010) (Risbud et al., 2004). The half-life of the HIF-1α protein at normoxic conditions is approximately 5 minutes (Wang et al., 1995). Thus the mRNA levels as well as the HIF-1α concentration is an indicator of the cellular oxygen level. The reason for this is that under normoxic conditions, the HIF-1α protein is hydroxylated by prolyl hydroxylase domain-containing enzymes (PHDs) resulting in the formation of a binding site for the von Hippel-Lindau (pVHL) tumor suppressor protein. The binding of pVHL results in the ubiquitination of HIF-1α which ultimately results in its degradation. Conversely at hypoxic conditions, HIF-1α is not degraded. The reason for this is that the rate at which the PHDs hydroxylate HIF-1α decreases as these enzymes are dependent on oxygen. Thus at low oxygen levels, HIF-1α accumulates and enters the nucleus where it together with HIF-1β and p300 forms a transcription factor. This transcription factor activates genes involved in processes needed to respond to the low oxygen level. These processes include angiogenesis and anaerobic metabolism (Kaelin and Ratcliffe, 2008).

Viability staining

Viability staining is used to distinguish between viable and dead cells. LIVE/DEAD® Cell Imaging Kit (Molecular Probes®, Life Technologies, Carlsbad, USA) is used in this study and is visualized using fluorescence microscopy. This staining is based on the fact that viable cells have intact cell membranes and an activity of the intracellular enzyme esterase. Esterase can convert the cell-permeable calcein AM ($C_{46}H_{46}N_2O_{23}$), a non-fluorescent molecule, into the green-fluorescent and cell-impermeable molecule calcein. The esterase cleaves the acetoxymethyl (AM) ester groups on the calcein AM yielding calcein. Thus, viable cells will be visualized having a uniform green fluorescence as the fluorescent product can be retained inside the cells. Damaged cell membranes are an indication of dying or dead cells. The impaired integrity of the cell membrane enables the molecule BOBO™-3 Iodide ($C_{45}H_{58}I_4N_6S_2$), which is impermeable to viable cells, to pass through the membrane resulting in a nuclear red fluorescence when the molecule binds to DNA (Life Technologies, 2014). LIVE/DEAD® Cell Imaging Kit is used in this project to qualitatively study the survival of MSC cultured in a hydrogel in cell culture medium with different pH levels.

Materials and Methods

In this section, the procedure, the experimental setup and methods used in the study are described.

Experimental plan and setup

Three different experiments were performed in this study; an oxygen experiment, a pH experiment and a control experiment of chondrogenesis in which pellet systems were cultured. In the control experiment of chondrogenesis, pellet mass systems were cultured to promote chondrogenic differentiation of the MSC (Johnstone et al., 1998). These will be used as control systems of chondrogenesis. The pellets were cultured in chondrogenic medium to induce chondrogenic differentiation at standard conditions (37°C, 21% O_2, 5% CO_2) and were harvested after 14 and 28 days. The differentiation of hMSC cultured as pellets was studied by IHC, ABvG staining and RT-PCR.

In the oxygen experiment, MSC from three different patients were studied and the patients were a female born in 1964 (referred to as patient F64), a male born in 1970 (referred to as patient M70) and a female born in 1982 (referred to as patient F82). Thus the oxygen experiment included three sub-experiments in which each of the patient's cells were studied. In this experiment, cells in passage 4 were encapsulated in hydrogels and cultured in an incubator providing low oxygen levels (37°C, 10% O_2, 5% CO_2). As a control system for this, cells encapsulated in the hydrogels was also cultured at standard cell culturing conditions (37°C, 21% O_2, 5% CO_2). As both the differentiation and proliferation was to be evaluated, the cell/gel mixture was cultured in a growth medium stimulating chondrogenic differentiation (referred to as chondrogenic medium). This medium consisted of Dulbecco's Modified Eagle Medium (DMEM) high glucose (4.5g/l) (PAA Laboratories GmbH, Pasching, Austria) with added 10^{-6} M dexamethasone (Sigma-Aldrich, St. Louis, USA), 14 µg/ml ascorbic acid (Merck, Darmstadt, Germany), insulin, transferrin and selenium (ITS) (Life technologies, Carlsbad, USA), 1% penicillin/streptomycin (PEST) (PAA laboratories GmbH, Pasching, Austria), 1mg/ml human serum albumin (HSA) (Equitech-Bio, Kerrville, USA) and 0.01µg/ml TGF-β1. The cells encapsulated in the hydrogels were harvested after 14 days and the methods used to analyze the chondrogenic differentiation of hMSC were IHC, ABvG staining and RT-PCR. The initial intention was to study the proliferation of the hMSC in the hydrogels by calculating the number of cells using Scepter™ 2.0 Cell Counter. However, to count the cells in this way was not optimal and thus the proliferation of hMSC cultured in hydrogels was studied by performing IHC studies of PCNA on sections of the hydrogel mixed with cells.

In the experiment studying the pH, MSC from one patient, F82, were used. In this experiment, four different pH levels were tested; pH 7.4, 7.1, 6.8 and 6.5 and hMSC in passage 4 were cultured in a hydrogel. The different pH levels were achieved by changing the pH in the growth medium using sterilized HCl (1M) (Sigma-Aldrich) and NaOH (1M) (Sigma-Aldrich). As the effect of pH and oxygen level on the survival of hMSC cultured in the hydrogel was to be studied, and not the differentiation, there was no need to use a medium inducing chondrogenesis. Thus the growth medium used in the pH experiment was DMEM with low

glucose (DMEM/LG) (Thermo Fisher Scientific, Waltham, USA) with added 1% PEST (PAA laboratories GmbH, Pasching, Austria) and 10% human serum (HSE). One well plate with the different pH levels was incubated at standard cell culturing conditions (37°C, 21% O_2, 5% CO_2) to only observe the effect of the pH on the survival of hMSC. A second well plate with the different pH levels was placed in the incubator providing low oxygen levels (37°C, 10% O_2, 5% CO_2) to study the combined effect of low pH and a decreased oxygen level on the survival of hMSC. The duration of the experiment was 6 days and after harvesting, the survival of hMSC in the hydrogel at the different pH levels was studied by performing viability staining using LIVE/DEAD® Cell Imaging Kit. For this experiment, only one hydrogel was studied. The hydrogel to be used was chosen by testing the viability staining on both hydrogels and observing which of the hydrogels the staining works the best with.

Cell culturing

The different protocols used for cell culturing including freezing and thawing of hMSC as well as cell passaging and harvesting are described in this section.

Freezing and thawing of MSC

Cells were frozen in freezing medium containing Dulbeccos's modified eagle medium (DMEM/F12) (Gibco®, Life technologies, Carlsbad, USA) with 20% human serum (HSE) and 10% dimethylsulphoxide (DMSO) (Sigma-Aldrich, St. Louis, USA).The cells that had been frozen in cryotubes were thawed in 37°C water and thereafter transferred to a centrifugation tube. The growth medium used for the hMSC was Dulbecco's Modified Eagle Medium with low glucose (HyClone® DMEM/LG) (Thermo Fisher Scientific, Waltham, USA) with added 1% PEST (PAA Laboratories GmbH, Pasching, Austria), basic fibroblast growth factor (bFGF) (Gibco®, Life technologies) and 10% human serum (HSE). The growth medium was added to the cell suspension tube which was centrifuged using Centrifuge 4-16KS (Qiagen, Hilden, Germany) at 1500 rpm for 5 minutes. The supernatant was thereafter removed while the pellet was resuspended in growth medium. The cell suspension was then transferred to a culture flask (Nunc™, Roskilde, Denmark) and growth medium was added to the flask. The presence of cells was then studied in a light microscope (Nikon ECLIPSE TE2000-E) and finally placed in the Heraeus BBD 6220 CO2 incubator (Thermo Scientific) providing 37°C, 21% O_2 and 5% CO_2.

Human serum production

Blood that had been tested for contagious diseases was collected from donators at the Blood Centre (Sahlgrenska University Hospital). A pipette was used to transfer the serum surrounding the coagulated blood clot to a Falcon tube. The tube containing the serum was centrifuged for 10 minutes at 3000rpm. The supernatant containing the serum was thereafter sterile filtered using water suction and the filtration construct GP Millipore Express® PLUS Membrane (Millipore Corporation, Billerica, USA) with a pore size of 0.22µm. The human serum had a yellow/orange color and was stored at −20°C.

Passaging and harvesting of MSC

The cells were first studied in a light microscope (Nikon ECLIPSE TE2000-E) to estimate how many flasks the cells should be split into. In order to wash the cells, the growth medium was removed from the culture flask and new growth medium (DMEM/LG+FGF+10% HSE) was added. The growth medium was removed and HyClone® Trypsin 0.05% (Thermo Fisher Scientific) was added to the cell culture flask which was then placed in the incubator providing 37°C, 21% O_2 and 5% CO_2 for 5 minutes. After incubation, the cells were studied by light microscopy to verify their detachment from the surface of the flask. If the cells had not detached, the cells were incubated at 37°C for a few more minutes. In order to arrest the trypsinization, a "stop-medium" consisting of DMEM/LG and 10% HSE was added to the cells and thereafter the homogenous cell suspension was transferred to a 15ml centrifuge tube. The cells were counted with a Scepter™ 2.0 Cell Counter (Millipore Corporation) which is a handheld automated cell counter. The cells were further centrifuged at 1500 rpm for 5 minutes and the pellet was resuspended in growth medium. The cells were thereafter seeded in new cell culture flasks and growth medium was added to the flasks. As a last step, the cells were studied by light microscopy to verify detachment and the cells were thereafter incubated at 37°C, 21% O_2 and 5% CO_2.

FACS

hMSC were cultured according to previous sections. Cells in passage 3 from the three patients (patient F64, patient M70 and patient F82) were characterized using FACS and the expressions of the cell surface markers CD105, CD166, CD90, CD34 and CD45 were measured. The fluorophores phycoerythrin (PE), fluorescein isothiocyanate (FITC), peridinin chlorofyll protein (PerCP) and allophycocyanin (APC) were used. The antibody for CD166 was conjugated to PE (BD Biosciences, New Jersey, USA) while the one for CD105 was conjugated to FITC (BD Biosciences). The antibodies for CD90 and CD34 were conjugated to APC (BD Biosciences) while the antibody for CD45 was conjugated to PerCP (BD Biosciences). To evaluate the amount of non-viable cells, the cell impermeable dye 7-amino actinomycin D (7-AAD) was used and conjugated to PerCP (BD Biosciences). This dye can enter non-viable cells due to their impaired membranes. As a negative control for the fluorophores FITC and PE, IgG1 was conjugated to both FITC and PE (BD Biosciences) as this shows the cells that have not bound to CD166-PE and CD105-FITC. In order to be characterized as MSC, the cells should show a positive expression for the stem cell markers CD105, CD166, CD90 and lack expression of the leukocyte marker CD45 and the hematopoietic marker CD34.

hMSC in passage 3 cultured in growth medium were centrifuged at 200g for 5 minutes and the supernatant was thereafter discarded. The cell suspensions for each of the three patients were divided into three tubes. One tube contained the cell impermeable dye 7AAD while the second tube contained the antibodies for CD34, CD45 and IgG1. The last tube contained the antibodies for CD166, CD105 and CD90. The tubes were incubated in dark at room temperature for 20 minutes and then washed with PBS. The tubes were further centrifuged at 200g for 5 minutes and the supernatant was discarded. The cells were then resuspended in PBS and this cell solution was used for the FACS analysis.

Pellet culture systems

Pellet mass systems were cultured in chondrogenic medium in order to induce chondrogenic differentiation. These were used as control systems of chondrogenesis. When the cells had reached a confluence of at least 80%, the medium was removed and the cells were washed twice with serum free medium. Trypsin was added to the culture flask and the cells were incubated (37°C, 21% O_2, 5% CO_2) for 5 minutes and then studied by light microscopy to verify detachment. In order to arrest the trypsinization, medium containing serum was added. The cell suspension was then placed in a Falcon tube and 100µl of it was used to calculate the number of cells/ml using the Scepter™ 2.0 Cell Counter. 200 000 cells were needed per pellet. After counting the cells, the cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded while the pellet was resuspended in chondrogenic medium without TGF-β1 (R&D Systems, Abingdon, UK) to a concentration of 400 000 cells/ml. The chondrogenic medium consisted of DMEM High Glucose (4.5g/l) (PAA Laboratories GmbH) with added 10^{-6} M dexamethasone (Sigma-Aldrich, St. Louis, USA), 14 µg/ml ascorbic acid (Merck, Darmstadt, Germany), insulin, transferrin and selenium (ITS) (Life technologies, Carlsbad, USA), 1% PEST, 1mg/ml human serum albumin (HSA) (Equitech-Bio, Kerrville, USA) and 0.01µg/ml TGF-β1. 0.5ml of the cell suspension was placed in tubes to reach 200 000 cells/tube and thereafter the tubes were centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 0.5 ml chondrogenic medium with TGF-β. The tubes were centrifuged once again at 1500 rpm for 5 minutes and thereafter detached from the bottom of the tube by tapping the tube gently. The pellets were then placed in the incubator (37°C, 21% O_2, 5% CO_2) and the medium was changed three times a week (Johnstone et al., 1998) (Tallheden et al., 2004).

Two pellets of cells from each patient were harvested after 14 and 28 days yielding a total of 4 pellets of cells per patient. The medium was first removed from the pellets and thereafter the pellets were washed with Dulbecco's Phosphate Buffered Saline DPBS (1X) (Gibco®, Life technologies). For harvesting of pellets cultured for RT-PCR studies, the pellets were transferred to cryotubes and RNA*later*™ RNA Stabilizing Reagent (Qiagen) was added to the pellets after the first PBS washing step to keep the RNA stabilized. The pellets were then stored at −80°C. Pellets cultured for histology studies were fixated with Histofix™ (HistoLab®, Göteborg, Sweden) for 15 minutes and thereafter washed with PBS. 70% ethanol (VWR Chemicals, Radnor, USA) was added to the pellets and the pellets were sent to Histo-Center AB (Göteborg, Sweden). Histo-Center AB dehydrated the samples in ethanol and isopropanol. The samples were then embedded in paraffin and cut into 5µm thick sections. A section from each sample was thereafter stained by the addition of Alcian Blue 8GX (Sigma Aldrich) with a pH of just below 3, basic fuchsin (Merck), acidic fuchsin (Merck) and hematoxylin (Merck). The unstained sections could be used for IHC studies.

Incubator providing decreased oxygen level

In order to study the effect of a decreased oxygen level on the proliferation and differentiation of hMSC, a CB 53 CO2/O2 incubator (BINDER GmbH, Tuttlingen, Germany) suitable for studies requiring low oxygen levels was purchased. In the first experiment where the effect of

a decreased oxygen level was studied, the cells cultured in the hydrogels were incubated at 37°C, 10% O_2, 5% CO_2. As a control, another setup was incubated at 37°C, 21% O_2, 5% CO_2. In the second experiment where the effect of a decreased pH was studied, one setup of the well plate with different pH levels in the wells were incubated at 37°C, 10% O_2, 5% CO_2 to study the combined effect of a decreased oxygen and pH level. To only study the effect of a decreased pH, one setup was incubated at 37°C, 21% O_2, 5% CO_2.

Cell culture inserts and well plates

In this study, 24-well plates (Thermo Scientific) were used for the culture of cells in the hydrogels. In each of the wells, a 0.4µm PET track-etched cell culture insert (BD Biosciences) was placed (**Figure 4**). The bottom of the cell culture insert consists of a polyethylene terephthalate (PET) membrane containing pores with the size of 0.4µm. Growth medium can diffuse through these pores and thus the cell/gel mixture will be surrounded by growth medium (**Figure 4B**).

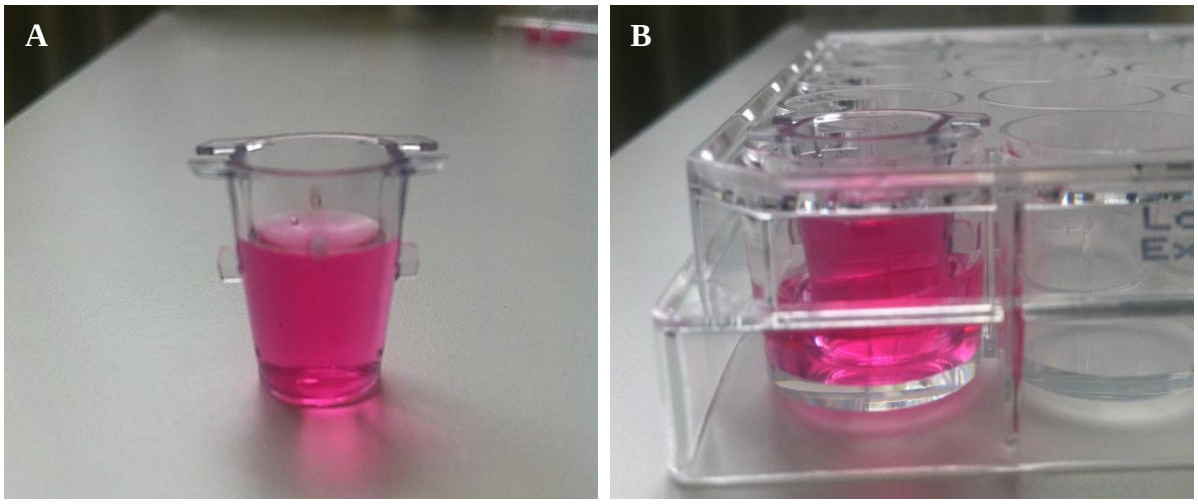

Figure 4. Images of the cell culture insert and 24-well plate. **A)** Image of a cell culture insert with growth medium. **B)** Image of a cell culture insert inside a well in a 24-well plate. Growth medium is placed in both the insert and the well so that cells cultured inside the insert are surrounded by growth medium.

Cell encapsulation in hydrogels

The cells were encapsulated in the hydrogels PuraMatrix™ (BD Biosciences) and HydroMatrix™ (Sigma-Aldrich). The PuraMatrix™ hydrogel was delivered as a liquid while the HydroMatrix™ was delivered as a powder and therefore it was dissolved in sterile water before usage. The stock solution of the hydrogels was first vortexed in order to decrease its viscosity. 200µl PuraMatrix™ hydrogel was transferred to each Eppendorf tube (one Eppendorf tube for each well that is needed). The HydroMatrix™ hydrogel was kept on ice until usage to avoid premature gelation. For the oxygen experiment, inserts were then placed in the well plates. For the pH experiment, no cell culture inserts were used and thus the cell/gel mixtures were cultured directly in the wells. Then cells in passage 4 were prepared by removing the medium in the culture flasks and the cells were washed twice with serum free medium. To detach the cells from the surface of the culture flask, trypsin was added and the cells were placed in the incubator (37°C, 21% O_2, 5% CO_2) for 5 minutes. After the incubation, the cells were studied by light microscopy to verify the detachment and thereafter

medium with serum was added to block trypsinization. The number of cells was then calculated using Scepter™ 2.0 Cell Counter. The cells were then centrifuged at 1500 rpm for 5 minutes and medium was added to the lower chambers of the inserts. After centrifugation, the supernatant was discarded and the pellet was resuspended in 10% sucrose solution. The sucrose solution was added to protect the cells from the acidic pH of the hydrogels.

The cells were then further centrifuged at 1500 rpm for 5 minutes and during these 5 minutes, 200µl HydroMatrix™ hydrogel was transferred to Eppendorf tubes (one Eppendorf tube for each well that was needed). After the centrifugation, the pellet was resuspended in 10% sucrose solution at a concentration that was double the concentration of the cells in the hydrogel (300 000 cells in the hydrogel). 100µl of the cell/sucrose solution was then rapidly added to a hydrogel in an Eppendorf tube and mixed by pipetting. This solution was then rapidly pipetted on the insert of a 24-well and was left there to settle for 10 minutes. During these 10 minutes, the next cell/sucrose solution was mixed with the hydrogel and added to the next insert. After this, medium was added to the hydrogel on the first insert and this was repeated for all the inserts. For the oxygen experiment, the samples were then put in the incubator providing low levels of oxygen (37°C, 10% O_2, 5% CO_2) for 1 hour and thereafter the medium was removed and new medium was carefully added to the inserts and to the wells. As a control system, cells were also put in the incubator providing standard cell culturing conditions (37°C, 21% O_2, 5% CO_2). After the medium change, the inserts were placed in their respective incubator for 30 minutes. The medium was then removed and 200µl medium was added to the inserts and 700µl medium was added to the wells. The well plates were placed in their respective incubator and medium was carefully changed every two days and the cells in this experiment were harvested after 14 days. For the pH experiment, no cell culture inserts were used and thus the cell/gel mixtures were cultured directly in the wells. In this experiment, one well plate was placed in the incubator providing standard cell culturing conditions (37°C, 21% O_2, 5% CO_2) while another well plate was put in the incubator providing low levels of oxygen (37°C, 10% O_2, 5% CO_2) for 90 minutes. Thereafter, the medium was changed and 300µl of new medium was added to each well. The medium was then changed again on day 3 and the cells in this experiment were harvested after 6 days.

Harvesting of cells encapsulated in hydrogels

After 14 days, the oxygen experiment in which cells were encapsulated in the hydrogels was harvested. For cells to be studied by RT-PCR, the cell/gel mixture was transferred into a cryotube containing RNA*later*™ RNA stabilizing reagent. The cryotube was thereafter placed in a Falcon tube and centrifuged at 1500rpm for 5 minutes. After the centrifugation, a thin film in the bottom of the tube was observed and the supernatant was discarded leaving about 2-3mm liquid above the film. The cryotubes containing the cells were stored at −80°C until RNA extraction was to be performed.

For the histology and IHC studies, a box containing dry ice and a metal bowl was used. A thin liquid layer of 2-propanol (Merck, Darmstadt, Germany) was poured in the metal bowl and a cryomold was placed there. A droplet of the mounting medium for cryotomy O.C.T. compound (VWR Chemicals, Radnor, USA) was put in the cryomold and thereafter the cell

culture insert containing the cell/gel mixture was placed on top of the droplet. Thereafter, O.C.T. compound was put on top of the cell/gel mixture inside the cell culture insert mixture and was allowed to completely freeze. The cell culture insert together with the cryomold was packaged using aluminum foil and stored at −20°C until sent to Histo-Center AB. Histo-Center AB cut the samples into sections with a thickness of 7µm and then fixated the sections by exposure to formalin and ethanol. Every twentieth section was thereafter stained by the addition of Alcian Blue 8GX (Sigma Aldrich) with a pH of just below 3, basic fuchsin (Merck), acidic fuchsin (Merck) and hematoxylin (Merck). The unstained sections could be used for IHC studies.

pH experiment

In the experiment studying the effect of pH on the survival of hMSC in a hydrogel, the cells were cultured at both standard cell culturing conditions (37°C, 21% O_2, 5% CO_2) and at a decreased oxygen level (37°C, 10% O_2, 5% CO_2) to study the combined effect of a decreased pH and oxygen level. To achieve different pH levels for the cells to grow in, the pH of the growth medium was changed. The growth medium used for the pH study was DMEM/LG supplemented with 1% PEST and 10% HSE. bFGF was not included in the growth medium composition as the effect of pH on the survival of hMSC was studied and not the differentiation. Four different pH levels were to be achieved, pH 7.4, 7.1, 6.8 and 6.5, thus four different tubes of growth medium were prepared. To achieve the different pH levels, sterilized HCl (1M) (Sigma-Aldrich) and NaOH (1M) (Sigma-Aldrich) were added to the growth media and the pH was measured using Jenway 3510 pH meter (Jenway, Staffordshire, UK) (3 decimal resolution). The color difference between the four different media can be visualized in **Figure 5**. The four different media were sterile filtered and then incubated (37°C, 21% O_2, 5% CO_2) for 3 days to obtain a stabilized pH (Li et al., 2012). On the day of the pH experiment, the pH was checked, adjusted and sterile filtered. The experiment with the cells cultured in a hydrogel spanned over 6 days and at day 3 the respective growth media were replaced with new media. Viability staining of the cells cultured in the hydrogel was performed after 6 days of culture according to the next section.

Cell culture inserts were not used in the pH experiment as fluorescence microscopy was used to visualize the viability staining. Thus to not interfere with the fluorescence microscopy, inserts were not used and the cells encapsulated in the hydrogel were therefore cultured directly in the well plate.

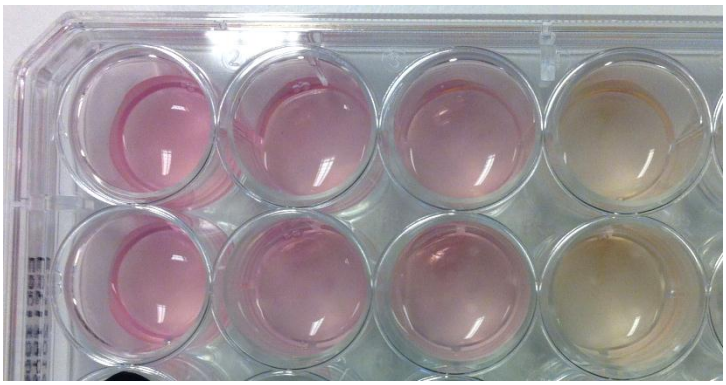

Figure 5. The growth media with different pH in a24-well plate. From left to right: pH 7.4, 7.1, 6.8 and 6.5 where each medium is present in two wells (the respective upper and bottom wells).

Viability staining

The viability staining was first tested on both hydrogels to observe which of these the staining worked the best for. As fluorescence microscopy was used to visualize the cells in the well plates, HydroMatrix™ was considered to be the most appropriate choice as the PuraMatrix™ hydrogel was thicker while HydroMatrix™ could be placed relatively flat on the bottom of the well facilitating imaging with fluorescence microscopy.

The LIVE/DEAD® Cell Imaging Kit was used for viability staining of cells cultured at different pH. First the vials containing the Live Green and Dead Red stains were thawed and the Live Green solution (1ml) was mixed with the Dead Red solution (1µl). This solution was then mixed with sterile PBS (1X) to create a 2X stock solution. Medium was removed from the wells containing the cells mixed in the hydrogels. 250µl of the 2X solution was then added to each well. The cells were then incubated for 15 minutes at 37°C and thereafter visualized using fluorescence microscopy.

Methods for studying proliferation and differentiation

In this section, the methods used to study the proliferation and chondrogenic differentiation of MSC are described.

Histology

Samples that were sent to Histo-Center AB were returned sectioned and every twentieth section had been stained with ABvG. These sections were 5-7µm thick and the ABvG stained sections were visualized by light microscopy (Nikon Eclipse E600). Unstained sections were also received which were used for IHC studies. The unstained sections used were chosen by studying the cell prevalence and integrity of the hydrogel in the ABvG stained sections. The unstained pellet sections were embedded in paraffin when received and it was therefore necessary to deparaffinize the sections before performing antibody staining. Frozen sections of hydrogel were received and these were fixated before antibody staining.

To evaluate chondrogenesis in pellet cultures and in the hydrogels by visualization in a light microscope, the Bern scoring system was used. The Bern score is a grading system designed for pellet cultures of chondrocytes stained with Safarin-O fast green stain (Grogan et al., 2006). However, the grading can be applied to Alcian Blue as well (Svanvik et al., 2010). The

cells in the pellets were scored by studying three different parameters; the darkness and uniformity of the Alcian blue stain, the distance between the cells and the cell morphology. A large distance between the cells indicate that matrix has been formed which creates a distance between the cells while a rounded and less fibrous cell indicate chondrogenic morphology. Immature MSC are given the score 0 as these have not differentiated. Due to the hydrogel affecting the cell distance as well as the cell morphology, the cells in the hydrogel were scored by only studying the darkness and uniformity of the Alcian blue stain. Each parameter can be scored 0-3 where 3 is the closest to the chondrocyte phenotype and the maximum score is thereby 3 for the cells in the hydrogel and 9 for the pellet cultures. A high score thus indicates that the cells are chondrocyte-like (Grogan et al., 2006).

Immunohistochemistry

IHC was used to study the differentiation of hMSC and antibodies for PCNA and SOX9 were used. On the first day of IHC studies, the cells grown as pellets were first deparaffinized by a consecutive exposure to the following solutions: xylene (HistoLab®) (2 times for 10 minutes), 99% ethanol (VWR Chemicals) (for 5 minutes), 95% ethanol (VWR Chemicals) (for 5 minutes), 70% ethanol (VWR Chemicals) (for 5 minutes) and finally PBS (for 5 minutes). The cells cultured in hydrogels were not embedded in paraffin but were placed in frozen blocks. Therefore these sections did not need to be deparaffinzed. These sections were instead placed in the fixative Histofix™ for 15 minutes and then in Tris-buffered saline (TBS) for 15 minutes. A pen containing liquid repellant (liquid blocker pen) was thereafter used to screen off the area around the samples by drawing circles. To retrieve the antigen in both the pellets and hydrogel sections, the sections were exposed to 0.1M citrate buffer (pH 6.0) and incubated in humidity chambers at 95°C for 15 minutes. After a few minutes in room temperature, the samples were washed twice with PBS for 3 minutes.

SOX9

In IHC studies of SOX9, the primary antibody used was mouse anti-SOX9 ab76997 (Abcam) diluted 1:500. The secondary antibody was Alexa Fluor 546 goat anti-mouse A11030 (Molecular Probes®, Life Technologies) diluted 1:250. After the antigen retrieval step described above, the samples were washed with PBS for 5 minutes and then permeabilized by the addition of 0.1% Triton-X (Merck, Darmstadt, Germany) for 15 minutes at room temperature. The samples were then washed three times with PBS for 5 minutes. To block the permeabilization, Triton block containing 2% bovine serum albumin (BSA), 0.1% Triton-X and 100mM glycine (Sigma-Aldrich) in PBS was added to the samples for 15 minutes at room temperature. The primary antibody diluted in Triton block was then added to each sample, except the negative control to which distilled water diluted in Triton block was added. The primary antibody was omitted as the control would be used to study the unspecific binding of the secondary antibody. The samples were incubated at 4°C in a humidity chamber.

On the second day, the samples were washed four times with PBS for 3 minutes. Triton block was thereafter added to the samples for 15 minutes at room temperature. The secondary antibody diluted in Triton block was then added to each sample and the samples were incubated in the dark for 2 hours in a humidity chamber. After this incubation, the samples were washed four times with PBS for 3 minutes. One droplet of the mounting medium

Prolong Gold antifade reagent with DAPI (Life technologies) was added to each sample to stain the nucleus and thereafter a cover glass was placed on each slide. The samples were stored in the dark at room temperature until studied by fluorescence microscopy using the filters Cy3 and DAPI.

PCNA

In IHC studies of PCNA, the primary antibody was mouse anti- PCNA ab29 (Abcam, Cambridge, USA) with a 1:500 dilution. The secondary antibody was Jackson HRP goat anti mouse (Abcam) which is an antibody that is conjugated to horseradish peroxidase (HRP). The dilution of the secondary antibody was 1:150. When using an antibody conjugated to HRP, a step where the samples are exposed to hydrogen peroxide is needed. This is because endogenous peroxidase is expressed in many cells and tissues and therefore this would result in background staining which is not specific. By treating the samples with hydrogen peroxide prior to the addition of the HRP-conjugated antibody, the background staining is minimized (Buchwalow, 2010). Thus following the wash with PBS, the samples to be stained with the PCNA antibody were exposed to 3% hydrogen peroxide (Merck, Darmstadt, Germany) in PBS. The samples were then washed twice with PBS for 3 minutes. Further, cells need to be permeabilized as the antibody needs to enter the cells. For this purpose 0.1% Triton-X was added to the samples for 15 minutes and the samples were washed twice with PBS for 5 minutes. Triton block was added to block the permeabilization for 15 minutes at room temperature. Lastly, the primary antibody diluted in Triton block was added to each sample except the negative control. Distilled water diluted in Triton block was added to the negative control as the control would be used to study the unspecific binding of the secondary antibody. The samples were then incubated at 4°C over night in a humidity chamber.

On the second day, the samples were washed three times in PBS for 2 minutes. To block the primary antibody, Triton block was added to the samples for 5 minutes. The secondary antibody diluted in Triton block was then added to each sample and the samples were incubated in the dark for 2 hours at room temperature. To amplify the signal from the HRP-conjugated antibody, a TSA™ Cyanine 3 System (PerkinElmer, Waltham, USA) was used. Tyramide signal amplification (TSA) works to amplify the signal as tyramide is a substrate for HRP. Tyramide is a tyramine conjugate and HRP catalyzes the oxidation of tyramine which can then bind to proteins which are near the site of HRP-antibody binding. In this way, the signal is amplified. The samples were first washed three times with 0.05% TWEEN20 (Sigma-Aldrich) in PBS for 3 minutes. Thereafter, the Cyanine3 reagent from the TSA kit was diluted 1:100 in amplification diluent also provided in the kit. The solution was added to each sample and placed in the dark for 10 minutes. The samples were then washed 3 times with 0.05% TWEEN20 in PBS for 3 minutes. After this wash, the samples were rinsed in PBS for 3 minutes. One droplet of the mounting medium Prolong® Gold antifade reagent with DAPI (Life technologies) was added to each sample to stain the nucleus. Lastly, a cover glass was placed on each slide and the samples were stored at room temperature until studied by fluorescence microscopy (Nikon Eclipse E600) using the filters Cy3 and DAPI.

Semi-quantification of SOX9 and PCNA staining

To semi-quantify the results of the SOX9 and PCNA staining of cells cultured in hydrogels, percentages of positive cells for SOX9 and PCNA were calculated respectively. Two sections were used from each sample and a total of 100 cells were randomly selected and counted. These were counted manually by observing the DAPI staining using fluorescence microscopy. NIS Elements Software was used which allowed to mark cells which had been counted. Out of this hundred, the number of cells positive for SOX9 or PCNA were counted and this represented the percentages of cells positive for the SOX9 or PCNA.

RNA extraction for RT-PCR

In order to use RT-PCR, RNA was first extracted from the cells in the hydrogels or from the cell pellets, and from this cDNA could be synthesized. RNA extraction was performed using RNeasy® Plus Mini Kit (Qiagen). The RNA extraction began with cleaning the work bench and pipettes with RNase Away® Reagent (Ambion®, Life technologies) to eliminate any RNase present. Thereafter, the cells (maximum 1×10^7 cells) were placed in a Safe-Lock Tube 2.0 ml (Eppendorf AG, Hamburg, Germany). A tungsten bead (Qiagen) with a diameter of 3mm was then added and thereafter the cells were washed twice with PBS. A thermose was filled with liquid nitrogen and the sample tubes were placed in the thermose. Thereafter, a metal rack was placed in the liquid nitrogen for 10 seconds and then the other side of the rack was exposed to liquid nitrogen for 10 seconds. The sample tubes were then placed in the rack and placed in liquid nitrogen for 10 seconds. This rack was placed in TissueLyser II (Qiagen) which is a machine that mechanically disrupts the samples. Empty tubes were placed opposite as counter-weights. The TissueLyser II was run at 20Hz for 4 minutes. The rack containing the sample tubes was once again placed in liquid nitrogen for 10 seconds and then the other side was also exposed for 10 seconds. Following this, the rack was put in the TissueLyser II again and run at 20Hz for 4 minutes and homogenization of the cells was checked. The tubes were inspected macroscopically and if not homogenized, the rack with the sample tubes were placed in liquid nitrogen and then run in the TissueLyser II for additional 4 minutes at 20Hz.

1ml QIAzol® Lysis Reagent (Qiagen) was then added to each sample tube and the tubes were placed in a metal rack that was not cooled and then run in the TissueLyser II for 2 minutes at 20Hz. The tube containing the homogenized cells was then placed on the work bench for 5 minutes at room temperature. 200µl LiChrosolv® Chloroform (Merck, Darmstadt, Germany) was added to the tubes and after capping them, the tubes were shaken for 15 seconds. The tubes were then placed on the work bench for 2-3 minutes and after this they were centrifuged at 13000 rpm for 15 minutes at 4°C in the microcentrifuge Sigma 1-14K (Sigma GmbH, Harz, Germany). The phase at the top was transferred to new tubes and 1 volume of 70% ethanol was added and mixed by pipetting. RNeasy® Plus Mini Kit was then used for the RNA extraction. A maximum of 700µl of the lysate was added to a RNeasy mini column in a tube. The tubes were centrifuged at 13000 rpm for 15 seconds at +20°C and the RNA had absorbed on the column after the centrifugation, thus the flow-through was discarded. To remove DNA, 350µl Buffer RW1 was added to the columns and centrifuged at 13000 rpm for 15 minutes. Further 80µl of DNase mix (10µl DNase I and 70µl Buffer RDD) was pipetted onto each column and the tubes were placed on the work bench for 5 minutes at room temperature.

350µl Buffer RW1 was then added to each column and the tubes were centrifuged once again at 13000 rpm for 15 seconds.

The columns were thereafter placed into a new collection tubes and 500µl Buffer RPE was added to each column for washing. The tubes were centrifuged at 13000 rpm for 15 seconds and the flow-through was discarded. Additional 500µl Buffer RPE was added to each column and the tubes were centrifuged at 13000 rpm for 2 minutes to dry the gel membrane. The columns were then carefully detached without touching the flow-through to avoid the transfer of ethanol. To absolutely dry the gel membrane, the columns were placed in new tubes and these were centrifuged at 13000 rpm for 1 minute. The columns were further transferred to new collection tubes and 30µl RNase-free water was added to the gel membranes. After 3-5 minutes, the tubes were centrifuged at 13000 rpm for 1 minute. To measure the RNA concentration in the tubes, NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific) was used. The tubes were stored at −80°C until cDNA synthesis was to be performed.

cDNA synthesis for RT-PCR

Extracted RNA which had been frozen was first thawed on ice. cDNA was prepared of minimum 133.4ng of total RNA using the The QuantiTect® Reverse Transcription Kit (Qiagen). This kit contained gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water which were thawed at room temperature. To exclude genomic DNA from the extracted RNA, a tube containing 2µl gDNA Wipeout Buffer (7X) and variable volumes of template RNA and RNase-free water was prepared and put on ice. 8.75ng of RNA made into cDNA through reverse transcription was to be used in each RT-PCR reaction (in each PCR-tube). Thus the volume of extracted RNA to place in the tube for gDNA elimination depended on the concentration of RNA in the frozen tube. After the gDNA elimination tube had been prepared, it was placed in the PCR machine Rotor-Gene Q (Qiagen) for 3 minutes at 42°C. A reverse-transcription master mix was then prepared containing 1µl Quantiscript Reverse Transcriptase, 4µl Quantiscript RT Buffer (5X) and 1µl RT Primer Mix. The extracted RNA in the gDNA elimination mixture was then mixed with this master mix to give a final volume of 20µl and the tube was placed on ice. The tube was then placed in the PCR machine and incubated for 15 min at 42°C. To inactivate the reverse transcriptase, the tube was then incubated for 3 min at 95°C. The obtained cDNA was then diluted with RNase-free water to yield a final concentration of 1.75ng/µl. The tube was stored on ice if RT-PCR was performed the same day or at −20°C for long-term storage (Qiagen, 2009).

RT-PCR

RT-PCR was performed to study the expression of genes involved in the chondrogenic differentiation; *SOX9* and *collagen IIA1*. RT-PCR was also used to analyze the expression *HIF-1α* which is a gene involved in the response to a decreased oxygen level. The reference genes used in the RT-PCR were CREBBP (TATAA Biocenter AB, Göteborg, Sweden) and PPIA (TATAA Biocenter AB). First the cDNA, primers, IPC cDNA, primer and probe provided in TATAA Interplate calibrator 250rxn Probe FAM (TATAA Biocenter AB) were placed on ice. RNase-free water and TaqMan® Universal Master Mix II (Applied Biosystems, Foster City, USA) were also placed on ice. For each primer, a gene-specific master mix was

made containing 1µl of the primer, 4µl RNase-free water and 10µl 2X TaqMan master mix for every sample. 7.5µl of this master mix was added twice to each of the tubes that contained the specific gene. After this, tubes containing the IPC were made by mixing 3µl RNase-free water, 1µl IPC primer, 1µl IPC Probe and 10µl of the 2X TaqMan master mix. Further a no template control (NTC) was made by mixing 9µl RNase-free water, 1µl reference gene primer (either CREBBP or PPIA) and 10µl of 2X TaqMan master mix. Then 5µl of the respective cDNA was placed in the tubes (8.75ng of RNA reverse transcribed into cDNA to each tube) designated for that specific cDNA. The tubes were placed in the PCR machine with the PCR program shown in **Table 1.**

Table 1. PCR program for real-time reverse transcription PCR (RT-PCR).

Temperature (°C)	Time	Number of cycles
95	10 min	Hold (1 cycle)
95 60	15 sec 60 sec	40

The IPC was used to observe variation between the different RT-PCR runs and plates to be able to compare between samples of different runs. Therefore, the IPC was included in all runs. Thereafter, the standard deviation of the IPC C_T-values was divided with the mean value of the IPC C_T and multiplied with 100 to obtain a percentage. This percentage is referred to as the coefficient of variation and is used to examine the series variation between different runs. A value below 10% is considered a common threshold value in laboratories following GMP procedures. In this thesis, a percentage of 6.9% was obtained for the IPC containing a FAM probe, which was used in the different RT-PCR runs.

Cell proliferation

The aim was to study both the differentiation and proliferation of the MSC cultured in the hydrogels. The proliferation of the cells was intended to be studied by using a cell counter (Scepter™ 2.0 Cell Counter) and a developed protocol for re-plating of cells was modified for this purpose (3DM Inc.). In the modified protocol, 0.5ml trypsin was added and the hydrogel was dissolved by repetitive pipetting of the content of the well. The content was then transferred to a sterile Eppendorf tube (VWR Chemicals) which was placed inside a Falcon tube and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in growth medium. The cells were then counted using the cell counter. However, it did not work to count the cells in the hydrogel using the cell counter. The cause for this could be there was still hydrogel left even after the pipetting and trypsinization steps. Thus, a hemocytometer was a potential option to use for calculating the number of cells; however by using this, it was not possible to distinguish individual cells as cell aggregates were present. Hence, the proliferation of MSC in the hydrogels was not studied using this method. Instead, IHC studies on sections of the cells encapsulated in the hydrogels were performed using the PCNA antibody which is expressed in proliferating cells. This gave an indication of the proliferating features of the cells.

Results

In this section, the results of the FACS analysis, ABvG staining, IHC studies and RT-PCR are presented for the pellet cultures and the cells cultured in the hydrogels. In addition, the results of the viability staining performed in the pH experiment are presented.

FACS analysis

FACS was performed in order to characterize the MSC population. Cells in passage 3 from the three patients were characterized using FACS. The percentage of cells expressing or lacking the respective cell surface markers are represented for the three patients in **Table 2**.

Table 2. Fluorescence-activated cell sorting (FACS) results of human mesenchymal stem cells (hMSC) in passage 3 from patients F64, M70 and F82. Percentages of hMSC expressing the stem cell markers CD105, CD166 and CD90 (marked as +) and lacking expression of the leukocyte marker CD45 and the hematopoietic marker CD34 (marked as -) are presented.

	Patient F64	**Patient M70**	**Patient F82**
CD34-/CD45- (%)	96.4	95.0	95.2
CD105+/CD166+ (%)	97.1	98.1	98.0
CD105+/CD90+ (%)	97.1	98.1	98.0
CD166+/CD90+ (%)	97.1	98.1	98.0

A high percentage of MSC from all patients showed an expression of the stem cells markers CD105, CD166 and CD 90. In addition, 95% of cells from all patients lacked expression of the leukocyte marker CD45 and the hematopoietic marker CD34. The results from the FACS analysis of cells from patient F82 are presented in **Figure 6**. The high number of viable cells which were used for the study of cell surface marker expression can be visualized as orange dots inside the P1 region in **Figure 6A**. Cells lacking the expression of CD45 and CD34 are present in the defined region in **Figure 6B** while cells expressing CD105, CD166 and CD90 are observed in the enclosed regions in **Figures 6C-E**. The negative control for the fluorophores PE and FITC is presented in **Figure 6F** in which cells that have not bound to the antibodies CD166-FITC and CD105-PE are present in the defined region.

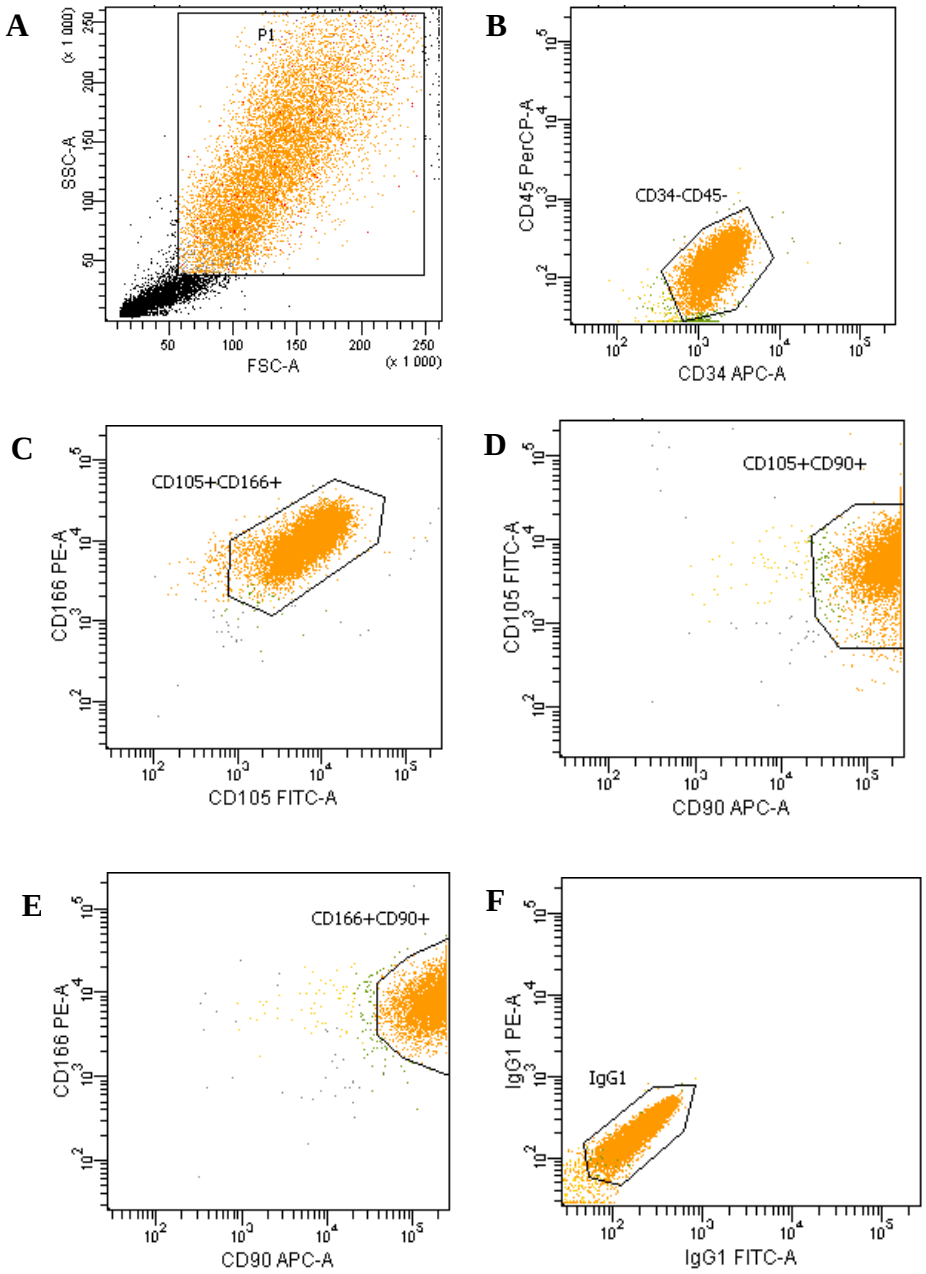

Figure 6. Results from fluorescence-activated cell sorting (FACS) of human mesenchymal stem cells in passage 3 from the iliac crest of patient F82**. A)** Cells in the region P1 are used for the measurement of expression of cell surface markers. **B**) A defined region containing cells lacking the expression of both CD34 and CD45. **C)** A defined region containing cells expressing both CD105 and CD166. **D)** A defined region containing cells expressing both CD105 and CD90**. E)** A defined region containing cells expressing both CD166 and CD90. **F)** Negative control of fluorophores FITC and PE.

Pellet culture systems

The pellet mass systems were cultured in chondrogenic medium in order to induce chondrogenic differentiation. These were used as control systems of chondrogenesis. Sections of the pellets were stained with ABvG staining by Histo-Center AB. PCNA and SOX9 staining was also performed to study the chondrogenic differentiation of MSC.

Alcian blue van Gieson staining

ABvG staining was performed on sections of all pellet cultures and these were studied using light microscopy. Pellets were harvested after 14 and 28 days. The ABvG staining of pellets of MSC from patient F82 are presented in **Figure 7.**

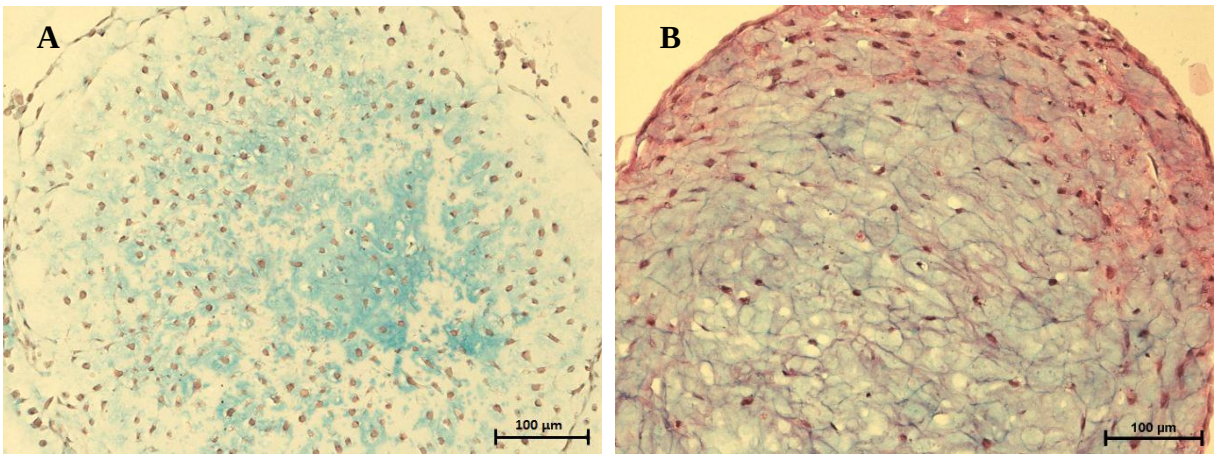

Figure 7. Alcian Blue van Gieson (ABvG) staining of sections of mesenchymal stem cell pellet systems from patient F82. **A)** Pellet system harvested after 14 days. The blue staining indicates sulfated glycosaminoglycans (GAGs) while the pink/red staining indicates the presence of collagens. Cell nuclei are stained brown/black. **B)** Pellet system harvested after 28 days. The areas stained blue indicate the presence of sulfated GAGs while the pink/red staining indicates the presence of collagens. Cell nuclei are stained brown/black.

Cell pellet cultures were scored using the Bern scoring system which examines chondrogenesis by studying three different parameters; the darkness and uniformity of the Alcian blue stain, the distance between the cells and their morphology. Two pellets from each patient were harvested after 14 and 28 days resulting in a total of 4 pellets per patient. The two pellets for each harvest day were studied and given a joint score using the scoring system developed by Grogan et al. (Grogan et al., 2006). In **Table 3**, the Bern scores are presented for the cell pellet cultures with the maximum score 9. A high Bern score indicates that the cells are chondrocyte-like.

Table 3. Bern scores of mesenchymal stem cell pellet cultures harvested after 14 and 28 days (D14 and D28). The pellets were scored studying three parameters; darkness and uniformity of the Alcian blue stain, the distance between the cells and the morphology of the cells. A high score indicates that the cells are chondrocyte-like. The maximum score is 9.

	Alcian blue	Cell distance	Cell morphology	**Total score**
MSC F64, D14	0	0	0	**0**
MSC F64, D28	1	1	1	**3**
MSC M70, D14	1	1	1	**3**
MSC M70, D28	0	1	0	**1**
MSC F82, D14	1	3	2	**6**
MSC F82, D28	2	3	2	**7**

As can be seen from **Table 3**, the pellets of MSC from patient F64 that were harvested on day 14 received a score of 0 as the pellets showed no Alcian blue staining and the cells were packed densely with a morphology resembling undifferentiated MSC. The pellets of MSC from patient F82 received the highest scores as the Alcian blue staining was dark and the cells had a morphology resembling chondrocytes. In addition, the cell densities in these pellets were low where more than one cell could fit in the gap between the cells.

SOX9 staining

Sections of the pellet cultures were stained with an antibody for SOX9 which is an early marker for chondrogenesis. The SOX9 staining of cell pellets from patient M70 is presented in **Figure 8**. The cell nuclei are visualized blue by DAPI staining. Yellow dots inside the cells indicate cells positive for SOX9. Positive cells could be observed at both time points (**Figures 8B and C**). This could also be observed in cell pellets from the other two patients at both harvest days (data not shown).

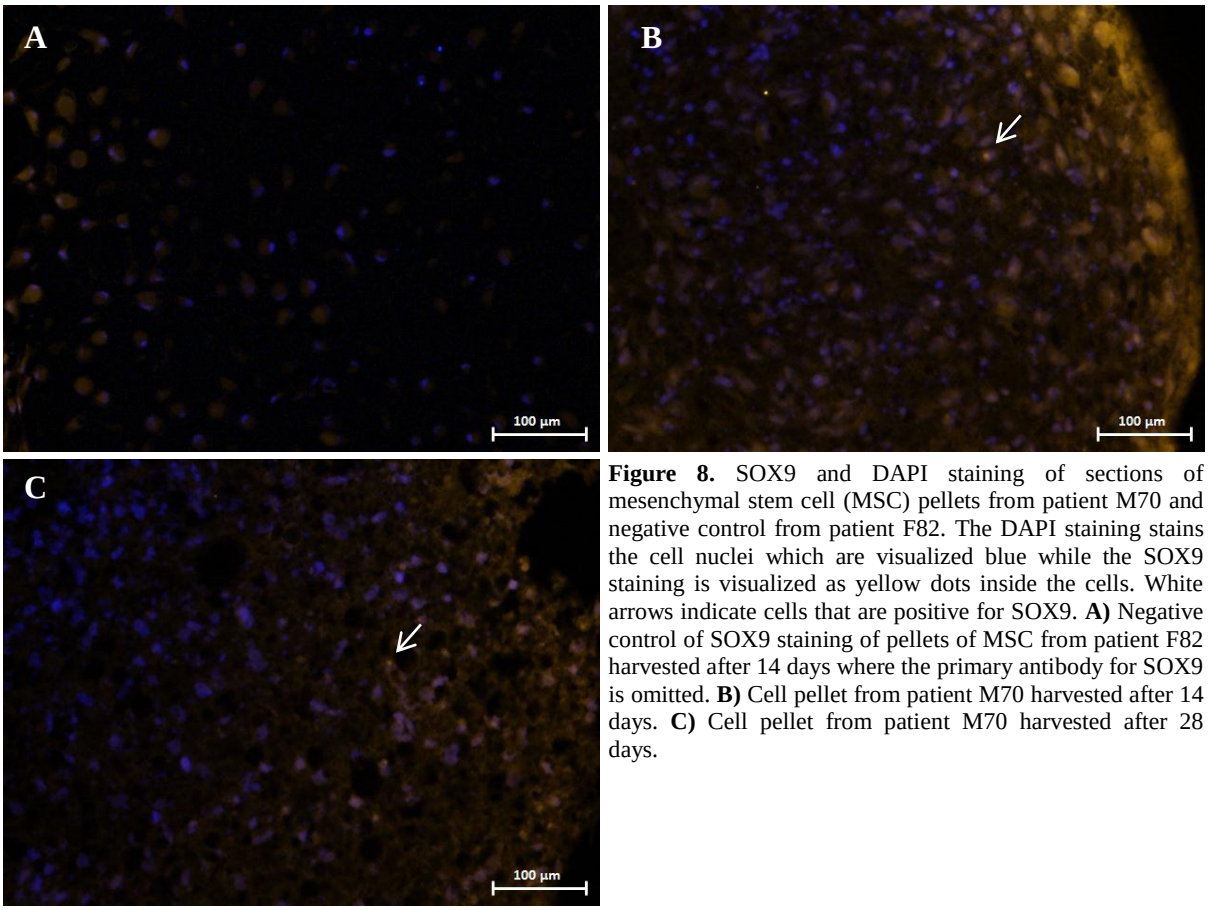

Figure 8. SOX9 and DAPI staining of sections of mesenchymal stem cell (MSC) pellets from patient M70 and negative control from patient F82. The DAPI staining stains the cell nuclei which are visualized blue while the SOX9 staining is visualized as yellow dots inside the cells. White arrows indicate cells that are positive for SOX9. **A)** Negative control of SOX9 staining of pellets of MSC from patient F82 harvested after 14 days where the primary antibody for SOX9 is omitted. **B)** Cell pellet from patient M70 harvested after 14 days. **C)** Cell pellet from patient M70 harvested after 28 days.

PCNA staining

PCNA staining was performed on sections of the pellet cultures to study if the cells were proliferating in the pellets. However, the negative control showed a high fluorescence signal and cells which were falsely positive for PCNA can be seen in **Figure 9**. Thus this staining was not optimal for the pellet systems.

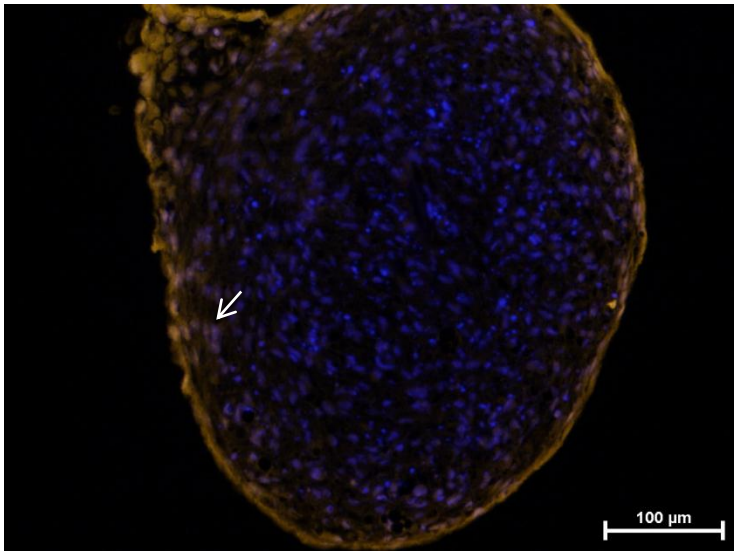

Figure 9. Negative control of the proliferating cell nuclear antigen (PCNA) staining where the primary antibody for PCNA is omitted. The cell nuclei are stained blue with DAPI. The negative control showed cells falsely positive for PCNA staining in which yellow dots are visualized. The white arrow specifies a cell falsely positive for PCNA staining.

RT-PCR

In this section, the results of the RT-PCR of the pellet cultures are presented. Three different genes were studied by RT-PCR; *collagen IIA1*, *SOX9* and *HIF-1α*. Relative values were obtained using the $\Delta\Delta C_T$ method and the relative expression of the reference sample is set to 1 (Livak and Schmittgen, 2001). The cell pellet from patient F82 that was harvested after 28 days was used as a reference sample in the analysis of RT-PCR results and is thus denoted RS in the figures. This sample was chosen to be used as a reference sample as it had an even average expression of the genes of interest i.e. the C_T values of the genes for this sample were close to the average C_T values of all samples. The results of the RT-PCR of the cells cultured in the hydrogels are interpreted in the Oxygen experiment section.

SOX9

The relative expressions of *SOX9* in the pellet cultures are presented in **Figure 10**. The highest relative expression of *SOX9* is observed in cell pellets from the youngest patient, F82, harvested after 14 days while the lowest is observed in cell pellets from the oldest patient patient, F64, harvested after 14 days. Furthermore, it can be seen that a higher relative expression of *SOX9* is present in pellets of MSC from patient F64 harvested after 28 days compared to 14 days. The opposite is observed in MSC from patients F82 and M70 where a higher relative expression *SOX9* is present after 14 days compared to 28 days.

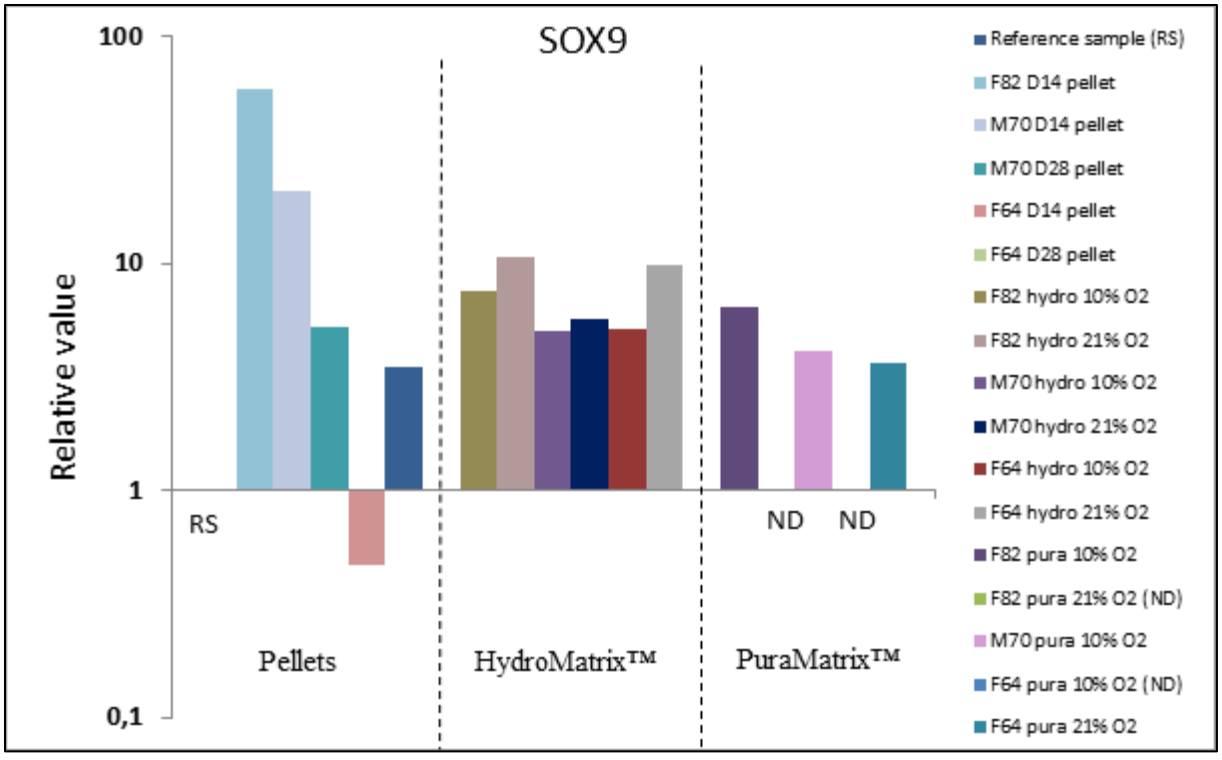

Figure 10. Relative expression of SOX9 in mesenchymal stem cells (MSC) presented with a logarithmic y-axis. The MSC were either cultured as pellets that were harvested after 14 and 28 days (D14 and D28) or in the hydrogels HydroMatrix™ and PuraMatrix™ at different oxygen levels. Each condition is based on two measurements. The reference sample is the cell pellet from patient F82 harvested at day 28 and the relative expression of this sample is set to 1. If *SOX9* expression in a sample obtained a C_T value in the range of 37-40 it was considered as barely detected (BD) while if it obtained a C_T value above 40 it was considered as not detected (ND).

Collagen IIA1

In **Figure 11**, the relative expressions of *collagen IIA1* in the pellet cultures are presented. The highest relative expression of *collagen IIA1* is observed in cell pellets from the youngest patient, F82, harvested after 14 days while the lowest is observed in cell pellets from the oldest patient, F64, harvested after 28 days. Furthermore, cell pellets from patient F64 harvested after 14 days has a slightly higher relative expression of *collagen IIA1* than the ones harvested after 28 days. The same is observed in MSC from patient F82 which showed a much higher relative expression of *collagen IIA1* after 14 days compared to 28 days (F82 D28 pellet is denoted RS in **Figure 11**). The same trend is seen in MSC from patient M70 where a higher relative expression of *collagen IIA1* is present after 14 days compared to 28 days.

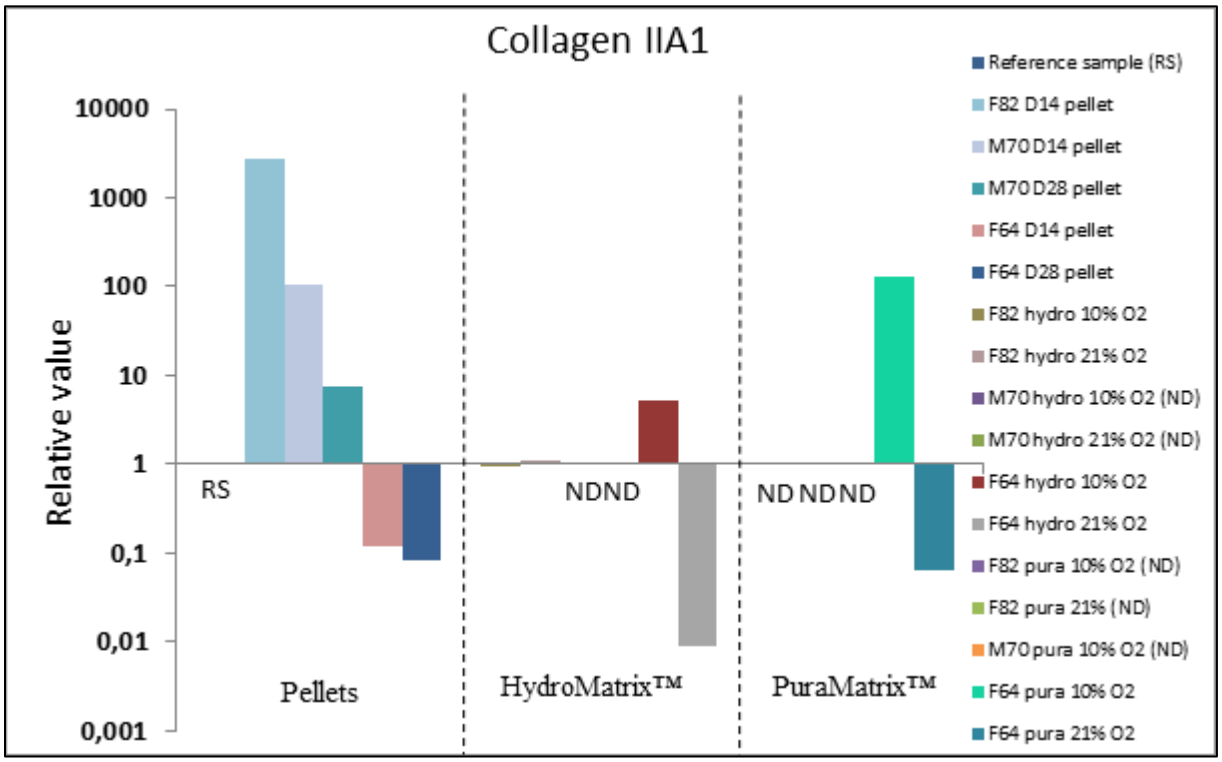

Figure 11. Relative expression of *collagen IIA1* in mesenchymal stem cells (MSC) presented with a logarithmic y-axis. The MSC were either cultured as pellets that were harvested after 14 and 28 days (D14 and D28) or in the hydrogels HydroMatrix™ and PuraMatrix™ at different oxygen levels. Each condition is based on two measurements. The reference sample is the cell pellet from patient F82 harvested at day 28 and the relative expression of this sample is set to 1. If *SOX9* expression in a sample obtained a C_T value in the range of 37-40 it was considered as barely detected (BD) while if it obtained a C_T value above 40 it was considered as not detected (ND).

HIF-1α

The relative expressions of *HIF-1α* in the pellet cultures are presented in **Figure 12**. It can be seen that *HIF-1α* expression is present in all pellet cultures. A higher relative expression of *HIF-1α* is present in pellets of MSC from patient F64 harvested after 28 days compared to 14 days. The opposite is observed in pellets of MSC from patient F82 where a higher expression of *HIF-1α* is present in pellets harvested after 14 days compared to 28 days. The same trend is observed in pellets of MSC from patient M70 where a higher expression of *HIF-1α* is observed in pellets harvested after 14 days compared to 28 days.

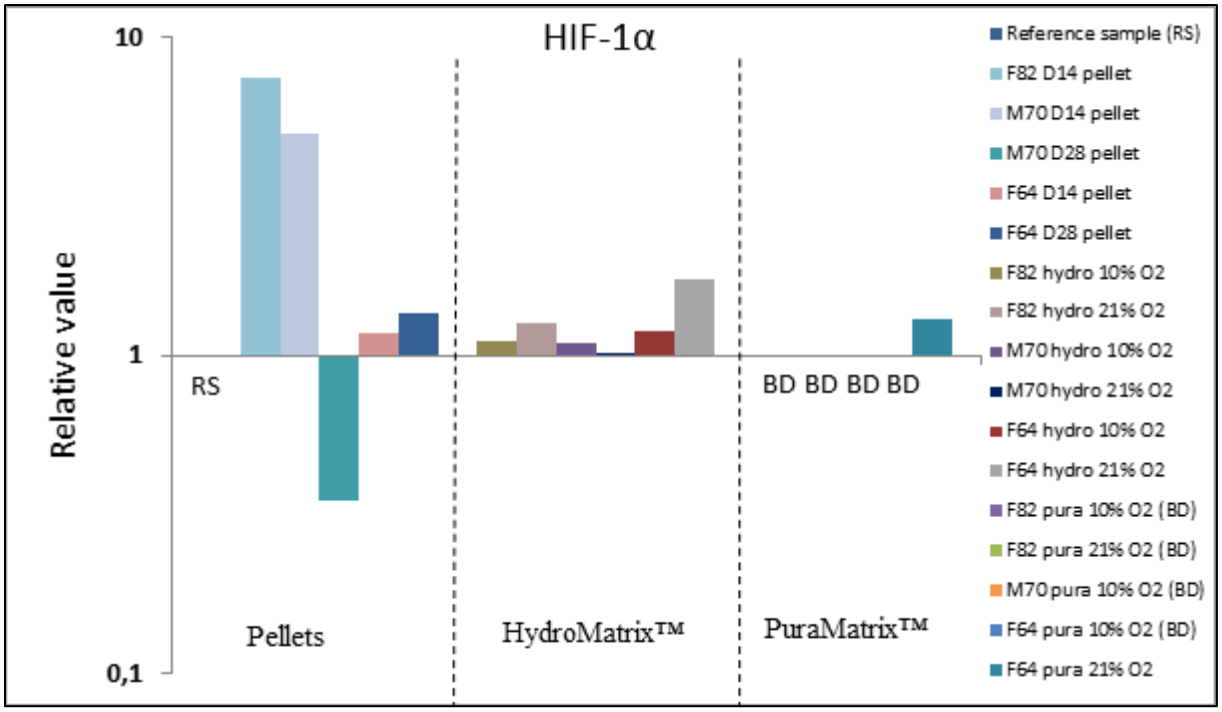

Figure 12. Relative expression of HIF-1α in mesenchymal stem cells (MSC) presented with a logarithmic y-axis. The MSC were either cultured as pellets that were harvested after 14 and 28 days (D14 and D28) or in the hydrogels HydroMatrix™ and PuraMatrix™ at different oxygen levels. Each condition is based on two measurements. The reference sample is the cell pellet from patient F82 harvested at day 28 and the relative expression of this sample is set to 1. If *SOX9* expression in a sample obtained a C_T value in the range of 37-40 it was considered as barely detected (BD) while if it obtained a C_T value above 40 it was considered as not detected (ND).

Cells encapsulated in hydrogels

The results from the experiments in which cells were encapsulated in HydroMatrix™ and PuraMatrix™ are presented in this section. The results from the oxygen experiment and the pH experiment are presented here.

Oxygen experiment

In this section, the ABvG staining, IHC studies and the RT-PCR results from the oxygen experiment are presented. In this experiment, hMSC in passage 4 were cultured in HydroMatrix™ and PuraMatrix™.

ABvG staining

ABvG staining was performed on sections of cells encapsulated in the hydrogels. ABvG stains sulfated GAGs blue while collagens are stained red/pink and cell nuclei black/brown. PuraMatrix™ was very scattered and not intact for cells from all three patients in this experiment as can be seen in **Figure 13A**. No macroscopic difference in appearance of the two hydrogels could be seen when culturing at the two different oxygen levels; 10% O_2 and 21% O_2. Example images of the two hydrogels are presented in **Figure 13**.

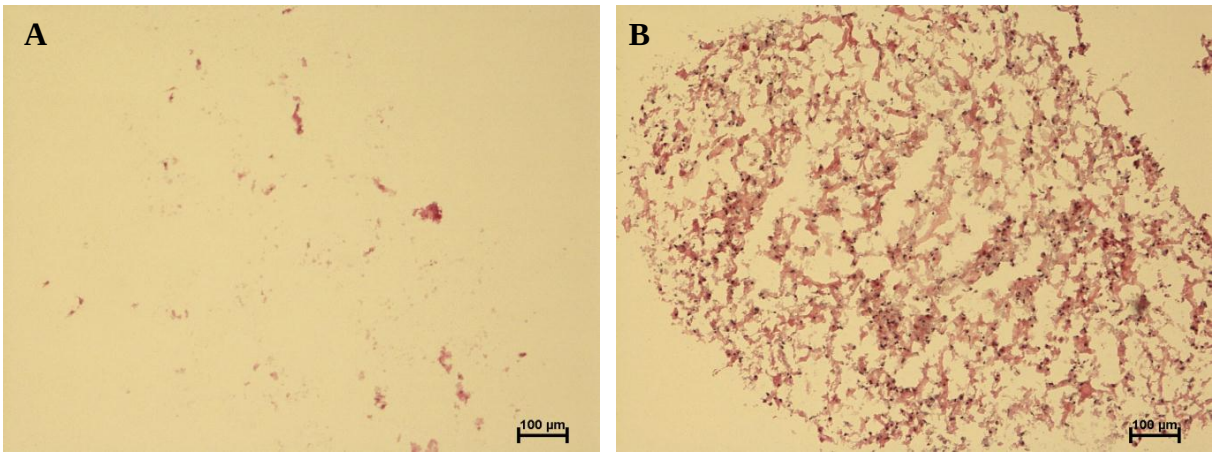

Figure 13. Example images of Alcian blue van Gieson (ABvG) stained sections of mesenchymal stem cells (MSC) from patient F82 cultured in PuraMatrix™ and HydroMatrix™. Sulfated glycosaminoglycans (GAGs) are stained blue while collagens are stained red/pink and cell nuclei are stained black/brown. **A)** MSC from patient F82 cultured in PuraMatrix™ at 21% O2. The hydrogel is scattered and some cells can be visualized in the hydrogel. **B)** MSC from patient F82 cultured in HydroMatrix™ at 21% O2. The hydrogel is intact and many cells can be visualized.

In **Figure 14**, the results from the ABvG staining of sections of MSC from patient F82 cultured in HydroMatrix™ at the two different oxygen levels are presented. As the hydrogel has a similar color as the collagen staining, it cannot be said for certain that collagens are present in the hydrogel. However, sulfated GAGs are present at the decreased oxygen level of 10%. This difference was only seen in HydroMatrix™ cultured with cells from patient F82 however, this hydrogel was not as intact for the MSC from the other two patients (data not shown). In the results of ABvG staining of cells cultured in PuraMatrix™, sulfated GAGs were not observed (data not shown). However, the PuraMatrix™ samples were very scattered and not intact.

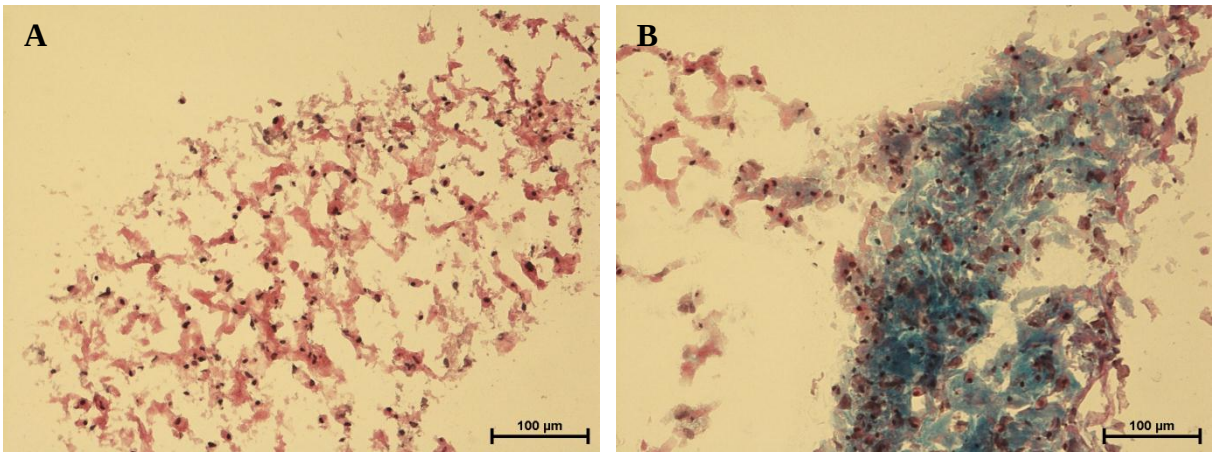

Figure 14. Alcian Blue van Gieson (ABvG) staining of sections of mesenchymal stem cells (MSC) from patient F82 cultured in the hydrogel HydroMatrix™ at different oxygen levels. Sulfated glycosaminoglycans (GAGs) are stained blue while collagens are stained red/pink and cell nuclei are stained black/brown. **A)** MSC cultured in HydroMatrix™ at 21% O_2. The hydrogel has a similar color as the collagen staining and therefore it cannot be said for certain if collagens are present. **B)** MSC cultured in HydroMatrix™ at 10% O_2. Sulfated GAGs are present at this oxygen level.

The cells cultured in the hydrogels were scored using the Bern scoring system. This system scores the cells by observing three parameters; darkness and uniformity of the Alcian blue stain, the cell distance and the cell morphology. However, cells cultured in the hydrogels could only be scored by studying one parameter; the darkness and uniformity of the Alcian

blue stain and thus an adjusted Bern score was given. This reason for this was because the hydrogel affected the cell distance in a way that the distance between the cells was not solely due to produced proteoglycans creating gaps between cells. The hydrogel also affected the third parameter, cell morphology, as the cells change their morphology when migrating in the hydrogel. Duplicates of each cell and hydrogel combination were harvested after 14 days and the duplicates were studied and given a joint score using the scoring system developed by Grogan et al. (Grogan et al., 2006). In the following table (**Table 4**), the adjusted Bern scores for the cells encapsulated in the hydrogels at different oxygen levels are presented with the maximum score of 3. A dark and uniform distribution of the Alcian blue stain results in a high adjusted Bern score indicating chondrogenesis. The only sample receiving a score above 0 was MSC from patient F82 cultured in HydroMatrix™ at 10% O_2.

Table 4. Adjusted Bern scores of mesenchymal stem cells (MSC) from three different patients cultured in the hydrogels HydroMatrix™ and PuraMatrix™ at two different oxygen levels; 21% O_2 and 10% O_2. The MSC cultured in the hydrogels were scored studying the darkness and uniformity of the Alcian blue stain. A dark and uniform distribution of the Alcian blue stain results in a high adjusted Bern score indicating chondrogenesis. The maximum score is 3.

Cell and hydrogel combination	**21% O_2**	**10% O_2**
MSC F64, HydroMatrix™	0	0
MSC F64, PuraMatrix™	0	0
MSC M70, HydroMatrix™	0	0
MSC M70, PuraMatrix™	0	0
MSC F82, HydroMatrix™	0	2
MSC F82, PuraMatrix™	0	0

SOX9 staining

MSC from the three patients were cultured in the hydrogels and thereafter sections of these were stained with an antibody for SOX9 to study chondrogenic differentiation. The results from the SOX9 staining of MSC from patient F82 cultured in PuraMatrix™ are presented in **Figure 15**. DAPI staining was performed and the cell nuclei are thus visualized blue. Yellow dots inside the cells indicate cells positive for SOX9. From **Figure 15A**, it can be seen that PuraMatrix™ is autofluorescent as the hydrogel gives a high fluorescence signal even though the primary antibody for SOX9 is omitted. In **Figure 15B**, MSC from patient F82 are cultured in PuraMatrix™ at 21% O_2 and stained with an antibody for SOX9. As the negative control showed that PuraMatrix™ is autofluorescent, it is not possible to evaluate and quantify the results from the SOX9 staining of MSC cultured in PuraMatrix™.

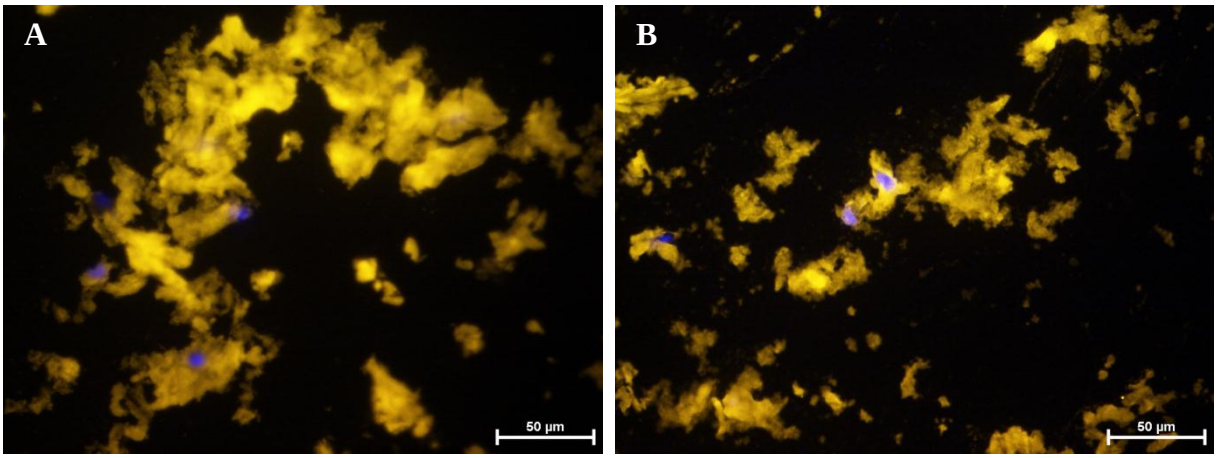

Figure 15. SOX9 and DAPI staining of sections of mesenchymal stem cells (MSC) cultured in PuraMatrix™. DAPI stains the cell nuclei blue while SOX9 can be visualized as yellow dots inside the cells. **A)** Negative control of SOX9 staining where MSC from patient F82 are cultured in PuraMatrix™ at 21% O_2 and the primary antibody for SOX9 is omitted. PuraMatrix™ is autofluorescent for SOX9 as the whole hydrogel is showing a high fluorescence signal. **B)** MSC from patient F82 cultured in PuraMatrix™ at 21% O_2. As the negative control showed a high fluorescence signal, it is not possible to evaluate the SOX9 staining of MSC cultured in PuraMatrix™.

The results of SOX9 staining of MSC from patient M70 cultured in HydroMatrix™ at the two different oxygen levels are presented in **Figure 16**. SOX9 can be visualized as yellow dots inside the cells while DAPI staining was performed to stain the cell nuclei blue. Cells positive for SOX9 are observed when cultured in HydroMatrix™ at 21% O_2 which can be seen in the magnified image in the upper right corner of **Figure 16B**. Cells positive for SOX9 are also observed cultured in HydroMatrix™ at 10% O_2 (**Figure 16C**). This can be compared to the negative control of SOX9 (**Figure 16A**) where MSC were cultured in HydroMatrix™ at 10% O_2 and the primary antibody for SOX9 is omitted.

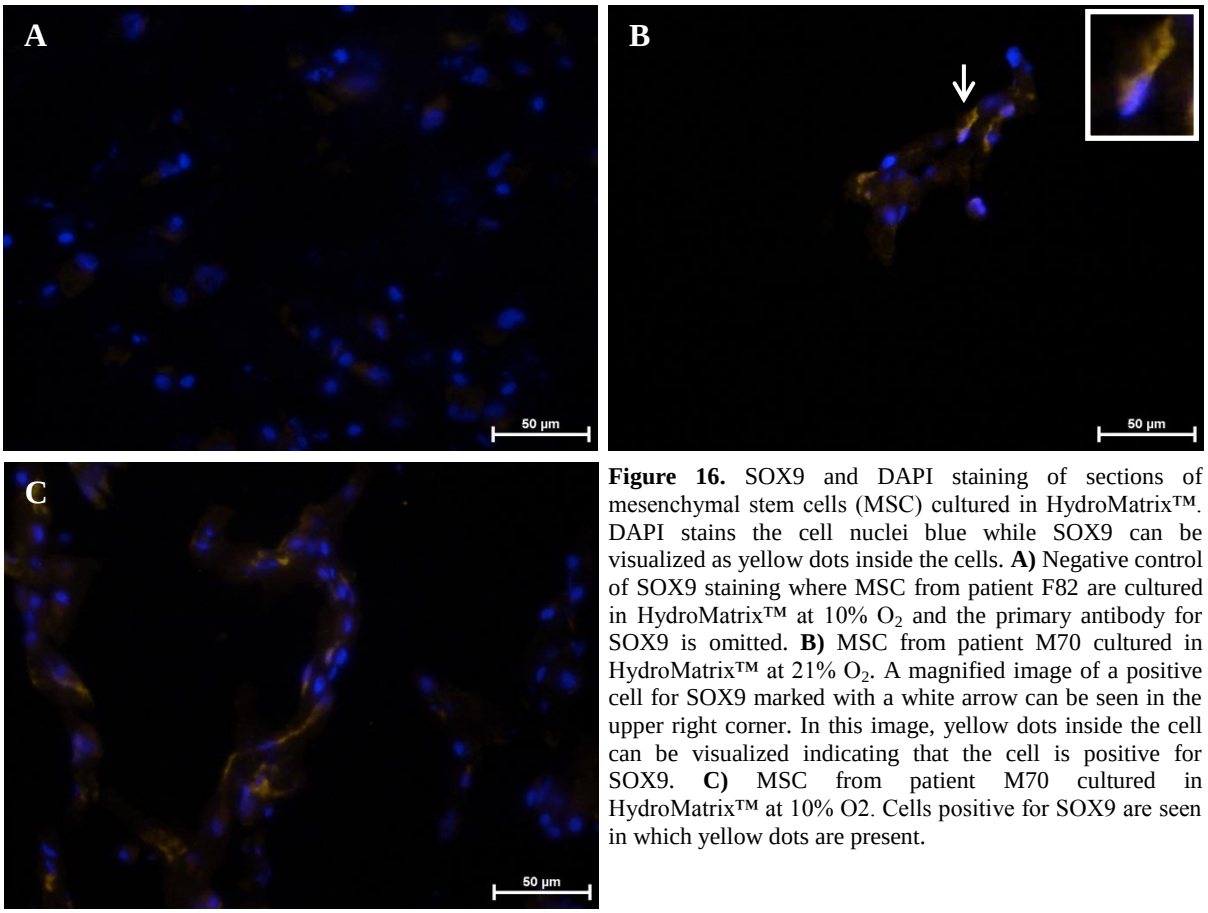

Figure 16. SOX9 and DAPI staining of sections of mesenchymal stem cells (MSC) cultured in HydroMatrix™. DAPI stains the cell nuclei blue while SOX9 can be visualized as yellow dots inside the cells. **A)** Negative control of SOX9 staining where MSC from patient F82 are cultured in HydroMatrix™ at 10% O_2 and the primary antibody for SOX9 is omitted. **B)** MSC from patient M70 cultured in HydroMatrix™ at 21% O_2. A magnified image of a positive cell for SOX9 marked with a white arrow can be seen in the upper right corner. In this image, yellow dots inside the cell can be visualized indicating that the cell is positive for SOX9. **C)** MSC from patient M70 cultured in HydroMatrix™ at 10% O2. Cells positive for SOX9 are seen in which yellow dots are present.

To semi-quantify the results of the SOX9 staining of cells cultured in HydroMatrix™, percentages of positive cells for SOX9 were calculated. This was done by studying the number of cells positive for SOX9 out of 100 randomly selected cells from two sections per sample. The percentages from each sample are presented in **Figure 17**. From this figure, it can be seen that cells positive for SOX9 were observed in all samples with the highest percentage observed for MSC from patient M70. For this patient, the highest percentage of cells positive for SOX9 was observed for cells cultured at 21% O_2 compared to 10% O_2. For MSC from patient F64, a higher percentage of positive cells was seen when cells were cultured at 10% O_2 compared to 21% O_2. For the cells from the third patient, F82, an equal percentage of positive cells was observed at the two different oxygen levels. From this figure, it can be seen that the number of cells positive for SOX9 decrease with increasing age of the patient when considering the cells cultured at 10% O_2.

Figure 17. Percentage of mesenchymal stem cells (MSC) cultured in HydroMatrix™ that are positive for SOX9. A total of 100 randomly selected cells are counted from two sections per sample and these are counted by observing the DAPI staining (cell nuclei staining) using fluorescence microscopy and NIS Elements Software. Cells that are positive for SOX9 are observed to have yellow dots inside the cells.

PCNA staining

Sections of cells cultured in the hydrogels were stained with an antibody for PCNA to study proliferation. Yellow dots inside the cells indicate cells positive for PCNA. DAPI staining was performed to stain cell nuclei blue. PuraMatrix™ was autofluorescent for PCNA (data not shown) and therefore it was not possible to evaluate and quantify the results of PCNA staining of MSC cultured in this hydrogel. HydroMatrix™ was also seen to be autofluorescent for PCNA. This was only seen in sections where the hydrogel was cultured with cells from patients F64 and M70 seen in **Figure 18**.

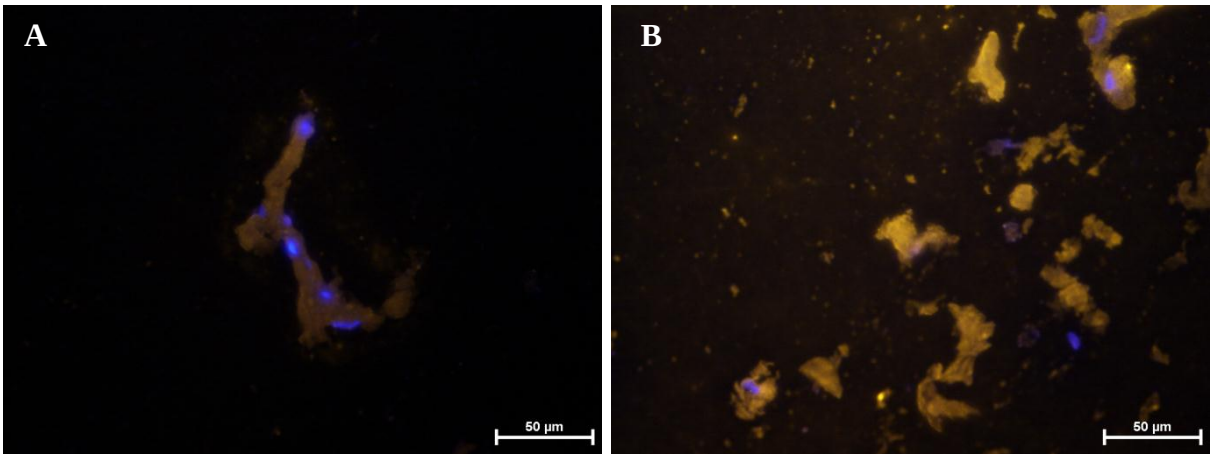

Figure 18. Proliferating cell nuclear antigen (PCNA) and DAPI staining of mesenchymal stem cells (MSC) cultured in HydroMatrix™. PCNA is visualized as yellow dots inside the cells while DAPI stains the cell nuclei blue. **A)** MSC from patient M70 cultured in HydroMatrix™ at 21% O2. The hydrogel is autofluorescent for PCNA and therefore it is not possible to evaluate the PCNA staining. **B)** MSC from patient F64 cultured in HydroMatrix™ at 10% O2. The hydrogel is autofluorescent for PCNA and therefore it is not possible to evaluate the PCNA staining.

Due to these results, MSC from patient F82 cultured in HydroMatrix™ at the two different oxygen levels were the only samples to be evaluated and semi-quantified in the PCNA study.

These results are presented in **Figure 19**. Cells positive for PCNA are seen in **Figure 19B** which presents MSC from patient F82 cultured in HydroMatrix™ at 21% O_2. The positive cells can be observed as having yellow dots present inside the cells. Cells positive for PCNA are also observed in **Figure 19C** in which MSC from patient F82 were cultured in HydroMatrix™ at 10% O_2. This can be compared to **Figure 19A** which is the negative control for PCNA in HydroMatrix™ in which the primary antibody is omitted.

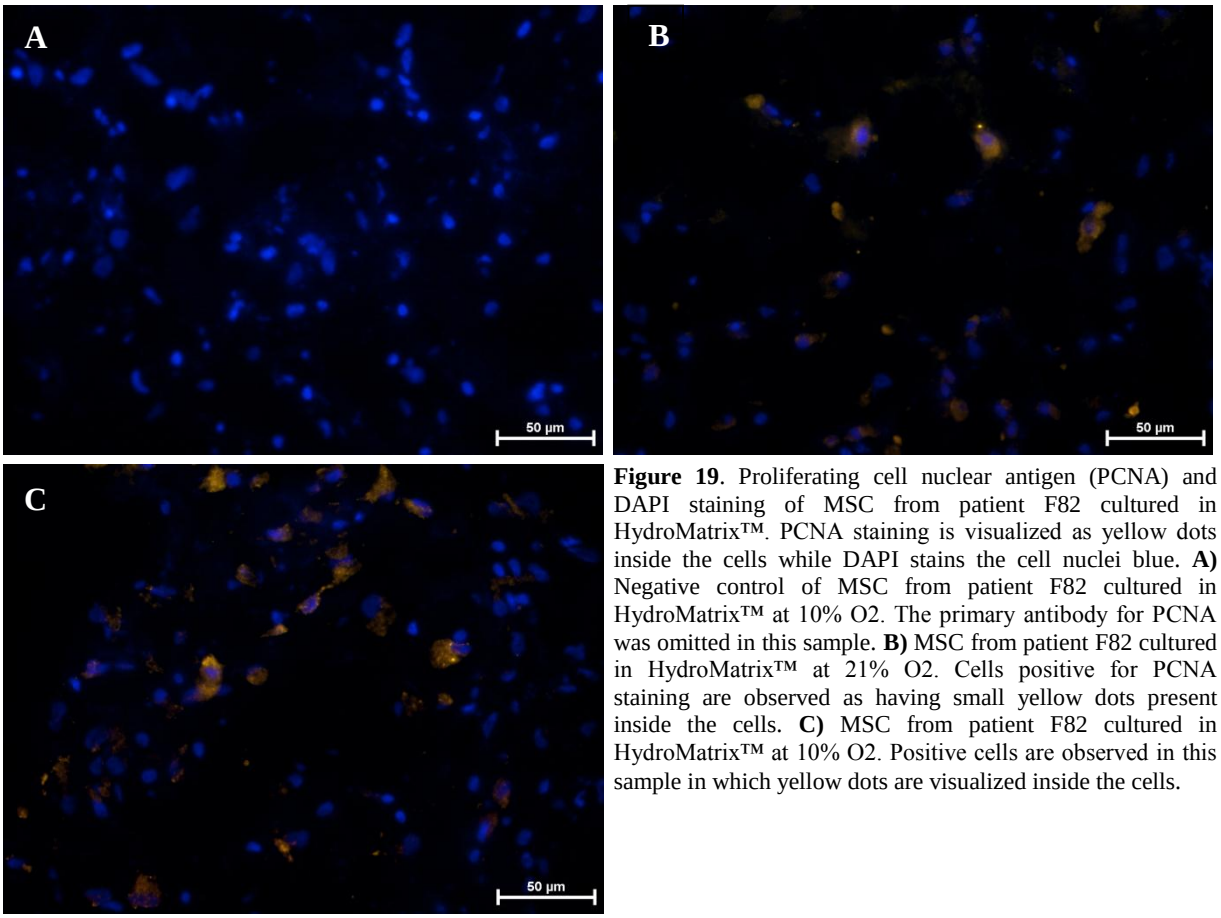

Figure 19. Proliferating cell nuclear antigen (PCNA) and DAPI staining of MSC from patient F82 cultured in HydroMatrix™. PCNA staining is visualized as yellow dots inside the cells while DAPI stains the cell nuclei blue. **A)** Negative control of MSC from patient F82 cultured in HydroMatrix™ at 10% O2. The primary antibody for PCNA was omitted in this sample. **B)** MSC from patient F82 cultured in HydroMatrix™ at 21% O2. Cells positive for PCNA staining are observed as having small yellow dots present inside the cells. **C)** MSC from patient F82 cultured in HydroMatrix™ at 10% O2. Positive cells are observed in this sample in which yellow dots are visualized inside the cells.

To semi-quantify the results of the PCNA staining, percentages of cells positive for PCNA were calculated for the two different samples; MSC from patient F82 cultured in HydroMatrix™ at 21% O_2 and 10% O_2 respectively. This was done by studying the number of cells positive for PCNA out of 100 randomly selected cells from two sections per sample. The percentages for the two different samples are presented in **Table 5**. A higher percentage of positive cells were observed when the cells were cultured at 10% O_2.

Table 5. Percentage of mesenchymal stem cells (MSC) cultured in HydroMatrix™ that are positive for PCNA. The percentages of cells positive for PCNA are calculated out of 100 randomly selected cells from two sections per sample. The 100 cells are counted by observing the DAPI staining (cell nuclei staining) using fluorescence microscopy and NIS Elements Software. Cells positive for PCNA are observed to have yellow dots inside the cells.

Sample	Percentage of cells positive for PCNA (%)
MSC from F82 in HydroMatrix™ at 21% O_2	22
MSC from F82 in HydroMatrix™ at 10% O_2	32

RT-PCR

RT-PCR was performed on all samples and the relative expressions of *SOX9* and c*ollagen IIA1* were studied to investigate the chondrogenic differentiation of MSC. In addition, the relative expression of *HIF-1α* was studied to investigate the response to the lowered oxygen level. Relative values were obtained using the $\Delta\Delta C_T$ method and the relative expression of the reference sample was set to 1 (Livak and Schmittgen, 2001). MSC from patient M70 cultured in PuraMatrix™ at 21% O_2 did not show expression of the two reference genes and was therefore excluded. If a gene in a sample obtained a C_T value in the range of 37-40 it was considered as barely detected (BD) while if it obtained a C_T value above 40 it was considered as not detected (ND).

SOX9

In **Figure 10**, the relative expressions of *SOX9* in the samples are presented. *SOX9* expression was observed in all HydroMatrix™ samples. In these samples, a higher relative expression of *SOX9* is observed in MSC from all three patients cultured at 21% O_2 compared to 10% O_2. In the PuraMatrix™ samples, a higher relative expression of *SOX9* is observed in MSC from patient F64 cultured at 21% O_2 compared to 10% O_2 (expression was not detected at 10% O_2). The opposite can be seen in MSC from patient F82 where the relative expression of *SOX9* is higher when the cells were cultured at 10% O_2 compared to 21% O_2 (expression was not detected at 21% O_2). *SOX9* expression is also observed in MSC from patient M70 cultured at 10% O_2.

By comparing the HydroMatrix™ and PuraMatrix™ samples in categories of same oxygen level and patient, it can be seen that a higher relative expression of *SOX9* is present in MSC from all three patients cultured in HydroMatrix™ compared to PuraMatrix™. This was seen at both oxygen levels.

Collagen IIA1

In **Figure 11**, the relative expressions of *collagen IIA1* in the samples are presented. In the HydroMatrix™ samples, it can be seen that a higher relative expression of *collagen IIA1* is present in MSC from patient F64 cultured at 10% O_2 compared to 21% O_2. The opposite is observed in MSC from patient F82 where a lower relative expression is present in the cells cultured at 10% O_2 compared to 21% O_2. In the MSC from the third patient, M70, the expression of *collagen IIA1* was not detected at either of the two oxygen levels. In the PuraMatrix™ samples, a much higher expression of *collagenIIA1* is observed in MSC from patient F64 cultured at 10% O_2 compared to 21% O_2 (**Figure 11**). In MSC from patient F82, expression of *collagen IIA1* was not detected at either of the oxygen levels. This was also the case for MSC from patient M70 where expression of *collagen IIA1* was not detected at 10% O_2 (MSC from patient M70 cultured in PuraMatrix™ at 21% O_2 was excluded).

By comparing the HydroMatrix™ and PuraMatrix™ samples in categories of same oxygen level and patient, it can be seen that a higher relative expression of *collagen IIA1* is seen in MSC from patient F64 cultured in PuraMatrix™ compared to HydroMatrix™. This was seen for both oxygen levels. The opposite is seen in MSC from patient F82 where a higher relative expression of the gene is present in cells cultured in HydroMatrix™ compared to

PuraMatrix™ (not detected in PuraMatrix™). This was observed at both oxygen levels. In MSC from patient M70, expression of *collagen IIA1* was not detected in either of the hydrogels.

HIF-1α

In **Figure 12**, the relative expressions of *HIF-1α* in the samples are presented. In the HydroMatrix™ samples, a higher relative expression of *HIF-1α* is present in MSC from patient F64 and F82 cultured at 21% O_2 compared to 10% O_2. The opposite is seen in MSC from patient M70 where a slightly higher expression of *HIF-1α* is observed in MSC cultured at 10% O_2 compared to 21% O_2. In the PuraMatrix™ samples, a higher relative expression of *HIF-1α* is present in MSC from patient F64 cultured at 21% O_2 compared to 10% O_2 (expression was barely detected at 10% O_2). The relative expressions of *HIF-1α* in the remaining PuraMatrix™ samples were barely detected as their corresponding C_T values ranged between 37 and 40.

pH experiment

The results of the viability staining that was used to study the survival of the MSC in HydroMatrix™ at different pH levels is presented in this section. MSC from patient F82 in passage 4 were cultured in HydroMatrix™ in growth media with different pH levels; 7.4, 7.1, 6.8 and 6.5. One setup of these was cultured at 10% O_2 to study the combined effect of a decreased pH and oxygen level. Another setup was cultured at 21% O_2 to only study the effect of pH on the survival of the MSC.

The results from the viability staining of cells cultured in HydroMatrix™ at different pH levels at 21% O_2 are presented in **Figure 20**. Some cells appear to fluoresce with a higher intensity than others which is due to that cells are located in different planes of the hydrogel. The results from the viability staining were not quantified as the cells were present in different planes of the hydrogel making the quantification difficult. The results indicate an increased presence of dead cells, which are visualized red, at the lower pH levels (**Figures 20C and D**) compared to pH 7.1 and 7.4 (**Figures 20A and B**). This was more clearly seen in the fluorescence microscope compared to in the images in **Figure 20**. In addition, the number of viable cells, which are visualized green, seems to decrease at the lower pH levels compared to pH 7.1 and 7.4. However, there are a number of viable cells present at pH 6.8 and 6.5.

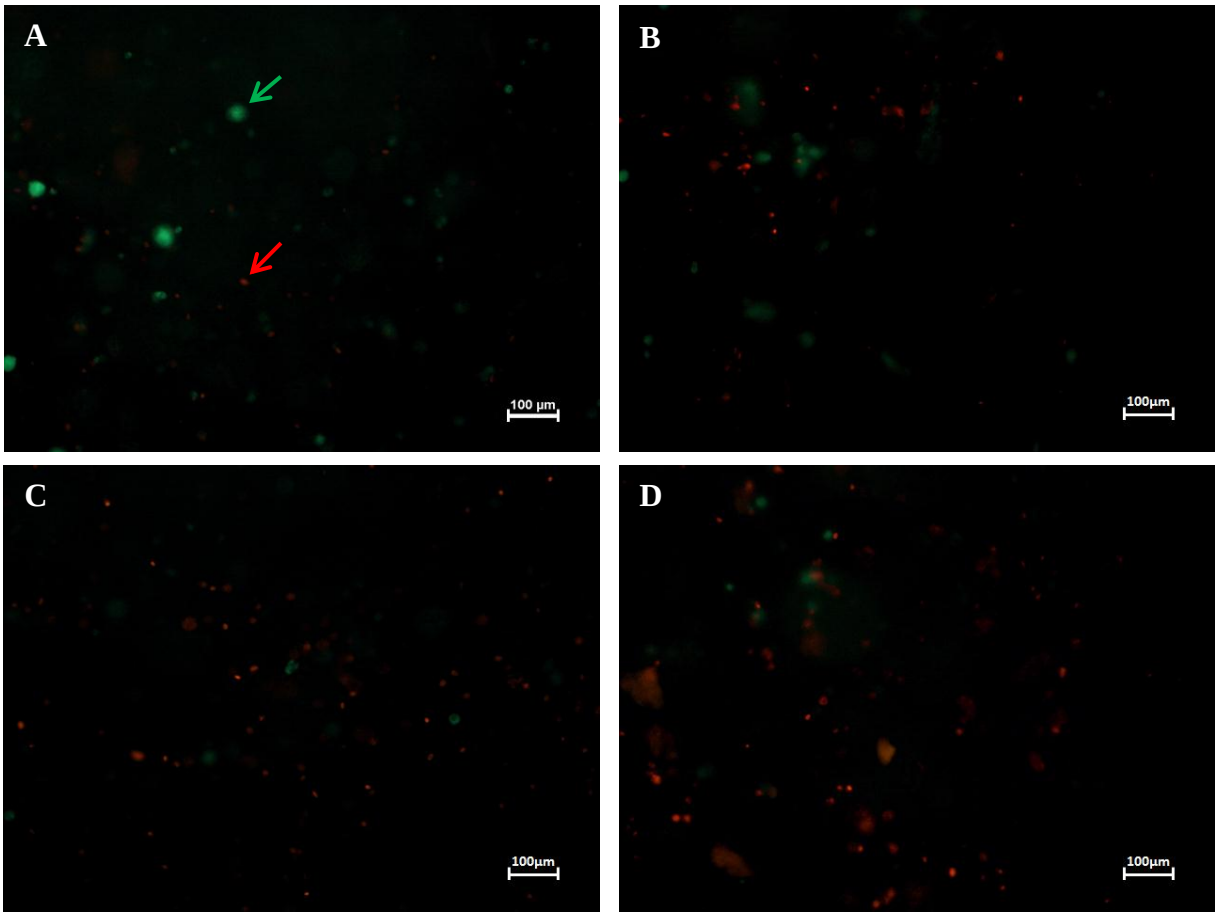

Figure 20. Viability staining of mesenchymal stem cells (MSC) cultured in HydroMatrix™ in media with different pH at 21% O_2 using a LIVE/DEAD® Cell Imaging Kit. Viable cells are visualized green and dead cells are visualized red by fluorescence microscopy. **A)** Visualization of cells cultured at pH 7.4 (standard cell culturing conditions) where viable and dead cells are observed. One viable cell is marked with a green arrow while one dead cell is marked with a red arrow. **B)** Visualization of cells cultured at pH 7.1 (normal IVD condition). Both viable and dead cells are observed. **C)** Visualization of cells cultured at pH 6.8 (mildly degenerated IVD condition) and both viable and dead cells are observed. **D)** Visualization of cells cultured at pH 6.5 (severely degenerated IVD condition) where viable and dead cells are observed.

The results from the viability staining of cells cultured in HydroMatrix™ at different pH levels at 10% O_2 are presented in **Figure 21**. As with the cells cultured at 21% O_2, the results indicate an increased presence of dead cells at the lower pH levels (**Figure 21C and D**) compared to pH 7.1 and 7.4. This was more clearly seen in the fluorescence microscope compared to in the images in **Figure 21**. It can also be seen that some MSC could survive at both pH 6.8 and pH 6.5. In the fluorescence microscope, it did not seem to be a large difference in the presence of viable and dead cells at pH 7.1 and 7.4 when comparing culturing the cells at 10% and 21% O_2. On the other hand, at the lower pH levels of 6.5 and 6.8 there seemed to be an increased presence of dead cells when cultured at 10% O_2. However, the total number of cells in **Figures 21C and D** also seem to be higher.

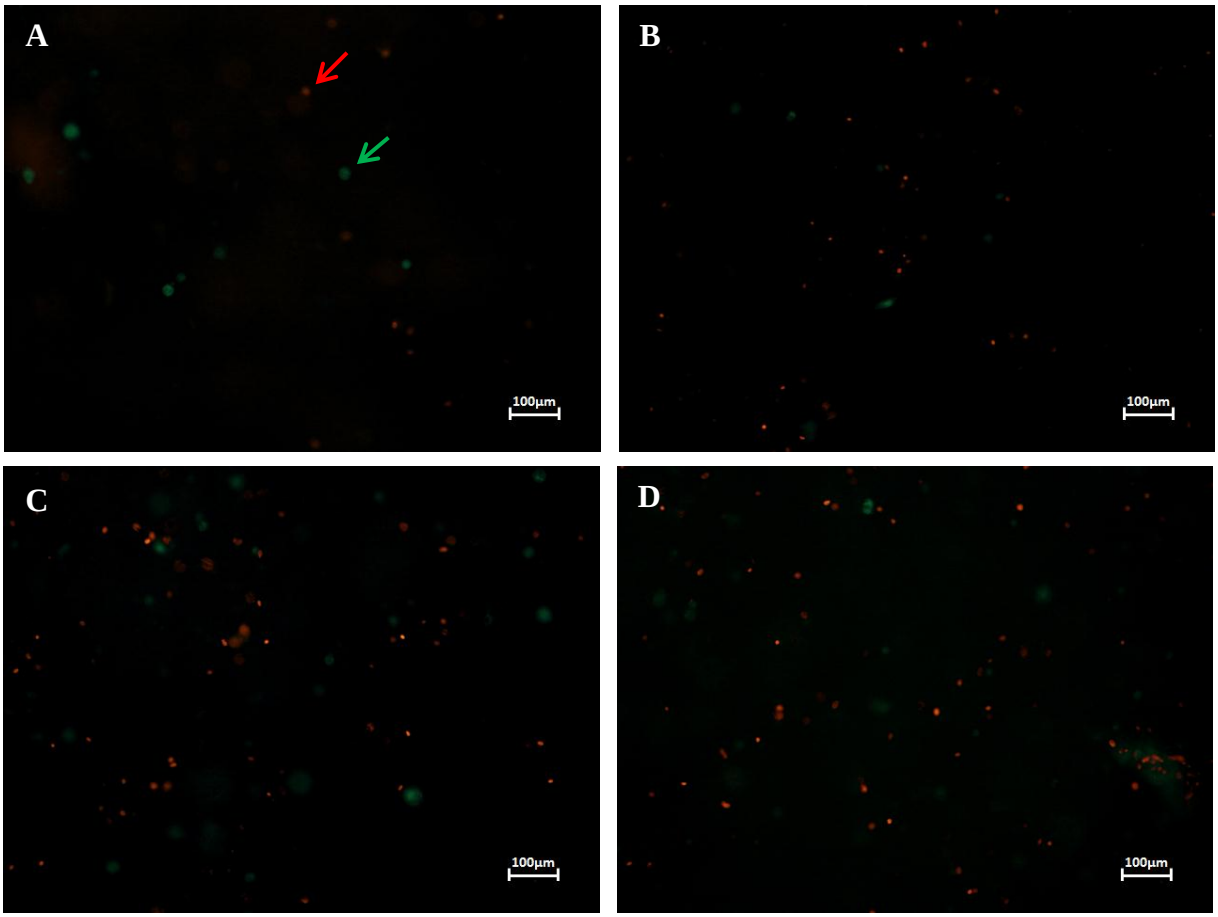

Figure 21. Viability staining of mesenchymal stem cells (MSC) cultured in HydroMatrix™ in growth media with different pH at 10% O_2 using a LIVE/DEAD® Cell Imaging Kit. Cells that are viable are visualized green while cells that are dead are visualized red by fluorescence microscopy. **A)** Visualization of **c**ells cultured at pH 7.4 (normal cell culturing condition). Both viable and dead cells are observed. One viable cell is marked with a green arrow while one dead cell is marked with a red arrow. **B)** Visualization of cells cultured at pH 7.1 (normal IVD condition) where viable and dead cells are observed. **C)** Visualization of cells cultured at pH 6.8 (mildly degenerated IVD condition) and both viable and dead cells are observed. **D)** Visualization of cells cultured at pH 6.5 (severely degenerated IVD condition) where viable and dead cells are observed.

Discussion

Current strategies for treating degenerated IVDs include physiotherapy often in combination with pain killers. However these treatments may not be so effective and surgical treatments could therefore be needed. The surgical approaches are very invasive and thus there is need for an approach which is minimally invasive. One potential strategy is to transplant hMSC together with a cell carrier into a degenerated disc to halt degeneration. The cell carriers studied in this thesis were the hydrogels HydroMatrix™ and PuraMatrix™. As the environment inside a degenerated IVD is hypoxic and acidic, the effects of a decreased oxygen level as well as a low pH on proliferation and chondrogenic differentiation of hMSC were studied in this thesis. These effects were evaluated using ABvG staining, IHC studying SOX9 and PCNA and RT-PCR studying the gene expression of *collagen IIA1*, *SOX9* and *HIF-1α*. In this section, the results from these studies are discussed and compared to other studies.

FACS analysis

In the results from the FACS analysis, it was observed that a high percentage of MSC from all three patients had an expression of CD105, CD166 and CD90. In addition, 95% of cells from all three patients lacked expression of the leukocyte marker CD45 and the hematopoietic marker CD34 (**Table 2**). From these results, it could be said that the vast majority of the cell populations in passage 3 were in fact MSC.

Pellet culture systems

In this section, the results of the RT-PCR, IHC studies and ABvG staining of the pellet culture systems are discussed.

Chondrogenic differentiation

Pellet mass systems were cultured as a control system for chondrogenesis (Johnstone et al., 1998). The expressions of two different chondrogenic markers were studied using RT-PCR; *SOX9* and *collagen IIA1*. SOX9 was in addition studied on a protein level by staining with an antibody for SOX9. The matrix production including the synthesis of collagen IIA1 and sulfated GAGs was studied by ABvG staining and graded using the Bern scoring system.

The ABvG stained sections of the cell pellets that were harvested after 14 days and 28 days, were scored using the Bern scoring system (**Table 3**). For the pellets systems, all three parameters were studied. From **Table 3**, it can be observed that the Bern score increases with increasing time in cells from two out of three patients (patients F82 and F64) indicating cells undergoing chondrogenesis over time. The pellets of MSC from the oldest patient, F64, harvested after 14 days received a score of 0 indicating that these cells are the least likely to be chondrocyte-like with a morphology resembling undifferentiated MSC. This suggests that the cells in these pellets were undifferentiated MSC. In addition, by studying the *SOX9* and *collagenIIA1* expressions for this particular sample it was observed that only very low expressions of *SOX9* and *collagen IIA1* were present (**Figures 10 and 11**). Specifically, the *SOX9* expression was lower in cells harvested after 14 days compared to 28 days while *collagen IIA1*expressionwas only slightly higher in cell pellets harvested after 28 days. This

could indicate that these cells differentiate slower into chondrocyte-like cells compared to cells from the two younger patients. Thus a higher *collagen IIA1* expression might have been observed at a later time point than day 14. A potential reason for the delayed chondrogenic differentiation in cells from patient F64 could be that this patient was the oldest of the three. An increased age of the donor seemed to affect the chondrogenic differentiation capacity negatively. In a study by Karlsson et al. in which MSC were cultured in pellet systems, the authors observed that an increased age of the donor correlated with a decreased ability for the MSC to produce matrix (Karlsson et al., 2007). This was also observed in another study in which MSC were cultured in pellet mass systems (Svanvik et al., 2010).

By further studying **Table 3**, it can be seen that the cell pellet from patient M70 harvested after 14 days received a higher Bern score than the corresponding cell pellet harvested after 28 days. The opposite was seen in cells from patients F82 and F64 as discussed above. This could be due to patient variation. However, a weak Alcian blue staining could be detected at day 14 as well as expressions of *SOX9* and *collagen IIA1* at both harvest days (**Figures 10 and 11**). This indicates chondrogenic differentiation of the MSC from patient M70.

The cell pellets receiving the highest Bern score were the cell pellets from the youngest patient, F82, harvested after 14 days and 28 days. This indicates that these cells were the most chondrocyte-like out of all cell pellets. This is supported by studying the *SOX9* and *collagen IIA1* expressions (**Figures 10 and 11**). From these figures, it can be seen that the highest relative expressions of *SOX9* and *collagen IIA1* were observed in cell pellets from patient F82 harvested after 14 days. As patient F82 was the youngest of the three patients, these cells could potentially have a better chondrogenic differentiation capacity (Karlsson et al., 2007) (Svanvik et al., 2010).

SOX9 was also studied on a protein level. SOX9 staining was performed on sections of cell pellets harvested after 14 days and 28 days (**Figure 8**). Cells positive for SOX9 were observed in all cell pellets at each time point indicating chondrogenic differentiation of MSC. The results from the SOX9 staining were not quantified and thus it cannot be said which time point had the highest number of cells positive for SOX9 present.

By comparing the relative expression of *collagen IIA1* between the harvest days, it could be seen that cell pellets from all three patients harvested after 14 days showed a higher relative expression of *collagen IIA1* than the respective cell pellets harvested after 28 days. As collagen IIA1 is a late marker for chondrogenesis but an early marker for ECM accumulation in the late stages of chondrogenesis, a possible explanation for this could be that the *collagen IIA1*gene is more expressed at day 14 when the cells are differentiating. Then at day 28 the gene expression has decreased while the collagen IIA1 protein level is high. This is as the gene expression is observed prior the protein expression. This can be supported by the results of the ABvG staining where collagens seem to be present at day 28 (**Figure 7B**). This indicates that the cells harvested after 28 days have reached a later stage in the chondrogenic differentiation process.

By comparing the *SOX9* expression between the harvest days (**Figure 10**), it can be seen that cell pellets from the two younger patients F82 and M70 harvested after 14 days showed a

higher relative expression of *SOX9* compared to the ones harvested after 28 days. This is in accordance with literature as SOX9 is an early marker for chondrogenesis and should thus be more expressed at earlier time points (Wright et al., 1995) (Ng et al., 1997) (Akiyama et al., 2002). The results of the ABvG staining, SOX9 staining and RT-PCR studying expressions of *collagen IIA1* and *SOX9* indicated that the cells from the three patients could undergo chondrogenic differentiation although it seemed to be delayed in cells from patient F64. The delayed chondrogenic differentiation of MSC from patient F64 could be seen in the ABvG staining in which no sulfated GAGs were observed in cell pellets harvested after 14 days. The delay could also be seen in the RT-PCR results of *SOX9* and *collagen IIA1* which showed very low expression of the genes in cell pellets harvested after 14 days.

Proliferation

To study proliferation of the cells in the pellet cultures, PCNA staining was performed on sections of cell pellets harvested after 14 days and 28 days. However, the negative control in which the primary antibody for PCNA had been omitted showed a high fluorescence signal and cells which were falsely positive for PCNA staining were visualized (**Figure 9**). This suggests that the PCNA staining procedure was not optimal for the pellet systems as it did not seem to work. Thus these results were not evaluated.

HIF-1α

RT-PCR was also used to study the expression of *HIF-1α* in the cell pellets (**Figure 12**) to observe how the gene was expressed in pellet cultures. *HIF-1α* expression was observed in all cell pellets. The pellets were cultured at 21% O_2 and thus the comparisons of the relative expression of *HIF-1α* were made between cell pellets harvested after 14 days and 28 days. A higher relative expression of *HIF-1α* was observed in cell pellets from the oldest patient, F64, harvested after 28 days compared to 14 days. The opposite trend was observed in cell pellets from patients F82 and M70 where a higher relative expression of *HIF-1α* was seen in pellets harvested after 14 days compared to 28 days. This correlates to the higher expressions of both *SOX9* and *collagen IIA1* observed after 14 days compared to 28 days in cell pellets from patients F82 and M70 (**Figures 10 and 11**). The opposite trend of *SOX9* expression was seen in cell pellets from patient F64. In addition, the relative *collagen IIA1* expression levels were very low in cell pellets from patient F64 where only a slightly higher expression was observed in pellets harvested after 14 days compared to 28 days. As the expressions of *SOX9*, *collagen IIA1* and *HIF-1α* seem to coincide, this could indicate that the *HIF-1α* gene is induced during chondrogenic differentiation. It could possibly be that the cells residing in the center of the pellet systems have a lower level of available oxygen and that more oxygen is needed during the chondrogenic differentiation process. This might induce the expression of *HIF-1α*. To the best of the author's knowledge, studies in which *HIF-1α* expression is investigated in hMSC cultured in pellet systems have not previously been performed.

Cells encapsulated in hydrogels

In this section, the results from the experiments in which MSC were cultured in the hydrogels are discussed.

Oxygen experiment

The results of the RT-PCR, IHC studies and ABvG staining of cells cultured at different oxygen levels in HydroMatrix™ and PuraMatrix™ are discussed in this section.

Chondrogenic differentiation

The expressions of two different chondrogenic markers were studied using RT-PCR; *SOX9* and *collagen IIA1*. SOX9 was in addition studied on a protein level by staining with an antibody for SOX9. The matrix production including the synthesis of collagen and sulfated GAGs was studied by ABvG staining and graded using the Bern scoring system.

By first studying the RT-PCR results of expression of *SOX9*, it could be seen that the relative expression of *SOX9* was higher in MSC cultured in HydroMatrix™ at 21% O_2 compared to 10% O_2 for all samples (**Figure 10**). As SOX9 expression is an early marker for chondrogenesis, this could suggest that cells cultured at 10% O_2 had a high expression of *SOX9* at a time point prior to day 14 and that the expression then decreased. *SOX9* expression was observed in all HydroMatrix™ samples indicating chondrogenic differentiation of MSC as undifferentiated MSC do not express SOX9 in high amounts.

In the PuraMatrix™ samples, a lower relative expression of *SOX9* was observed in MSC from patient F64 cultured at 10% O_2 compared to 21% O_2 (expression was not detected at 10% O_2). This follows the same trend as the HydroMatrix™ samples. However, the opposite was observed in MSC from patient F82 where the relative expression of *SOX9* was higher in cells cultured at 10% O_2 compared to 21% O_2 (expression was not detected at 21% O_2). The differences in the trend of *SOX9* expression in cells from the patients cultured in PuraMatrix™ could possibly be due to patient variation. *SOX9* expression was also observed in MSC from patient M70 cultured in PuraMatrix™ at 10% O_2 indicating chondrogenic differentiation of MSC. The RT-PCR results of the sample in which MSC from patient M70 was cultured in PuraMatrix™ at 21% O_2 did not show expressions of the reference genes and was thus excluded. A duplicate of this sample was also run in RT-PCR but no expressions of the reference genes were observed. A possible reason for this could be RNase contamination of the sample after the RNA had been extracted and the concentration had been measured. This reason is the most probable as RNA was measured and detected in both duplicates after the RNA extraction.

By finally comparing the *SOX9* expression in HydroMatrix™ and PuraMatrix™ samples in categories of same oxygen level and patient, it could be seen that a higher relative expression of *SOX9* was present in MSC from all three patients cultured in HydroMatrix™ compared to PuraMatrix™. This was seen at both oxygen levels. This could either indicate a higher induction of chondrogenesis in the HydroMatrix™ samples or that cells in the PuraMatrix™ samples are further along the chondrogenic differentiation process. The former explanation is the most probable as expression of *SOX9* was not detected for some of the PuraMatrix™ samples. In addition, by studying the *collagen IIA1* expressions it could be seen that expression was only detected in two out of five PuraMatrix™ samples (**Figure 11**).

SOX9 was also studied by IHC to observe if the *SOX9* expression had yielded the SOX9 protein. The results of this staining of MSC from the youngest patient, F82, cultured in HydroMatrix™ showed an equal percentage of cells positive for SOX9 at the two different oxygen levels (**Figure 17**). In MSC from the oldest patient, F64, cultured in HydroMatrix™, a higher number of cells positive for SOX9 were observed when cultured at 10% O_2 compared to 21% O_2. The opposite was observed in MSC from patient M70 cultured in Hydromatrix™ where a higher number of cells positive for SOX9 were seen when cultured at 21% O_2 compared to 10% O_2. The differences in results of SOX9 staining for the three patients are possibly due to patient variation. However, cells positive for SOX9 were present in all HydroMatrix™ samples indicating chondrogenic differentiation of MSC at both oxygen levels. From this figure, it could also be seen that the number of cells positive for SOX9 decreased with increasing age of the patient when considering the cells cultured at 10% O_2. This could be due to patient variation or it could indicate that the chondrogenic differentiation capacity decreases with increasing age as discussed previously. The results of SOX9 staining of MSC cultured in PuraMatrix™ showed that this hydrogel was autofluorescent for SOX9 (**Figure 15**). Thus cells positive for SOX9 could not be distinguished from the autofluorescence of the hydrogel and the results could not be evaluated or quantified. The autofluorescence of the PuraMatrix™ hydrogel could possibly be due its amino acids components. However, no references have been found to support an intrinsic fluorescence created by these specific amino acids components.

To study the production of proteoglycans and collagens in the hydrogels, ABvG staining was performed and the samples were graded using the Bern scoring system. Only one parameter could be used to score the cells cultured in the hydrogels as the hydrogel could affect the cell distance and morphology. Thus the darkness and uniformity of the Alcian blue stain was the only parameter studied. In MSC from the youngest patient, F82, cultured in HydroMatrix™ it could be observed that sulfated GAGs were only present at the lower oxygen level (10%) (**Figure 14**). This sample was the only sample receiving a Bern score above 0 out of all hydrogel samples (**Table 4**). It received a score of 2 with the maximum being 3. This indicates that the cells are chondrocyte-like and thus suggests chondrogenic differentiation of MSC. This is because MSC cannot produce high levels of sulfated GAGs and thus the result implies chondrogenic differentiation of MSC.

This suggests that a lower oxygen level of 10% either induces chondrogenic differentiation of MSC or that it accelerates the differentiation process so that a later stage is reached faster. This cannot be said for certain as only one time point (14 days) was considered in this thesis and the MSC cultured at 21% O_2 might synthesize sulfated GAGs after 14 days. The RT-PCR results of *SOX9* expression in these samples supports these results as a lower *SOX9* expression was observed at 10% O_2 compared to 21% O_2. This indicates, as mentioned earlier, that the cells might have had a higher *SOX9* expression prior to day 14 and then the expression decreased. This in turn suggests that cells cultured at 10% O_2 were further along the chondrogenic differentiation process. In a study performed by Stoyanov et al. hMSC were cultured in alginate beads at 2% O_2 (Stoyanov et al., 2011). The authors studied the expression levels of the chondrogenic markers aggrecan, collagen IIA1, and SOX9 after 7, 14 and 28

days of culture using quantitative RT-PCR. The cells were cultured in different media with different formulations. One of these formulations was chondrogenic medium containing TGF-β1 and dexamethasone. The authors could conclude that hypoxia promoted the differentiation of MSC into chondrocyte-like cells when cultured in chondrogenic medium. In another study conducted by Baumgartner et al. hMSC were embedded in a fibrin sealant and cultured at 3% O_2 (Baumgartner et al., 2010). The cells were cultured in a chondrogenic differentiation medium containing TGF-β1 and were harvested after 42 days. The authors performed RT-PCR studying the expression of *collagen IIA1* and histology studies including Alcian blue staining. Baumgartner et al. concluded that hypoxia induced the chondrogenic differentiation of hMSC.

As mentioned earlier, the presence of sulfated GAGs was only observed in MSC from the youngest patient, F82, cultured in HydroMatrix at 10% O_2 (**Table 4**). In the samples with MSC from the older two patients, the HydroMatrix™ hydrogel was not as intact and somewhat scattered. The same was observed in all the PuraMatrix™ samples where the hydrogel was very scattered. This could possibly be due to problems with sectioning of the hydrogels or the nature of the molecular bonds inside the hydrogel. The scattered appearance could have interfered with the ABvG staining which could be a reason for the absence of sulfated GAGs in these samples. Another possible reason for the lack of sulfated GAGs in the PuraMatrix™ samples could be that the cells are not located as close to each other in this hydrogel resulting in a decreased cell signaling between the cells. This could interfere with the signaling pathways that are needed to induce chondrogenesis of MSC (DeLise et al., 2000). All the hydrogel samples except MSC from the youngest patient, F82, cultured in HydroMatrix™ at 10% O_2 received a score of 0 as no sulfated GAGs were present. This indicated that MSC in these samples had not differentiated into chondrocyte-like cells after 14 days of culture. When considering the collagen staining, the hydrogels had a similar color as the collagens (red/pink) and thus it could not be said for certain whether collagens had been synthesized in the hydrogel samples or not.

The *collagen IIA1* expression was also studied with RT-PCR (**Figure 11**). The relative expression of *collagen IIA1* was observed to be higher in MSC from the youngest patient, F82, cultured in HydroMatrix™ at 21% O_2 compared to 10% O_2 although only very low expressions were present. By considering the ABvG staining of these samples in which sulfated GAGs were only present at 10% O_2, the results could indicate that cells cultured at 10% O_2 have had their peak in *collagen IIA1* expression prior to day 14 and are now synthesizing matrix. Therefore, a lower expression of *collagen IIA1* could suggest that the cells have matured further in the chondrogenic differentiation pathway. *SOX9* expression was also observed in this sample at both oxygen levels indicating chondrogenic differentiation of MSC. However in the study by Stoyanov et al., higher relative expressions of *collagen IIA1* and *SOX9* were observed in hMSC cultured in alginate beads at 2% O_2 compared to 20% O_2 after 14 days of culture in chondrogenic medium (Stoyanov et al., 2011). This trend in *collagen IIA1* expression was also seen in the study by Baumgartner et al. in which hMSC were embedded in a fibrin sealant and cultured in chondrogenic medium at 3% O_2 and 21% O_2 (Baumgartner et al., 2010). Baumgartner et al. could detect *collagen IIA1* at day 3 in cells

cultured at 3% O_2 while it was detected after 14 days in cells cultured at 21% O_2. A possible explanation for these deviating results could be that the MSC were cultured in different scaffolds. MSC cultured in HydroMatrix™ could result in a different gene expression pattern of *collagen IIA1* compared to culturing in alginate beads or fibrin sealant. This is as the scaffolds provide different environments for the MSC which affects the interactions between the MSC and the scaffold. This could in turn affect the chondrogenesis of MSC (Welter and Baskaran, 2012). To the best of the author's knowledge, other studies in which hMSC have been cultured in HydroMatrix™ at a decreased oxygen level have not been performed.

In MSC from the oldest patient, F64, cultured in HydroMatrix™, the relative expression of *collagen IIA1* was higher in cells cultured at 10% O_2 compared to 21% O_2. This was in accordance with the studies conducted by Stoyanov et al. and Baumgartner et al. *SOX9* expression was present at both oxygen levels in these samples however, sulfated GAGs were not observed in the samples. The gene expressions of *SOX9* and *collagen IIA1* in these samples at both oxygen levels could indicate chondrogenic differentiation of MSC. In cells from the third patient, M70, the expression of *collagen IIA1* was not detected at either of the two oxygen levels. A possible reason for the difference in the trend of *collagen IIA1* expression in cells from the three patients cultured in HydroMatrix™ could be patient variation.

In the PuraMatrix™ samples, a much higher relative gene expression of *collagen IIA1* was observed in MSC from the oldest patient, F64, cultured at 10% O_2 compared to 21% O_2 (**Figure 11**). This was also in accordance with the studies performed by Stoyanov et al. and Baumgartner et al. In the cells cultured at 21% O_2, the relative expression of *collagen IIA1* was lower than the expression observed for the corresponding cell pellet harvested after 14 days. On the other hand, in the cells cultured at 10% O_2, the relative expression of *collagen IIA1* was much higher than in the cell pellet. This shows indications of chondrogenic differentiation of MSC cultured in PuraMatrix™ at 10% O_2. However, taking the *SOX9* expression as well as the ABvG staining into consideration for this sample, it is not likely that the MSC are differentiating in this sample. In the cells from the two younger patients, *collagen IIA1* gene expression was not detected at either of the two oxygen levels. A possible reason for this could be that the cells have not yet reached the stage where SOX9 has been synthesized and can act as a transcription factor to induce expression of the *collagen IIA1* gene.

By finally comparing the relative *collagen IIA1* expression in the HydroMatrix™ and PuraMatrix™ samples in categories of same oxygen level and patient, it could be seen that a higher relative expression of *collagen IIA1* was observed in MSC from oldest patient, F64, cultured in PuraMatrix™ compared to HydroMatrix™. This was seen for both oxygen levels. This could suggest that cells cultured in Hydromatrix™ have had their peak in expression of *collagen IIA1* prior to day 14 and that it then decreased. This could possibly mean that cells cultured in HydroMatrix™ have matured further in the chondrogenic differentiation process than cells cultured in PuraMatrix™. The opposite trend is observed in MSC from the youngest patient, F82, where a higher relative expression of *collagen IIA1* was seen in HydroMatrix™ compared to PuraMatrix™ at both oxygen levels. However, the *collagen IIA1*

expression was not detected in the PuraMatrix™ samples which could indicate that these cells have not yet reached the stage where the *collagen IIA1* gene is induced by SOX9. In the cells from the third patient, M70, *collagen IIA1* expression was not detected in either of the hydrogels at both oxygen levels. The difference in trends of *collagen IIA1* expression could be due to patient variation.

hMSC have previously been cultured in PuraMatrix™. In a study performed by Florine et al. hMSC were cultured in PuraMatrix™ in medium containing TGF-β and dexamethasone (Florine et al., 2013). Sulfated GAGs as well as collagen types I and II were studied after 21 days of culture using toluidine blue dye staining and immunohistochemistry. It was observed that the sulfated GAG content increased after 21 days of culture compared to 14 days of culture in hMSC cultured in PuraMatrix™. This could possibly mean that if the duration of the experiment in this thesis had been extended beyond 14 days it could result in stronger indications of chondrogenic differentiation in the PuraMatrix™ hydrogel. In this thesis, no sulfated GAGs were observed in the PuraMatrix™ samples which could be due to the scattered appearance of the hydrogel. Bovine MSC have also been cultured in PuraMatrix™ (Kopesky et al., 2010). Kopesky et al. studied chondrogenic differentiation of the MSC by toluidine blue staining, immunohistochemistry and RT-PCR. The authors observed high gene expressions of *SOX9, collagen IIA1* and *aggrecan*. In addition, the sulfated GAG content increased over time in cells cultured in PuraMatrix™ indicating chondrogenic differentiation of MSC. In another study performed by Henriksson et al. hMSC were cultured in PuraMatrix™ and transplanted into injured discs of xenogenic porcine (Henriksson et al., 2009b). IHC studies and RT-PCR were conducted after 6 months and the authors could observe expression of chondrogenic markers including collagen IIA1, aggrecan and SOX9. This indicated chondrogenic differentiation of the MSC cultured in PuraMatrix™. As the sectioning of the PuraMatrix™ hydrogel was an issue in this thesis, the ABvG staining was difficult to assess. In addition, there was an issue with autofluorescence of the hydrogel and thus the protein level of SOX9 could not be studied. However, by observing the RT-PCR results it could be seen that expression of *SOX9* was detected in some PuraMatrix™ samples (**Figure 10**) indicating chondrogenic differentiation of the MSC. As discussed previously, stronger indications of chondrogenic differentiation might have been observed in the PuraMatrix™ samples if the duration of the experiment had been extended beyond 14 days. *Collagen IIA1* expression was detected in two of the five PuraMatrix™ samples also indicating chondrogenic differentiation of MSC in these samples (**Figure 11**).

Proliferation

PCNA staining of cells cultured in the hydrogels was performed to study proliferation. Cells positive for PCNA were observed in MSC from the youngest patient, F82, cultured in HydroMatrix™ at both oxygen levels (**Figure 19**). This indicates that the cells could proliferate in HydroMatrix™ at both oxygen levels. A higher number of cells positive for PCNA were observed in the samples cultured at 10% O_2 compared to 21% O_2 (**Table 4**). This suggests that more cells were proliferating when cultured at 10% O_2 compared to 21% O_2. Considering the results from the ABvG staining where sulfated GAGs were only observed at 10% O_2, there could be two potential reasons for a higher PCNA expression at 10% O_2. One

could be that there are both many proliferating as well as differentiating cells present at 10% O_2. Another reason could be that cells might have differentiated into fully matured chondrocytes at 10% O_2 and thereafter started to proliferate again, however as the cells were only cultured for 14 days the former explanation seems more likely.

The PCNA staining of MSC from the two older patients cultured in HydroMatrix™ showed an autofluorescence of the hydrogel for PCNA (**Figure 18**). A potential cause for this could be that the hydrogel had entwined and layered itself in stacks on top of each other resulting in thicker sections. A higher autofluorescence signal will therefore be seen compared to if only one stack or plane of the hydrogel was visualized in the microscope. Thus these samples were not evaluated. The PCNA staining of MSC from all three patients cultured in PuraMatrix™ showed an autofluorescence of the hydrogel for PCNA. Thus these results could not be evaluated. The autofluorescence of the PuraMatrix™ hydrogel could possibly be due to the amino acids it contains. However, no references have been found to support an intrinsic fluorescence created by these specific amino acids components.

Response to decreased oxygen level

In order to study whether the cells responded to the decreased oxygen level of 10%, the expression of *HIF-1α* was studied using RT-PCR. The results of *HIF-1α* expression in MSC cultured in the hydrogels are presented in **Figure 12**. In the HydroMatrix™ samples, a higher relative expression of *HIF-1α* was observed in MSC from patients F64 and F82 cultured at 21% O_2 compared to 10% O_2. In a study performed by Wagegg et al. bone marrow-derived hMSC were cultured at below 2% O_2 and at 18% O_2 for 14 days (Wagegg et al., 2012). By studying the RT-PCR results, the authors observed an increase in *HIF*-1α expression over time in cells cultured at 2% O_2 compared to cells cultured at 18% O_2. A possible explanation to observing the opposite trend in cells from two out of three patients in this thesis could be that an oxygen level of 10% is not low enough to induce the gene expression of *HIF-1α*. Another difference from the article by Wagegg et al. is that the cells were cultured in a hydrogel in this thesis which could possibly affect the response to the lowered oxygen level. However, the most probable reason is the difference in oxygen levels as the cells were cultured at 2% O_2 in the article. The trend seen in the article by Wagegg et al. was observed in cells from patient M70 where a slightly higher relative expression of *HIF-1α* was observed in cells cultured at 10% O_2 compared to 21% O_2. This could possibly be due to patient variation.

There have also been studies performed showing that the expression level of *HIF-1α* may not depend on the oxygen level (Wang et al., 1995) (Kallio et al., 1997). However in these studies, immortal cell lines including Hep 3B human hepatoma cells and HeLa cells were studied which are very different from normal cells as these are pathogenic and immortal. This could explain why the *HIF-1α* expression was observed to not be oxygen-dependent in these studies while the opposite was observed in the study by Wagegg et al.

In the PuraMatrix™ samples, a higher relative expression of *HIF-1α* was observed in MSC from patient F64 cultured at 21% O_2 compared to 10% O_2 (expression was barely detected at 10% O_2). In the remaining PuraMatrix™, expression of *HIF-1α* was barely detected as the C_T values of the gene ranged between 37 and 40 for these samples. A potential reason for this

could be that the PuraMatrix™ hydrogel affects the cells' response to the lowered oxygen level. The expression of *HIF-1α* could either have been speeded up so that the expression was high prior to day 14 or the expression was delayed. Another possible reason could be that chondrogenesis is not as upregulated in the PuraMatrix™ samples. As was seen in the pellet cultures, the *HIF-1α* expression seemed to coincide with high *SOX9* and *collagen IIA1* expressions suggesting that *HIF-1α* is more expressed during chondrogenic differentiation. However, *SOX9* and *collagenIIA1* expressions could be observed for some of the PuraMatrix™ samples indicating that the former explanation is more likely.

pH experiment

Viability staining using a LIVE/DEAD cell imaging kit was performed to study the survival of MSC from the youngest patient, F82, cultured in HydroMatrix™ at different pH and oxygen levels. The results from the viability staining of cells cultured at different pH levels at 21% O_2 indicated an increased presence of dead cells at the lower pH levels (pH 6.8 and 6.5) compared to pH 7.1 and 7.4 (**Figure 20**). The number of viable cells also seemed to decrease at the lower pH levels. This indicates that a decreased pH affects the survival of hMSC by resulting in a lower cell viability. This was also observed in a study by performed by Li et al. in which human adipose-derived MSC were cultured at the same four pH levels as in this thesis (Li et al., 2012). The viability of the human adipose-derived MSC decreased with decreasing pH level. This was also seen in a study performed by Wuertz et al. in which bone marrow-derived MSC from rats were cultured at the same four different pH levels as in this thesis (Wuertz et al., 2009). The authors observed that the viability of the bone marrow-derived MSC decreased with decreasing pH level. Viable cells could be seen at the lower pH levels but their morphology had changed showing a decreased cell size. As in the study by Wuertz et al., a number of viable cells were observed at pH 6.8 and 6.5 indicating that some hMSC could survive at these pH levels (**Figure 20**)**.** However, the morphology of these cells could not be observed in the images from the viability staining.

The results of the viability staining of MSC cultured at different pH levels at 10% O_2 indicate an increased presence of dead cells at the lower pH levels compared to pH 7.1 and 7.4 (**Figure 21**). It could also be seen that some hMSC could survive at both pH 6.8 and pH 6.5. This suggests that MSC would be able to survive at the pH levels of mildly (pH 6.8) and severely degenerated discs (pH 6.5) at a decreased oxygen level of 10%. However it cannot be said whether the morphology of the cells changed at the decreased pH levels.

By finally comparing the viability staining of cells cultured at different pH levels at 21% O_2 (**Figure 20**) and 10% O_2 (**Figure 21)** it could be seen that there was not a large difference in the presence of viable and dead cells at pH 7.1 and 7.4. This indicates that a decreased oxygen level of 10% does not affect the survival of MSC when cultured in HydroMatrix™ at pH 7.1 and 7.4. On the other hand, when considering the lower pH levels of 6.5 and 6.8, the results indicated an increased presence of dead cells when cultured at 10% O_2 compared to 21% O_2. However, the total number of cells in **Figures 21C and D** also seemed to be higher but quantification could not be performed due to the fact that the cells were located in different planes in the hydrogel. Thus from these results, it cannot be said whether the combination of a

decreased oxygen level of 10% and a decreased pH has a stronger negative effect on the survival of hMSC than a decreased pH alone.

In a study performed by Collins et al. human osteoarthritic chondrocytes (HOAC) were cultured in alginate beads at different oxygen levels at pH 7.2 and 6.2 (Collins et al., 2013). The authors observed a decreased cell viability in cells cultured at 2% O_2 in a medium with pH 6.2 after 4 days of culture. This decrease was not observed in cells cultured at 2% O_2 in medium with pH 7.2. However when the cells were cultured at 5% O_2, no decrease in cell viability was observed in medium with pH 6.2 compared to culturing in medium with pH 7.2. This suggests that there is a synergistic effect of an acidic pH and a decreased oxygen level on the cell viability when the oxygen level is below a certain threshold value. In the study by Collins et al. the threshold value was indicated to be below 5% O_2 and approximately 2% O_2. However, this specific value was observed for HOAC and thus it could be different for hMSC. Nonetheless, this could support the results from the pH experiment in this thesis as no clear difference in cell viability was observed between culturing MSC in HydroMatrix™ at 10% O_2 and 21% O_2 in media with acidic pH. As quantification of the pH experiment could not be performed in this thesis, it is difficult to state whether there was in fact a difference in cell viability between the two oxygen levels or not. However, a qualitative assessment was done and a clear difference could not be observed.

An important factor to consider when assessing the results of the pH experiment is the buffering capacity of the growth media. A buffered growth medium can buffer the pH back to 7.4 after some time. The equilibrium of the pH in the growth medium used was CO_2 dependent and it thus equilibrated in the incubator. Due to these factors, the pH of the different growth media was adjusted at the day of the experiment and the media was changed at day 3 with new medium with the respective pH level. However, it cannot be said for certain that the exact pH level was maintained through the 6 days of culture. To avoid the issues concerning the buffering capacity of growth medium, one can instead utilize growth medium which is CO_2 independent or a growth medium which is not buffered.

Summary

By FACS analysis, a high percentage of cells from all three patients were observed to express certain cell surface markers specific for MSC indicating that the vast majority of cells were in fact MSC. The control systems of chondrogenesis used were the pellet mass systems and the results from this indicated that MSC from all three patients could undergo chondrogenic differentiation. This was observed by studying the ABvG staining as well as the gene and protein expression of SOX9 and gene expression of *collagen IIA1*. An interesting observation seen in the results, using the different methods, was that the chondrogenic differentiation capacity seemed to decrease with increasing donor age. E.g. the chondrogenic differentiation of MSC from the oldest patient seemed to be delayed in the pellet system. This was observed in the ABvG staining as well as in the gene expressions of *SOX9* and *collagen IIA1*. In the hydrogel samples, this trend was not as prominent. However, the trend was seen in the IHC studies of SOX9 where the number of cells positive for SOX9 decreased with increasing age of the patient in cells cultured in HydroMatrix™ at 10% O_2.

By studying the hydrogel samples, it could be observed that MSC from the youngest patient could proliferate in the HydroMatrix™ hydrogel at both oxygen levels. More cells positive for PCNA were observed in the sample cultured at 10% O_2 compared to 21% O_2 indicating that more cells were proliferating when cultured at a decreased oxygen level. The cells from this patient could also undergo chondrogenic differentiation in HydroMatrix™ when cultured at 10% O_2 in chondrogenic medium. A lower oxygen level of 10% seemed to induce chondrogenic differentiation of MSC or it might accelerate the differentiation process so that a later stage was reached faster. The proliferation and chondrogenic differentiation of MSC was observed in IHC studies of PCNA and SOX9 as well as in the ABvG staining and RT-PCR results of *SOX9* and *collagen IIA1* expressions. In the PuraMatrix™ samples, indications of chondrogenic differentiation of MSC were observed when studying the *SOX9* and *collagen IIA1* expressions in some of the samples. If the duration of the experiment had been extended beyond 14 days, stronger indications might have been observed. However, the protein level of SOX9 could not be studied due to autofluorescence and there was an issue with the sectioning of the hydrogel which could have interfered with the ABvG staining.

Next, the cells' response to the oxygen level was studied by investigating the *HIF-1α* expression. In the pellet systems, which were all cultured at 21% O_2, a high *HIF-1α* expression seemed to coincide with high expressions of *SOX9* and *collagenIIA1*. This could indicate that cells undergoing chondrogenic differentiation in the pellet systems have an induction of the *HIF-1α* gene. In the HydroMatrix™ samples, the expected outcome was only observed in cells from one patient. This could potentially indicate that an oxygen level of 10% O_2 is not low enough to induce the *HIF-1α* gene. *HIF-1α* expression was only observed in one of the PuraMatrix™ samples while it was barely detected in the others. A possible explanation for this could be that the PuraMatrix™ hydrogel in some way affects the cells' response to the lowered oxygen level. Considering the *HIF-1a* expression in the pellet cultures where *HIF-1α* seemed to be induced during chondrogenic differentiation, another explanation could be that chondrogenesis is not upregulated as much in the PuraMatrix™ hydrogel, at least not during the 14 days of culture.

From the pH experiment in which MSC from the youngest patient were cultured in HydroMatrix™, a decreased pH seemed to affect the survival of MSC by resulting in a lower cell viability. From the experiment studying the combined effect of a decreased pH and an oxygen level of 10% O_2, it could not be said whether the combination had a stronger negative effect on the cell viability compared to a decreased pH alone. This is since quantification could not be performed due to the fact that the cells were located in different planes of the three-dimensional hydrogel. However, a qualitative assessment was done and a clear difference could not be observed. Viable MSC could be observed at pH 6.8 suggesting that some cells could be able to survive at a pH found in mildly degenerated discs at an oxygen level of 10%. However, the morphology of the viable cells was not assessed.

Conclusions

The chondrogenesis in the pellet systems seemed to be delayed in MSC from the oldest patient indicating that the chondrogenic differentiation capacity decreases with increasing donor age. By studying the *HIF-1α* expression in the pellet systems, the results indicated that cells undergoing chondrogenic differentiation in pellet systems have an induction of the *HIF-1α* gene.

At an oxygen level of 10%, MSC from the youngest patient could both proliferate and undergo chondrogenic differentiation when cultured in HydroMatrix™. More cells seemed to proliferate when cultured at a decreased oxygen level of 10% compared to 21% O_2. In the PuraMatrix™ samples, MSC cultured at 10% O_2 showed indications of chondrogenic differentiation.

In the pH experiment, the results indicated that a decreased pH results in a lower MSC viability. Some MSC from the youngest patient could survive at a pH level found in mildly degenerated discs at a decreased oxygen level of 10% O_2 when cultured in HydroMatrix™. However, it cannot be said whether the combination of a decreased oxygen level of 10% and a decreased pH has a stronger negative effect on the survival of MSC than a decreased pH alone.

Future studies

For future *in vitro* studies, a lower oxygen level than 10% needs to be studied to further mimic the environment found in degenerated IVDs. In addition, the chondrogenic differentiation of MSC when cultured in a combination of low pH and low oxygen level needs to be studied where the expression of chondrogenic markers are evaluated. This would give an indication to whether MSC would be able to survive in a mildly degenerated disc. In addition, the morphology of the cells needs to be assessed. This could be done by performing viability staining and imaging with higher magnification to be able to view a change in cell size.

Due to time limitations, the protein expressions of collagen IIA1 and HIF-1α were not studied and thus this should be included in future studies. In addition, more chondrogenic markers including aggrecan could be investigated both on a protein and genetic level. In order to observe clearer patterns in the expressions of the markers, a larger sample size is needed i.e. cells from more patients need to be studied. This would also make the effect of patient variation less prominent. These studies would first and foremost be conducted with cells cultured in HydroMatrix™ as this hydrogel seemed to be the better choice of the two. However, experiments with longer duration need to be performed in order to further evaluate the suitability of PuraMatrix™ as a potential cell carrier for transplantation of MSC into degenerated IVD.

To further mimic the *in vivo* environment, additional factors can be included in the *in vitro* culture. For instance MMPs can be included as these enzymes degrade ECM and a nutrient deficient environment can be created to mimic the *in vivo* situation. *In vivo* studies also need

to be performed as an *in vitro* environment cannot fully mimic the actual response of MSC cultured in a hydrogel at low pH and low oxygen level. In the *in vivo* situation, additional factors are to be studied, including the effect of mechanical loading.

An *in vivo* aspect that needs to be considered when developing cell therapy treatments for disc degeneration is the acidic pH. In healthy IVDs, the pH ranges between 7.0-7.2 while an acidic pH is found in degenerated IVDs (Li et al., 2012) (Razaq et al., 2003). The low pH is a consequence of a decreased nutrient supply to the degenerated IVD resulting in low oxygen and glucose concentrations. Urban et al. proposed that a method needs to be developed to characterize the nutrient supply in patients with degenerated IVDs. By this characterization, one could select patients with a proper nutrient supply to the IVD and treat the IVD degeneration using cell therapy strategies. This characterization and selection is needed as cells would not be able to survive in a degenerated IVD with a decreased nutrient supply (Urban et al., 2004). The low oxygen level inside the IVD may not be as harmful to the survival of the bone marrow-derived MSC compared to a low pH. This is as the oxygen tension inside bone marrow has been estimated to range between 1-7 % through modeling (Chow et al., 2001). However in a degenerated IVD there is combination of a decreased oxygen level and an acidic pH and thus the effect of this combination on the chondrogenic differentiation of MSC needs to be further studied.

References

3DM INC. *PuraMatrix Cell Recovery Protocol* [Online]. Available: http://puramatrix.com/protocol_pdfs/PuraMatrix_CellRecovery.pdf [Accessed 2014-05-02.

ADAMS, M. A., FREEMAN, B. J., MORRISON, H. P., NELSON, I. W. & DOLAN, P. 2000. Mechanical initiation of intervertebral disc degeneration. *Spine (Phila Pa 1976),* 25**,** 1625-36.

ADAMS, M. A., MCNALLY, D. S. & DOLAN, P. 1996. 'Stress' distributions inside intervertebral discs. The effects of age and degeneration. *J Bone Joint Surg Br,* 78**,** 965-72.

ADAMS, M. A. & ROUGHLEY, P. J. 2006. What is intervertebral disc degeneration, and what causes it? *Spine (Phila Pa 1976),* 31**,** 2151-61.

AKIYAMA, H., CHABOISSIER, M. C., MARTIN, J. F., SCHEDL, A. & DE CROMBRUGGHE, B. 2002. 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,* 16**,** 2813-28.

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. & WALTER, P. 2008. *Molecular Biology of the Cell,* New Tork, Garland Science: Taylor & Francis group.

ANDERSSON, G. B. 1999. Epidemiological features of chronic low-back pain. *Lancet,* 354**,** 581-5.

ANNUNEN, S., PAASSILTA, P., LOHINIVA, J., PERALA, M., PIHLAJAMAA, T., KARPPINEN, J., TERVONEN, O., KROGER, H., LAHDE, S., VANHARANTA, H., RYHANEN, L., GORING, H. H., OTT, J., PROCKOP, D. J. & ALA-KOKKO, L. 1999. An allele of COL9A2 associated with intervertebral disc disease. *Science,* 285**,** 409-12.

ASADA, M., SHINOMIYA, M., SUZUKI, M., HONDA, E., SUGIMOTO, R., IKEKITA, M. & IMAMURA, T. 2009. Glycosaminoglycan affinity of the complete fibroblast growth factor family. *Biochim Biophys Acta,* 1790**,** 40-8.

BASSOLS, A. & MASSAGUE, J. 1988. Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. *J Biol Chem,* 263**,** 3039-45.

BATTIE, M. C., VIDEMAN, T., KAPRIO, J., GIBBONS, L. E., GILL, K., MANNINEN, H., SAARELA, J. & PELTONEN, L. 2009. The Twin Spine Study: contributions to a changing view of disc degeneration. *Spine J,* 9**,** 47-59.

BATTULA, V. L., TREML, S., ABELE, H. & BUHRING, H. J. 2008. Prospective isolation and characterization of mesenchymal stem cells from human placenta using a frizzled-9-specific monoclonal antibody. *Differentiation,* 76**,** 326-36.

BAUMGARTNER, L., ARNHOLD, S., BRIXIUS, K., ADDICKS, K. & BLOCH, W. 2010. Human mesenchymal stem cells: Influence of oxygen pressure on proliferation and chondrogenic differentiation in fibrin glue in vitro. *J Biomed Mater Res A,* 93**,** 930-40.

BIBBY, S. R. S., JONES, D. A., LEE, R. B., YU, J. & URBAN, J. P. G. 2001. The pathophysiology of the intervertebral disc. *Joint Bone Spine,* 68**,** 537-542.

BRAVO, R., FEY, S. J., BELLATIN, J., LARSEN, P. M. & CELIS, J. E. 1982. Identification of a nuclear polypeptide ("cyclin") whose relative proportion is sensitive to changes in the rate of cell proliferation and to transformation. *Prog Clin Biol Res,* 85 Pt A**,** 235-48.

BUCHWALOW, I. B. 2010. *Immunohistochemistry: Basics and Methods* Springer.

BUCKWALTER, J. A. 1995. Aging and degeneration of the human intervertebral disc. *Spine (Phila Pa 1976),* 20**,** 1307-14.

BUSTIN, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol,* 25**,** 169-93.

BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem,* 55**,** 611-22.

CAWSTON, T., BILLINGTON, C., CLEAVER, C., ELLIOTT, S., HUI, W., KOSHY, P., SHINGLETON, B. & ROWAN, A. 1999. The regulation of MMPs and TIMPs in cartilage turnover. *Ann N Y Acad Sci,* 878**,** 120-9.

CHAMBERLAIN, G., FOX, J., ASHTON, B. & MIDDLETON, J. 2007. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells,* 25**,** 2739-49.

CHOW, D. C., WENNING, L. A., MILLER, W. M. & PAPOUTSAKIS, E. T. 2001. Modeling pO2 Distributions in the Bone Marrow Hematopoietic Compartment. I. Krogh's Model. *Biophysical Journal,* 81**,** 675-684.

CIAPETTI, G., GRANCHI, D., DEVESCOVI, V., LEONARDI, E., GREGGI, T., DI SILVESTRE, M. & BALDINI, N. 2012. Ex vivo observation of human intervertebral disc tissue and cells isolated from degenerated intervertebral discs. *Eur Spine J,* 21 Suppl 1**,** S10-9.

COLLINS, J. A., MOOTS, R. J., WINSTANLEY, R., CLEGG, P. D. & MILNER, P. I. 2013. Oxygen and pH-sensitivity of human osteoarthritic chondrocytes in 3-D alginate bead culture system. *Osteoarthritis and Cartilage,* 21**,** 1790-1798.

DANG, S. M., KYBA, M., PERLINGEIRO, R., DALEY, G. Q. & ZANDSTRA, P. W. 2002. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol Bioeng,* 78**,** 442-53.

DELISE, A. M., FISCHER, L. & TUAN, R. S. 2000. Cellular interactions and signaling in cartilage development. *Osteoarthritis and Cartilage,* 8**,** 309-334.

DENNIS, J. E., CARBILLET, J. P., CAPLAN, A. I. & CHARBORD, P. 2002. The STRO-1+ marrow cell population is multipotential. *Cells Tissues Organs,* 170**,** 73-82.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-7.

DORAK, M. T. 2006. *Real-time PCR* [Online]. Taylor & Francis.

DRAZIN, D., ROSNER, J., AVALOS, P. & ACOSTA, F. 2012. Stem cell therapy for degenerative disc disease. *Adv Orthop,* 2012**,** 961052.

DRURY, J. L. & MOONEY, D. J. 2003. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials,* 24**,** 4337-4351.

ESSERS, J., THEIL, A. F., BALDEYRON, C., VAN CAPPELLEN, W. A., HOUTSMULLER, A. B., KANAAR, R. & VERMEULEN, W. 2005. Nuclear dynamics of PCNA in DNA replication and repair. *Mol Cell Biol,* 25**,** 9350-9.

FLORINE, E. M., MILLER, R. E., PORTER, R. M., EVANS, C. H., KURZ, B. & GRODZINSKY, A. J. 2013. Effects of Dexamethasone on Mesenchymal Stromal Cell Chondrogenesis and Aggrecanase Activity: Comparison of Agarose and Self-Assembling Peptide Scaffolds. *Cartilage,* 4**,** 63-74.

GERECHT-NIR, S. & ITSKOVITZ-ELDOR, J. 2004. Cell therapy using human embryonic stem cells. *Transplant Immunology,* 12**,** 203-209.

GLICK, B. R., PASTERNACK, J. J. & PATTEN, C. L. 2009. *Molecular Biotechnology: Principles and Applications of Recombinant DNA,* ASM Press.

GROGAN, S. P., BARBERO, A., WINKELMANN, V., RIESER, F., FITZSIMMONS, J. S., O'DRISCOLL, S., MARTIN, I. & MAINIL-VARLET, P. 2006. Visual histological grading system for the evaluation of in vitro-generated neocartilage. *Tissue Eng,* 12**,** 2141-9.

GRUNHAGEN, T., WILDE, G., SOUKANE, D. M., SHIRAZI-ADL, S. A. & URBAN, J. P. 2006. Nutrient supply and intervertebral disc metabolism. *J Bone Joint Surg Am,* 88 Suppl 2**,** 30-5.

HARICHANDAN, A. & BUHRING, H. J. 2011. Prospective isolation of human MSC. *Best Pract Res Clin Haematol,* 24**,** 25-36.

HARICHANDAN, A. S., K; BURING H.J. 2012. Prospective Isolation and Characterization of Human Bone Marrow-Derived MSC. *In:* WEYAND, B. D., M; HASS, R; JACOBS, R; CASPER, C (ed.) *Mesenchymal Stem Cells- Basics and Clinical Applications I.* Springer Berlin Heidelberg.

HENRIKSSON, H., THORNEMO, M., KARLSSON, C., HAGG, O., JUNEVIK, K., LINDAHL, A. & BRISBY, H. 2009a. Identification of cell proliferation zones, progenitor cells and a potential stem cell niche in the intervertebral disc region: a study in four species. *Spine (Phila Pa 1976),* 34**,** 2278-87.

HENRIKSSON, H. B., SVANVIK, T., JONSSON, M., HAGMAN, M., HORN, M., LINDAHL, A. & BRISBY, H. 2009b. Transplantation of human mesenchymal stems cells into intervertebral discs in a xenogeneic porcine model. *Spine (Phila Pa 1976),* 34**,** 141-8.

HOLMES, T. C., DE LACALLE, S., SU, X., LIU, G., RICH, A. & ZHANG, S. 2000. Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. *Proc Natl Acad Sci U S A,* 97**,** 6728-33.

HOY, D., MARCH, L., BROOKS, P., BLYTH, F., WOOLF, A., BAIN, C., WILLIAMS, G., SMITH, E., VOS, T., BARENDREGT, J., MURRAY, C., BURSTEIN, R. & BUCHBINDER, R. 2014. The global burden of low back pain: estimates from the Global Burden of Disease 2010 study. *Ann Rheum Dis*.

HUANG, S. S. & HUANG, J. S. 2005. TGF-beta control of cell proliferation. *J Cell Biochem,* 96**,** 447-62.

IGNOTZ, R. A. & MASSAGUE, J. 1986. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem,* 261**,** 4337-45.

INNIS, M. A., GELFAND, D. H. & SNINSKY, J. J. 1995. *PCR Strategies,* Academic Press.

ISHIHARA, H. & URBAN, J. P. 1999. Effects of low oxygen concentrations and metabolic inhibitors on proteoglycan and protein synthesis rates in the intervertebral disc. *J Orthop Res,* 17**,** 829-35.

JOHNSTONE, B., HERING, T. M., CAPLAN, A. I., GOLDBERG, V. M. & YOO, J. U. 1998. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res,* 238**,** 265-72.

JONES, E. A., ENGLISH, A., KINSEY, S. E., STRASZYNSKI, L., EMERY, P., PONCHEL, F. & MCGONAGLE, D. 2006. Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow. *Cytometry B Clin Cytom,* 70**,** 391-9.

KAELIN, W. G., JR. & RATCLIFFE, P. J. 2008. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell,* 30**,** 393-402.

KALLIO, P. J., PONGRATZ, I., GRADIN, K., MCGUIRE, J. & POELLINGER, L. 1997. Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc Natl Acad Sci U S A,* 94**,** 5667-72.

KARLSSON, C., BRANTSING, C., SVENSSON, T., BRISBY, H., ASP, J., TALLHEDEN, T. & LINDAHL, A. 2007. Differentiation of human mesenchymal stem cells and articular chondrocytes: analysis of chondrogenic potential and expression pattern of differentiation-related transcription factors. *J Orthop Res,* 25**,** 152-63.

KAWAGUCHI, Y., OSADA, R., KANAMORI, M., ISHIHARA, H., OHMORI, K., MATSUI, H. & KIMURA, T. 1999. Association between an aggrecan gene polymorphism and lumbar disc degeneration. *Spine (Phila Pa 1976),* 24**,** 2456-60.

KELSEY, J. L., GITHENS, P. B., WHITE, A. A., 3RD, HOLFORD, T. R., WALTER, S. D., O'CONNOR, T., OSTFELD, A. M., WEIL, U., SOUTHWICK, W. O. & CALOGERO, J. A. 1984. An epidemiologic study of lifting and twisting on the job and risk for acute prolapsed lumbar intervertebral disc. *J Orthop Res,* 2**,** 61-6.

KOPESKY, P. W., VANDERPLOEG, E. J., SANDY, J. S., KURZ, B. & GRODZINSKY, A. J. 2010. Self-assembling peptide hydrogels modulate in vitro chondrogenesis of bovine bone marrow stromal cells. *Tissue Eng Part A,* 16**,** 465-77.

LAI, W. T., KRISHNAPPA, V. & PHINNEY, D. G. 2011. Fibroblast growth factor 2 (Fgf2) inhibits differentiation of mesenchymal stem cells by inducing Twist2 and Spry4, blocking extracellular regulated kinase activation, and altering Fgf receptor expression levels. *Stem Cells,* 29**,** 1102-11.

LE MAITRE, C. L., FREEMONT, A. J. & HOYLAND, J. A. 2006. Human disc degeneration is associated with increased MMP 7 expression. *Biotech Histochem,* 81**,** 125-31.

LE MAITRE, C. L., HOYLAND, J. A. & FREEMONT, A. J. 2007. Interleukin-1 receptor antagonist delivered directly and by gene therapy inhibits matrix degradation in the intact degenerate human intervertebral disc: an in situ zymographic and gene therapy study. *Arthritis Res Ther,* 9**,** R83.

LEFEBVRE, V., HUANG, W., HARLEY, V. R., GOODFELLOW, P. N. & DE CROMBRUGGHE, B. 1997. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol Cell Biol,* 17**,** 2336-46.

LEIST, M., BREMER, S., BRUNDIN, P., HESCHELER, J., KIRKEBY, A., KRAUSE, K. H., POERZGEN, P., PUCEAT, M., SCHMIDT, M., SCHRATTENHOLZ, A., ZAK, N. B. & HENTZE, H. 2008. The biological and ethical basis of the use of human embryonic stem cells for in vitro test systems or cell therapy. *ALTEX,* 25**,** 163-90.

LI, H., LIANG, C., TAO, Y., ZHOU, X., LI, F., CHEN, G. & CHEN, Q. X. 2012. Acidic pH conditions mimicking degenerative intervertebral discs impair the survival and biological behavior of human adipose-derived mesenchymal stem cells. *Exp Biol Med (Maywood),* 237**,** 845-52.

LIFE TECHNOLOGIES. 2014. *LIVE/DEAD® Cell Imaging Kit (488/570)* [Online]. Available: http://www.lifetechnologies.com/order/catalog/product/R37601 [Accessed 2014-04-04.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods,* 25**,** 402-408.

LUOMA, K., RIIHIMAKI, H., LUUKKONEN, R., RAININKO, R., VIIKARI-JUNTURA, E. & LAMMINEN, A. 2000. Low back pain in relation to lumbar disc degeneration. *Spine (Phila Pa 1976),* 25**,** 487-92.

MASSEY, C. J., VAN DONKELAAR, C. C., VRESILOVIC, E., ZAVALIANGOS, A. & MARCOLONGO, M. 2012. Effects of aging and degeneration on the human intervertebral disc during the diurnal cycle: a finite element study. *J Orthop Res,* 30**,** 122-8.

MASUDA, K. 2008. Biological repair of the degenerated intervertebral disc by the injection of growth factors. *Eur Spine J,* 17 Suppl 4**,** 441-51.

MCLAREN, A. 2001. Ethical and social considerations of stem cell research. *Nature,* 414**,** 129-31.

MELROSE, J., SMITH, S., LITTLE, C. B., KITSON, J., HWA, S. Y. & GHOSH, P. 2002. Spatial and temporal localization of transforming growth factor-beta, fibroblast growth factor-2, and osteonectin, and identification of cells expressing alpha-smooth muscle actin in the injured anulus fibrosus: implications for extracellular matrix repair. *Spine (Phila Pa 1976),* 27**,** 1756-64.

MINGUELL, J. J., ERICES, A. & CONGET, P. 2001. Mesenchymal stem cells. *Exp Biol Med (Maywood),* 226**,** 507-20.

MINTEER, D. M., K.G; RUBIN, P.J 2012. Adipose-Derived Mesenchymal Stem Cells: Biology and Potential Applications. *In:* WEYAND, B. D., M; HASS, R; JACOBS, R; CASPER, C (ed.) *Mesenchymal Stem Cells- Basics and Clinical Applications I.* Springer Berlin Heidelberg.

MIURA, Y., PARVIZI, J., FITZSIMMONS, J. S. & O'DRISCOLL, S. W. 2002. Brief Exposure to High-Dose Transforming Growth Factor-β1 Enhances Periosteal Chondrogenesis in Vitro A Preliminary Report. *The Journal of Bone & Joint Surgery,* 84**,** 793-799.

NG, L. J., WHEATLEY, S., MUSCAT, G. E., CONWAY-CAMPBELL, J., BOWLES, J., WRIGHT, E., BELL, D. M., TAM, P. P., CHEAH, K. S. & KOOPMAN, P. 1997. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev Biol,* 183**,** 108-21.

NOLAN, T., HANDS, R. E. & BUSTIN, S. A. 2006. Quantification of mRNA using real-time RT-PCR. *Nat Protoc,* 1**,** 1559-82.

OH, C. D., CHANG, S. H., YOON, Y. M., LEE, S. J., LEE, Y. S., KANG, S. S. & CHUN, J. S. 2000. Opposing role of mitogen-activated protein kinase subtypes, erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymes. *J Biol Chem,* 275**,** 5613-9.

PITTENGER, M. F., MACKAY, A. M., BECK, S. C., JAISWAL, R. K., DOUGLAS, R., MOSCA, J. D., MOORMAN, M. A., SIMONETTI, D. W., CRAIG, S. & MARSHAK, D. R. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science,* 284**,** 143-7.

PRESTA, M., DELL'ERA, P., MITOLA, S., MORONI, E., RONCA, R. & RUSNATI, M. 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev,* 16**,** 159-78.

PROKHOROVA, T. A., HARKNESS, L. M., FRANDSEN, U., DITZEL, N., SCHRODER, H. D., BURNS, J. S. & KASSEM, M. 2009. Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of Matrigel. *Stem Cells Dev,* 18**,** 47-54.

PURAMATRIX. *PuraMatrix™ Protocol 2-D Surface plating and 3-D Encapsulation in Cell Culture Plates* [Online]. Available: http://www.puramatrix.com/protocol_pdfs/PuraMatrix_2D3DMultiwell.pdf [Accessed 2014-03-19.

PURAMATRIX. 2013. *Clinical Trials* [Online]. Available: http://www.puramatrix.com/clinical_trials.html [Accessed 2014-05-27.

QIAGEN 2009. *QuantiTect Reverse Transcription Handbook*.

QUINTARELLI, G. & DELLOVO, M. C. 1965. The chemical and histochemical properties of Alcian blue. *Histochemie,* 5**,** 196-209.

RAZAQ, S., URBAN, J. P. & WILKINS, R. J. 2000. Regulation of intracellular pH by bovine intervertebral disc cells. *Cell Physiol Biochem,* 10**,** 109-15.

RAZAQ, S., WILKINS, R. J. & URBAN, J. P. 2003. The effect of extracellular pH on matrix turnover by cells of the bovine nucleus pulposus. *Eur Spine J,* 12**,** 341-9.

RISBUD, M. V., ALBERT, T. J., GUTTAPALLI, A., VRESILOVIC, E. J., HILLIBRAND, A. S., VACCARO, A. R. & SHAPIRO, I. M. 2004. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy. *Spine (Phila Pa 1976),* 29**,** 2627-32.

RISBUD, M. V., GUTTAPALLI, A., TSAI, T. T., LEE, J. Y., DANIELSON, K. G., VACCARO, A. R., ALBERT, T. J., GAZIT, Z., GAZIT, D. & SHAPIRO, I. M. 2007. Evidence for skeletal progenitor cells in the degenerate human intervertebral disc. *Spine (Phila Pa 1976),* 32**,** 2537-44.

ROBERTS, S., CATERSON, B., MENAGE, J., EVANS, E. H., JAFFRAY, D. C. & EISENSTEIN, S. M. 2000. Matrix metalloproteinases and aggrecanase: their role in disorders of the human intervertebral disc. *Spine (Phila Pa 1976),* 25**,** 3005-13.

ROBERTS, S., EVANS, H., TRIVEDI, J. & MENAGE, J. 2006. Histology and pathology of the human intervertebral disc. *J Bone Joint Surg Am,* 88 Suppl 2**,** 10-4.

SAKAI, D., MOCHIDA, J., YAMAMOTO, Y., NOMURA, T., OKUMA, M., NISHIMURA, K., NAKAI, T., ANDO, K. & HOTTA, T. 2003. Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc: a potential therapeutic model for disc degeneration. *Biomaterials,* 24**,** 3531-41.

SCHIZAS, C., KULIK, G. & KOSMOPOULOS, V. 2010. Disc degeneration: current surgical options. *Eur Cell Mater,* 20**,** 306-15.

SHANKAR, H., SCARLETT, J. A. & ABRAM, S. E. 2009. Anatomy and pathophysiology of intervertebral disc disease. *Techniques in Regional Anesthesia and Pain Management,* 13**,** 67-75.

SHI, Y. & MASSAGUE, J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell,* 113**,** 685-700.

SIEMINSKI, A. L., SEMINO, C. E., GONG, H. & KAMM, R. D. 2008. Primary sequence of ionic self-assembling peptide gels affects endothelial cell adhesion and capillary morphogenesis. *J Biomed Mater Res A,* 87**,** 494-504.

SIGMA-ALDRICH 2009. 3-D Culture Systems.

SIGMA-ALDRICH 2013. Material Safety Data Sheet: HydroMatrix™ Peptide Cell Culture Scaffold.

SIGMA-ALDRICH. 2014. *HydroMatrix™ Peptide Cell Culture Scaffold* [Online]. Available: http://www.sigmaaldrich.com/catalog/product/sigma/a6982?lang=en&region=SE.

SINGH, K., MASUDA, K., THONAR, E. J., AN, H. S. & CS-SZABO, G. 2009. Age-related changes in the extracellular matrix of nucleus pulposus and anulus fibrosus of human intervertebral disc. *Spine (Phila Pa 1976),* 34**,** 10-6.

STOPPOLONI, D., POLITI, L., DALLA VEDOVA, P., MESSANO, M., KOVERECH, A., SCANDURRA, R. & SCOTTO D'ABUSCO, A. 2013. L-carnitine enhances extracellular matrix synthesis in human primary chondrocytes. *Rheumatol Int,* 33**,** 2399-403.

STOYANOV, J. V., GANTENBEIN-RITTER, B., BERTOLO, A., AEBLI, N., BAUR, M., ALINI, M. & GRAD, S. 2011. Role of hypoxia and growth and differentiation factor-5 on differentiation of human mesenchymal stem cells towards intervertebral nucleus pulposus-like cells. *Eur Cell Mater,* 21**,** 533-47.

SVANVIK, T., HENRIKSSON, H. B., KARLSSON, C., HAGMAN, M., LINDAHL, A. & BRISBY, H. 2010. Human disk cells from degenerated disks and mesenchymal stem cells in co-culture result in increased matrix production. *Cells Tissues Organs,* 191**,** 2-11.

TAKAHASHI, M., HARO, H., WAKABAYASHI, Y., KAWA-UCHI, T., KOMORI, H. & SHINOMIYA, K. 2001. The association of degeneration of the intervertebral disc with 5a/6a polymorphism in the promoter of the human matrix metalloproteinase-3 gene. *J Bone Joint Surg Br,* 83**,** 491-5.

TALLHEDEN, T., KARLSSON, C., BRUNNER, A., VAN DER LEE, J., HAGG, R., TOMMASINI, R. & LINDAHL, A. 2004. Gene expression during redifferentiation of human articular chondrocytes. *Osteoarthritis Cartilage,* 12**,** 525-35.

THOMPSON, J. P., OEGEMA, T. R., JR. & BRADFORD, D. S. 1991. Stimulation of mature canine intervertebral disc by growth factors. *Spine (Phila Pa 1976),* 16**,** 253-60.

TOLONEN, J., GRONBLAD, M., VANHARANTA, H., VIRRI, J., GUYER, R. D., RYTOMAA, T. & KARAHARJU, E. O. 2006. Growth factor expression in degenerated intervertebral disc tissue. An immunohistochemical analysis of transforming growth factor beta, fibroblast growth factor and platelet-derived growth factor. *Eur Spine J,* 15**,** 588-96.

TSUTSUMI, S., SHIMAZU, A., MIYAZAKI, K., PAN, H., KOIKE, C., YOSHIDA, E., TAKAGISHI, K. & KATO, Y. 2001. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun,* 288**,** 413-9.

TULI, R., TULI, S., NANDI, S., HUANG, X., MANNER, P. A., HOZACK, W. J., DANIELSON, K. G., HALL, D. J. & TUAN, R. S. 2003. 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,* 278**,** 41227-36.

URBAN, J. P., MAROUDAS, A., BAYLISS, M. T. & DILLON, J. 1979. Swelling pressures of proteoglycans at the concentrations found in cartilaginous tissues. *Biorheology,* 16**,** 447-64.

URBAN, J. P. & ROBERTS, S. 2003. Degeneration of the intervertebral disc. *Arthritis Res Ther,* 5**,** 120-30.

URBAN, J. P., SMITH, S. & J.C., F. 2004. Nutrition of the Intervertebral Disc. *Spine,* 29**,** 2700-2709.

URBAN, J. P. G. & ROBERTS, S. 1995. Development and degeneration of the intervertebral discs. *Molecular Medicine Today,* 1**,** 329-335.

WAGEGG, M., GABER, T., LOHANATHA, F. L., HAHNE, M., STREHL, C., FANGRADT, M., TRAN, C. L., SCHONBECK, K., HOFF, P., ODE, A., PERKA, C., DUDA, G. N. & BUTTGEREIT, F. 2012. Hypoxia promotes osteogenesis but suppresses adipogenesis of human mesenchymal stromal cells in a hypoxia-inducible factor-1 dependent manner. *PLoS One,* 7**,** e46483.

WANG, B., WU, Y., SHAO, Z., YANG, S., CHE, B., SUN, C., MA, Z. & ZHANG, Y. 2012. Functionalized self-assembling peptide nanofiber hydrogel as a scaffold for rabbit nucleus pulposus cells. *J Biomed Mater Res A,* 100**,** 646-53.

WANG, G. L., JIANG, B. H., RUE, E. A. & SEMENZA, G. L. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A,* 92**,** 5510-4.

WEILER, C., NERLICH, A. G., ZIPPERER, J., BACHMEIER, B. E. & BOOS, N. 2002. 2002 SSE Award Competition in Basic Science: expression of major matrix metalloproteinases is associated with intervertebral disc degradation and resorption. *Eur Spine J,* 11**,** 308-20.

WELTER, J. F. S., L.A. & BASKARAN, H. 2012. Chondrogenesis from human mesenchymal stem cells: role of culture conditions. *In:* HAYAT, M. A. (ed.) *Stem cells and cancer stem cells.* Springer Inc.

WRIGHT, E., HARGRAVE, M. R., CHRISTIANSEN, J., COOPER, L., KUN, J., EVANS, T., GANGADHARAN, U., GREENFIELD, A. & KOOPMAN, P. 1995. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nat Genet,* 9**,** 15-20.

WUERTZ, K., GODBURN, K. & IATRIDIS, J. C. 2009. MSC response to pH levels found in degenerating intervertebral discs. *Biochemical and Biophysical Research Communications,* 379**,** 824-829.

YANG, F., LEUNG, V. Y., LUK, K. D., CHAN, D. & CHEUNG, K. M. 2009. Mesenchymal stem cells arrest intervertebral disc degeneration through chondrocytic differentiation and stimulation of endogenous cells. *Mol Ther,* 17**,** 1959-66.

ZANNETTINO, A. C., PATON, S., ARTHUR, A., KHOR, F., ITESCU, S., GIMBLE, J. M. & GRONTHOS, S. 2008. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J Cell Physiol,* 214**,** 413-21.

ZAVAN, B., CORTIVO, R. & ABATANGELO, G. 2009. Hydrogels and Tissue Engineering. *Hydrogels.* Springer Milan.

ZHANG, S., HOLMES, T., LOCKSHIN, C. & RICH, A. 1993. Spontaneous assembly of a self-complementary oligopeptide to form a stable macroscopic membrane. *Proc Natl Acad Sci U S A,* 90**,** 3334-8.

ZHANG, S., HOLMES, T. C., DIPERSIO, C. M., HYNES, R. O., SU, X. & RICH, A. 1995. Self-complementary oligopeptide matrices support mammalian cell attachment. *Biomaterials,* 16**,** 1385-93.

ZHANG, S. Z., X 2007. *Designed Self-assembling Peptide Nanobiomaterials* Springer.

ZHANG, S. Z., X; ELLIS-BEHNKE, R; SPIRIO, L 2006. *PuraMatrix: Self-assembling Peptide Nanofiber Scaffolds. In "Scaffolding in Tissue Engineering",* Boca Raton, FL, CRC Press.

ZVAIFLER, N. J., MARINOVA-MUTAFCHIEVA, L., ADAMS, G., EDWARDS, C. J., MOSS, J., BURGER, J. A. & MAINI, R. N. 2000. Mesenchymal precursor cells in the blood of normal individuals. *Arthritis Res,* 2**,** 477-88.