Cartilage Tissue Engineering;
the search for chondrogenic progenitor cells
and associated signalling pathways

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To my family

ABSTRACT

The socioeconomic cost of articular cartilage related diseases in the Western world is very high. The suffering of the individual patient is even more problematic. Different methods are used today to treat this large, heterogeneous group of patients, one of which has been in use for more than 20 years: cell based autologous chondrocyte implantation (ACI). Irrespective of treatment method, a great deal remains to be done to improve our knowledge of what occurs at molecular and cellular levels. The overall aim of this thesis was therefore to find chondrocytes with stem cell properties in cartilage used for ACI, and to study the associated molecular signalling pathways.

The helix-loop-helix (HLH) transcription factor Id1 was demonstrated to play a role in regulating proliferation of cultured human articular chondrocytes, indicating a role for the family of HLH proteins in chondrocytes. Human articular chondrocytes cultured in agarose suspension demonstrated subpopulations with different growth potential. Some showed mesenchymal stem cell (MSC) properties. To be able to locate potential stem cells *in vivo,* a rabbit BrdU model, identifying slow cycling cells was used. Stem cells were not only identified in the articular cartilage but also in the groove of Ranvier located in the periphery of the epiphyseal growth plate. The groove of Ranvier also exhibited properties as a stem cell niche structure. Further biopsies from human normal articular cartilage, as well as regenerated and repaired cartilage after ACI were studied. The human normal articular cartilage demonstrated expression of the stem cell associated markers STRO-1 and Bcrp1 in cells in the superficial zone, and activity of the fundamental Wnt (Wingless-related proteins) and Notch signalling pathways. The distribution showed a distinct zonal pattern in the normal cartilage. In biopsies from regenerated cartilage with almost normal histological architecture, the markers and pathways studied demonstrated a distinct zonal pattern similar to that in normal cartilage. Biopsies taken from repaired cartilage with more or less fibrocartilage formation and a disorganized matrix, showed increased Stro-1 expression and activity for the Wnt pathway throughout the biopsies. The supposed stem cell marker Bcrp1 was expressed in a sparse population of cells independently of cartilage tissue studied.

This thesis demonstrates that in articular cartilage there are subpopulations of cells with mesenchymal stem cell properties, and that it is possible to identify and select these populations for further study of their properties as stem cells and their usefulness for transplantation. The HLH, Wnt- and Notch signalling pathways are closely involved in articular cartilage regeneration and repair. The stem cells and signalling pathways may represent potential drug targets or valuable tools in the tissue engineering of joint tissue.

Key words: stem cell, progenitor cell, tissue engineering, articular cartilage, chondrocytes
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Populärvetenskaplig sammanfattning på svenska

Broskskador är ett vanligt problem och får inte sällan stora konsekvenser för den enskilda individen, som begränsad rörlighet, kronisk smärta och sänkt livskvalitet. På sikt kan broskskadorna leda till en utveckling av osteoartros (ledsvikt) och bli till ett stort handikapp för patienten. Genom åren har olika behandlingsmetoder för skador i ledbrosket använts; olika implantat, borrning och i vissa utvalda fall även transplantation av patientens egna broskceller. Ingen av dessa metoder har varit helt tillfredsställande då brosk är en vävnad som är mycket svår att reparera. Förklaringar har varit att det inte finns god blodförsörjning till broskvävnaden och att det är en cellfattig vävnad. Den sannolikt viktigaste faktorn som angetts har varit att brosket till synes inte har några vuxna stamceller som kan reparera uppkommen skada.

Detta avhandlingsarbete har visat att brosk hos vuxna innehåller celler med olika förmåga att dela sig och där en liten andel av cellerna uppvisar samma egenskaper som riktiga s.k. mesenkymala stamceller vilket ger bättre förutsättningar för brosket att delta i en reparationsprocess. Vi har också kunnat se var dessa celler med god förmåga att dela sig och potential att bilda nya celler finns i leden. De finns både i vissa delar av ledbrosket men också i ett område som kallas perikondrieringen som ligger strax nedom leden. Vissa primitiva reglersystem som är tätt kopplade till normal broskutveckling hos fostret har i vår studie visat sig att vara i obalans när brosket hos vuxna är drabbat av skada men också vid sjukdom såsom osteoartros.

Sammanfattningsvis kan man säga med detta avhandlingsarbete, att det finns populationer av celler i brosk hos vuxna som har potential att reparera skador med framgång. Med denna kunskap tillsammans med ökad förståelse för de involverade reglersystemen i reparation och regeneration av broskvävnad kan vi i första hand förbättra nuvarande metoder som vi har idag för att laga broskskador men också på sikt utveckla nya metoder som är både enklare och fungerar bättre än dagens metoder.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Julia Asp*, **Maria Thornemo***, Sven Inerot, Anders Lindahl. The helix-loop-helix transcription factors Id1 and Id3 have a functional role in control of cell division in human normal and neoplastic chondrocytes. *FEBS Letters* 1998;438: 85-90. *These authors contributed equally and should both be considered first authors.

II. Brittberg M, Sjögren-Jansson E, **Thornemo M**, Faber B, Tarkowski A, Peterson L, Lindahl A. Clonal growth of human articular cartilage and the functional role of the periosteum in chondrogenesis. *Osteoarthritis Cartilage* 2005;Feb;13(2):146-53.

III. **M Thornemo**, T Tallheden, E Sjögren Jansson, A Larsson, K Lövstedt, U Nannmark, M Brittberg, A Lindahl. Clonal populations of chondrocytes with progenitor properties identified within human articular cartilage. *Cells Tissues Organs* 2005;180(3): 141-50.

IV. Camilla Karlsson*, **Maria Thornemo***, Helena Barreto Henriksson, Anders Lindahl. Identification of a stem cell niche in the zone of Ranvier. An experimental study in the rabbit. *Submitted to Journal of Anatomy, under revision.* *These authors contributed equally and should both be considered first authors.

V. **Thornemo M**, Henriksson BH, Karlsson C, Concaro S, Stenhamre H, A Lindahl. Expression of the stem cell associated proteins Stro-1 and Bcrp1 and the Wnt and Notch signalling pathways in regenerated and repaired human articular cartilage. *Manuscript.*

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LIST OF ABBREVIATIONS

ACI	autologous chondrocyte implantation
ACS	adult stem cells
ACT	autologous chondrocyte transplantation
Bcrp1	breast cancer resistance protein 1
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
bp	base pairs
BrdU	5-bromo-2-deoxy-uridine
CD105	Endoglin
CD166	Alcam
CDMP-1	Cartilage-derived morphogenetic protein 1, same as GDF5
cDNA	complementary deoxy ribonucleic acid
COMP	Cartilage oligomeric protein
D	Differentiated cell cluster
3D	three-dimensional
DAPI	4´.6-Diamidino-2-phenylindole
DM	Differentiated Matrix cell cluster
DMEM	Dulbeccos modified eagle medium
DMEM-HG	Dulbeccos modified eagle medium-high glucose
DNA	deoxy ribonucleic acid
E12	Splice variant of the E2-alpha gene
E47	Splice variant of the E2-alpha gene
ECM	extracellular matrix
ESC	embryonic stem cells
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FSC	fetal stem cells
GADPH	glyceraldehyde-3-phosphate
GAG	glucosaminoglycan
GDF5	growth and differentiation factor 5
GM-CSF	granulocyte-monocyte colony-stimulating factor
H	Homogenous cell cluster
HA	hyaluronic acid
HES	Hairy and enhancer of split
HLH	helix-loop-helix
HM	Homogenous Matrix cell cluster
HRP	horseradish peroxidase
ICRS	International Cartilage Repair Society
ID	inhibitor of differentiation/ inhibitor of DNA binding

IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
ITS	insulin transferrin selenium
kb	kilo bases
kDa	kilo Dalton
LGT	low gel temperature agarose
MMPs	matrix metalloproteinases
MR	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
N-cadherin	Neural cadherin
N-CAM	Neural cell adhesion molecule
OA	osteoarthritis
PBS	phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	polymerase chain reaction
RBP-J	recombinant recognition sequence binding protein at the J kappa site
RNA	ribonucleic acid
Rnasin	ribonuclease inhibitor
RPA	ribonuclease protection assay
RT-PCR	reverse transcription PCR
18S	18S ribosomal RNA
SP	Side Population
SLG	standard low melting point agarose
Sox9	SRY-box containing gene 9
SRY	sex-determining region of the Y-chromosome
Tac	Thermus aquaticus
TEM	transmission electron microscopy
TGF-β	Transforming growth factor-β
TSA	tyramide signal amplification
Wnt	Wingless-related proteins
Wnt9A	Wingless-related protein type 9A, same as Wnt-14
Wnt-14	Wingless-related protein type 14

INTRODUCTION

The development of the vertebrate limb is a complex process dependent on the interaction of various factors, including growth factors, morphogens, transcription factors, extracellular matrix, and intricate signalling between these pathways. Although the adult articular cartilage is seemingly a morphological non-complex tissue, the understanding of the intricate interplay between the factors mentioned in adult cartilage is a puzzle necessary to understand. Articular cartilage defects are a common problem in the Western world and represent high medical costs (McGowan, 2003). William Hunter, a Scottish physician, is quoted as saying to the Royal Society as early as 1743: "From Hippocrates to the present age, it is universally allowed that ulcerated cartilage is a troublesome thing and that, once destroyed, is not repaired" (Hunter, 1743). Today the articular cartilage remains a troublesome thing thought to be post-mitotic tissue, with virtually no cellular turnover, and the process of repair and regeneration of articular cartilage also remains a challenge to scientists. In the field of regenerative medicine and tissue engineering the patterns and complex relationships between the signalling pathways and the existence of potential progenitor cells needs to be further understood to be able to regenerate adult articular cartilage tissue in the twenty-first century.

This thesis aims to give a deeper understanding of the chondrocytes in adult articular cartilage, from a molecular and cellular point of view.

BACKGROUND

The cartilage tissue

The joint consists of different tissues such as bone, ligament, synovium, fibrous capsule and cartilage. The importance of cartilage tissue in the human body is indisputable. It is entirely decisive for the development of the embryo as well as in the adult human being. The cartilage provides the initial parts of the skeleton in the embryo, and in the adult cartilage is implicated, for example, in breathing, articulation, locomotion and hearing (Lefebvre et al., 2005).

Formation of the synovial joint

It has been proposed that understanding embryonic tissue formation can help us to understand and control the processes of repair and regeneration in adult tissue. In 1925, Fell described a theory of the early development of the joint, and his description was later accepted by others. The early stages of skeletal and synovial joint formation in the developing vertebrate limb show a complex pattern involving different signalling molecules and pathways expressed in a temporal-spatial manner (Hall and Miyake, 2000; DeLise et al., 2000; Olsen et al., 2000). During embryonal development, gastrulation begins during the third week of pregnancy and converts the bilaminar embryo into a trilaminar embryo consisting of mesoderm, ectoderm and endoderm (Keller et al., 2005). From an articular cartilage perspective, the mesoderm is of interest.

The developing processes during joint formation consist of condensation, interzone formation and cavitation (figure 1).

Condensation

From the lateral plate mesoderm, undifferentiated mesenchymal cells begin to migrate to areas destined to become bone, followed by tight packing of the cells, known as mesenchymal condensation. The cartilage anlagen for the future skeletal elements have now formed. Cellular condensation is associated with increased cell to cell contact and increased cell to matrix interaction. Molecules taking part in the intercellular communication are e.g. neural cell adhesion molecule (N-CAM), N-cadherin, tenascin, versican, fibronectin and gap junctions (connexin 42 and 43), (DeLise et al., 2000; Hall and Miyake, 2000; Archer et al., 2003; Lefebvre et al., 2005). Characteristic limb skeletal abnormalities have been described when this communication between cells and matrix is disturbed as in mutations in encoding genes (Mundlos and Olsen, 1997a and b).

The matrix formed prior to condensation consists mainly of collagen type I, collagen type IIA and hyaluronan. Thereafter there is an increase in hyaluronidase and a decrease in hyaluronan that allows close cell to cell interactions. During further condensation there is a change in matrix composition to collagen type II, collagen types IX and XI, Gla protein, aggrecan and link protein while, collagen type I is turned off (reviewed in DeLise et al., 2000; Pitsillides and Ashhurst, 2008).

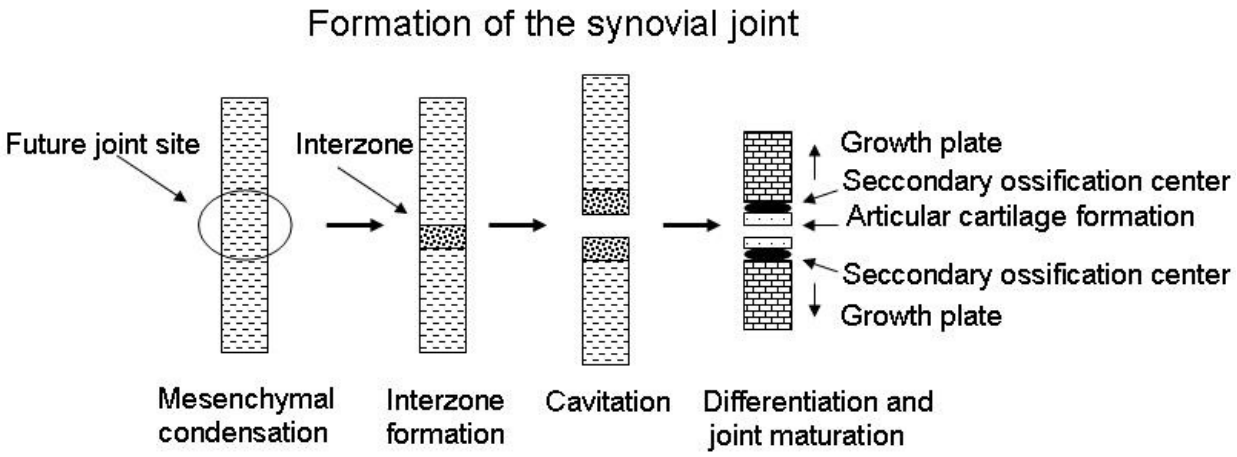


Figure 1. Schematic drawing of the development of a synovial joint

Interzone formation

The first sign of joint formation is the appearance of an interzone. The interzone cells gives rise to the articular layer of the future long bones while the chondrocytes developing from the mesenchymal condensation are assumed to be a part of epiphyseal growth plate and to take part in endochondral ossification, these cells are called transient chondrocytes. It has been unclear whether the interzone cells derive from transdifferentiation of local prechondrocytes into interzone cells or if there is migration of mesenchymal cells into the joint site, or a combination (Pacifici et al., 2000; Archer et al., 2003). The prechondrocytes in the interzone differentiate to early chondroblasts, thereafter becoming and remaining articular chondrocytes. However, recently new studies have presented data about the role of this population of cells in the interzone, expressing GDF5 (growth and differentiation factor 5; also known as CDMP-1 cartilage-derived morphogenic protein 1), a member of the transforming growth factor-beta family. It is supposed that GDF5 expressing cells give rise to the articular chondrocytes, synovial lining and ligaments in the joint, but that they do not contribute to the growth plate formation. Instead, the GDF5 positive population consists of a distinct cohort of limb skeletal progenitor cells. This was demonstrated by Koyama et al., (2008) using ROSA-LacZ-reporter mice where GDF5 reporter expressing cells from the interzone from E13.5 were followed postnatally and remain present in structures

that appear to arise from the interzone, namely the articular cartilage, synovium and other joint tissues (Koyama et al., 2008). This strengthens the idea that there are two populations of cells with distinct phenotypes, the transient chondrocytes forming the epiphysis and the GDF5 expressing cells forming the articular cartilage during interzone formation.

Cavitation

The cavitation process is essential to formation of a functional synovial joint. Cell death along the joint line in the interzone has previously been described as an important event in the cavitation, but this has lately been questioned (Pacifici et al., 2006; Pitsillides and Ashhurst, 2008). Instead local changes in composition of the extracellular matrix are suggested to be involved in the cavitation process. A mechanism of importance currently studied during joint cavitation is mechanical stimulation, in which hyaluronan and its receptor CD44 play significant roles in tissue separation (Pitsillides et al., 1995; Dowthwaite et al., 1998, Pitsillides and Ashhurst, 2008).

Molecular signalling

Molecular signalling in joint formation is complex. Several transcription factors, growth factors and other molecules have been implicated in joint development (Lefebvre et al., 2005; Archer et al., 2003; Pacifici et al., 2006; Pitsillides and Ashhurst, 2008). This introduction will only highlight some of them.

Sox9 is a transcription factor and a member of the SRY (sex determing region of the Y-chromosome) family, and has been demonstrated to play an important role in the commitment of the mesenchymal cells to the chondrogenic lineage. Sox9 is turned on prior condensation and is highly expressed in chondroblasts, but disappears when the cells undergo hypertrophy. The main role of Sox9 is to ensure cell survival and to activate collagen type IIA and other early cartilage markers (Olsen et al., 2000; Mori-Akiyama et al., 2003; Lefebvre et al., 2005).

Important factors involved in the cavitation process are the glycoprotein Wnt9A (previously called Wnt-14), GDF5 and Noggin. Wnt9A contributes to the cavitation process by inducing GDF5 (Hartmann and Tabin, 2001; Archer et al., 2003). Wnt9A also appears to promote the induction of CD44, the hyaluronan-binding protein (Hartmann and Tabin, 2001). In the lining joint area a bone morphogenic antagonist Noggin exerts an inhibiting effect on GDF5 to maintain the cavitation process. The decisive role of Noggin has been presented in at least two human syndromes characterized by the absence of joints due to mutations in Noggin (Gong et al., 1999; Hirshoren et al., 2008).

Wnts (Wingless-related proteins) are found throughout the developing limb. There have been many studies concerning the roles of Wnts, supporting their role in the developing limb (Später et al., 2006). The family consists of at least 15 cystein rich

secreted glycoprotein members, involved in cell fate, and axis determination in early embryos (Hill et al., 2005). One of the Wnts effectors is beta-catenin, known to play multifactorial roles in development. Beta-catenin is known to be membrane bound and to interact with adhesion molecules during embryonal development, but it also exists as a cytoplasmic pool and a nuclear pool. Its variable localisation and function has been suggested to play an intricate role during mesenchymal condensation and partly via a Sox9 dependent pathway (DeLise et al., 2000; Yano et al., 2005).

Growth factors sequentially involved in the patterning in chondrogenesis and cell fate specification are members of the transforming growth factor-beta family (TGF-β), such as GDF5 discussed above. The TGF-β family consists of various members and isoforms of TGF-β and the bone morphogenetic proteins (BMPs), with different roles (Massagu´e, 1998; Reddi, 2001). Members of the Fibroblast growth factor family (FGFs) are cytokines closely involved in chondrogenic differentiation, of special importance are their activation of Sox9. FGFs also induce the production of both fibronectin (Leonard et al., 1991) and the cell surface-adhesion protein N-cadherin (Tsonis et al., 1994) in limb bud mesenchyme.

Although knowledge about limb bud patterning and embryonal chondrogenesis has been well studied, it is too early to draw the conclusion that the epiphyseal chondrocytes and articular chondrocytes are separate populations of cells from the onset. But it has long been suggested that articular cartilage, and other joint tissues are separate not only in functionality but also in origin. Although there is no complete answer yet as described above new data from recent years support this idea and give us new insight into the formation of articular cartilage.

Anatomical structures in the joint

The groove of Ranvier

The perichondrial groove of Ranvier is an anatomical structure first described by Ranvier 1873 (figure 2 b) (Shapiro et al., 1977 and references therein). It is a circumferential anatomical structure in the periphery of the epiphyseal growth plate and consists of the zone of Ranvier and the ring of LaCroix. It is a well defined structure in the growing skeleton. In the adult it is assumed to be integrated with the periosteum however, this has not been well explored in the adult human being. Shapiro et al., (1977) characterized this structure thoroughly in the rabbit and described the morphology and matrix composition. Three different groups of cells could be distinguished at different locations: first fibroblasts within fibre bundles, second densely packed cells located in the innermost portions of the groove adjacent to the cartilage, and third between these two first groups of cells widely dispersed connective tissue cells, both poorly differentiated and more mature. The area of densly packed cells has been demonstrated to contain proliferating cells

(Shapiro et al., 1977). The ring of LaCroix, the fibrous band surrounding the zone of Ranvier and continuous with the periosteum of the metaphysis, has also been suggested to serve as a reservoir for precartilaginous cells in the germinal layer of the epiphyseal growth plate (Fenichel et al., 2006). The important role of an intact epiphyseal growth plate, and especially an intact perichondrial zone, for longitudinal bone growth is well documented. Fractures in the epiphyseal growth plate and Salter-Harris type IV fractures in the groove of Ranvier have both resulted in severe growth disturbances (Salter and Harris, 1963; Riseborough et al., 1983; Ilharreborde et al., 2006).

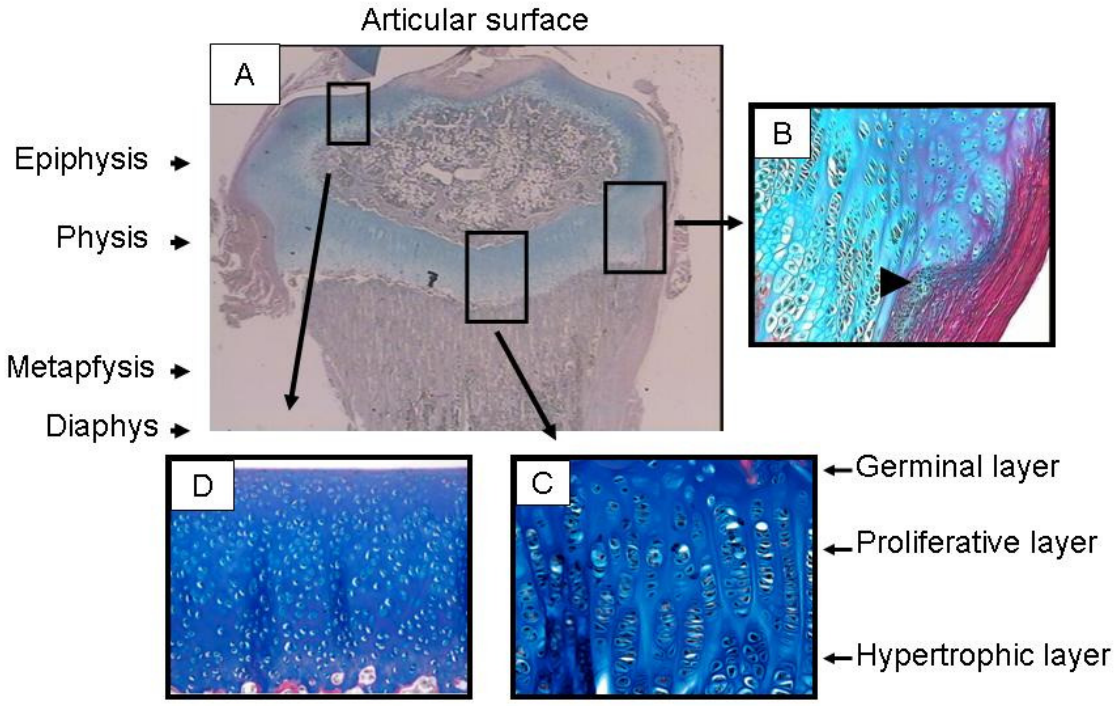


Figure 2. A. Rat tibia showing the location of epiphysis, physis, metaphysis and diaphysis. Magnification 10x. Marked areas represents: B. Groove of Ranvier. Arrowhead points at the groove of Ranvier. C. The epiphyseal growth plate. D. The articular cartilage. B, C and D are from rabbit. Magnification 20x. Alcian Blue van Gieson staining.

The epiphyseal growth plate

The epiphyseal growth plate is located between the epiphysis and metaphysis in the growing long bones (figure 2 c), and is resorbed following puberty, resulting in a fusion of the epiphysis to the metaphysis, known as closure of the physis. The epiphyseal growth plate is responsible for the longitudinal growth of the skeleton (Ballock and O'Keefe RJ, 2003). The growth plate can be divided into different layers: the germinal, proliferative and hypertrophic cell layers. The germinal layer, also known as the reserve zone, resting zone or stem cell layer, consists of cells

next to the epiphysis. These cells are scattered rather than organized, and embedded in the cartilage matrix. They have been demonstrated to function as stem cells (Kember and Lambert, 1981; Ohlsson et al., 1992; Hunziger, 1994; Abad et al., 2002). Underneath, there are columns of cells organized parallel to the long axis of the long bone. This zone plays a crucial role in longitudinal growth because these cells proliferate, hence the name proliferative layer. Thereafter, cell division ceases and the chondrocytes increases in volume and become hypertrophic. Here, in the hypertrophic layer, the hypertrophic chondrocytes attracts vascular in growth and bone cell invasion. The hypertrophic chondrocytes die through programmed cell death in the zone of calcification, a process suggested to be called chondroptosis instead of apoptosis (Roach et al., 2004; Bush et al., 2008). All these events described are the enchondral bone formation.

The articular hyaline cartilage

There are three basic forms of cartilage depending on the composition of the extracellular matrix: hyaline, elastic and fibrous. Hyaline cartilage is the most common form. The articular joints contain hyaline cartilage (figure 2 d), hyaline cartilage is also found in rib bone, nose, trachea and larynx. This thesis focuses on articular cartilage. The adult articular cartilage differs from young articular cartilage in a reduction of cell density and thickness of cartilage. There is also a shift to anisotropic structure from immature isotropic structures (Stockwell, 1978; Buckwalter and Mankin, 1997; Poole et al., 2001; Hunziker et al., 2006).

The articular cartilage tissue is avascular, non-innervated and alymphatic. The nutrition of the chondrocytes comes from passive diffusion. The only cells in articular cartilage are the chondrocytes, constituting about 2-5% of the tissue. The function of chondrocytes is to build, maintain, and remodel the extracellular matrix composed of collagens, proteoglycans, noncollagenous proteins and water (Stockwell, 1978; Buckwalter and Mankin, 1997; Olsen et al., 2000; Poole, 2003). Fresh articular cartilage contains about 75% water, the rest being matrix proteins. The articular cartilage has specialised load-bearing properties, ability to withstand compressive, tensile and shear forces due to the composition and structural integrity of its extracellular matrix (Grodzinsky et al., 2000). Under normal conditions it is a fine balance between chondrocytes and extracellular matrix but an imbalance can lead to the destruction of the articular cartilage as in OA.

Matrix composition of articular cartilage

The molecular organisation in articular cartilage is complex. This background focuses on the macromolecules mentioned in this thesis (collagen type II, collagen type X, COMP and aggrecan). Collagen type II accounts for 90-95% of the collagen in articular cartilage, it forms a three dimensional cross-linked network (together with smaller amounts of other minor collagens such as collagen type IX and XI). The collagen type II molecule is fibril forming and composed of three

identical polypeptide chains, forming a triple helix. Collagen type II can be found in two different splice variants: collagen types IIA and IIB. Collagen type IIB is uniquely expressed in differentiated chondrocytes, while type IIA is expressed by prechondrocytes (Ryan and Sandell, 1990; Sandell et al., 1991). Collagen type X is exclusively produced by prehypertrophic and hypertrophic chondrocytes in the calcified layer and has a role in mineralization (Buckwalter and Mankin, 1997).

A major constitute of articular cartilage is aggrecan. Aggrecan is a large macromolecule having a central core protein with negatively charged chondroitin sulphate and keratin sulphate glucosaminoglycans (GAGs) bound to it. The negatively charged GAGs can attract and bind water groups this leads to osmotic swelling and contributes to the compressive stiffness of the articular cartilage (Heinegård and Oldberg, 1989; Klippel et al., 2001; Bhosale et al., 2008). Via the link protein the large proteoglycan aggregates are stabilised, and the link proteins simultaneously bind to the aggregan molecule and hyaluronan acid (HA) (Franzén et al., 1981) (figure 3). HA then binds to CD44 to the surface of the chondrocyte (Chow et al., 1998). The large aggrecan molecule constitutes as much as 95% of the total proteoglycan mass in articular cartilage. Articular cartilage also consists of small proteoglycans including decorin, biglycan and fibromodulin (Klippel et al., 2001, Buckwalter and Mankin, 1997). They seem not to contribute directly to the mechanical behaviour of the tissue. Instead they bind to other macromolecules and probably influence cell function (Buckwalter and Mankin, 1997).

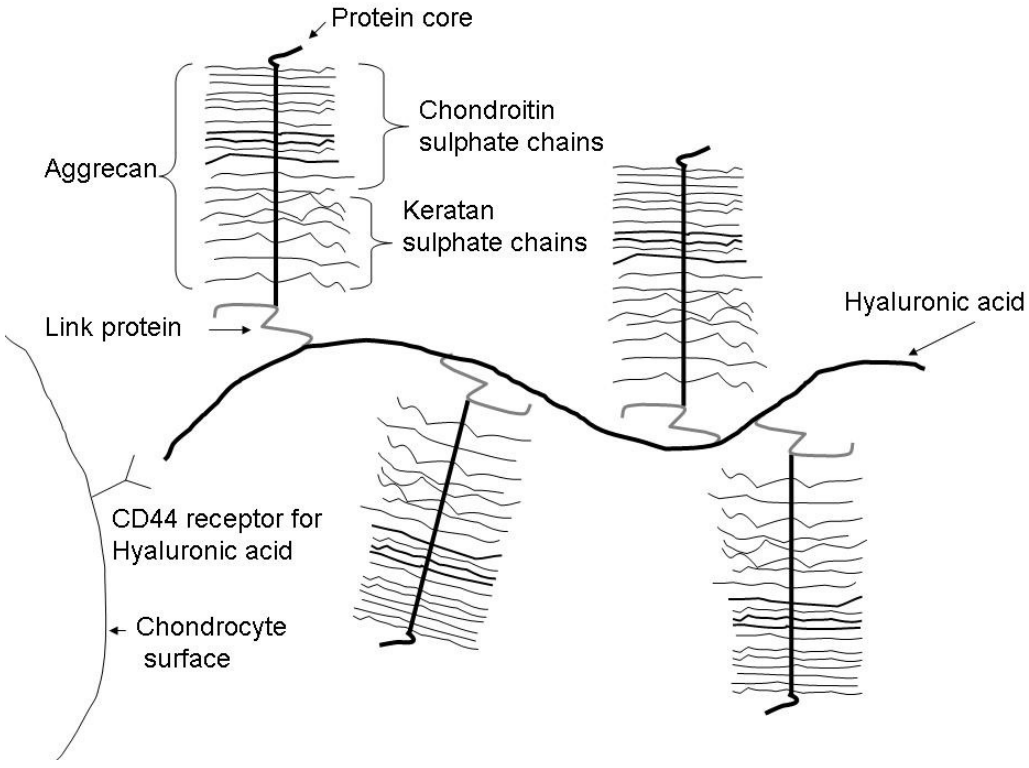


Figure 3. Schematic drawing of a proteoglycan aggregate.

Articular cartilage also consists of non-collagenous matrix proteins which are important for the interaction and assembly of the various macromolecules (Heinegård and Oldberg, 1989). Cartilage oligomeric protein (COMP) is one of these proteins. COMP is a glycoprotein belonging to the thrombospondin family also named thrombospondin 5. Its function is not fully understood but it interacts with collagen type I, II and IX, and it has been used as a diagnostic marker in serum for the progress of matrix degradation in OA (Saxne and Heinegård, 1992).

Morphology of articular cartilage

Depending on matrix composition and cellular appearance the articular cartilage is divided into several zones with different functional roles: superficial, transitional, radial and calcified zones (figure 4) (Bhosale et al., 2008; Poole et al., 2001; Hunziker et al., 2002). Facing the joint cavity is the superficial zone/tangential layer with small, flattened cells parallel to the surface. The matrix consists of mainly collagen type I fibres arranged tangentially to the articular surface, and small amounts of proteoglycans. The surface is covered with a thin sheet of fine fibrils and a thin layer of synovial fluid, sometimes called the “lamina splendens” (Buckwalter and Mankin, 1997). The production of the lubricin protein providing the frictionless surface of articular cartilage is specific for the cells at the surface of cartilage (Swann et al., 1981; Poole et al., 2001). Beyond the superficial zone is the transitional zone with larger and more rounded chondrocytes arranged both in groups and singly. The matrix is rich of proteoglycans and in collagens. The collagen fibrils are more randomly arranged than in the superficial zone. This zone displays the typical morphological features of hyaline cartilage. Then comes the radial zone of the articular cartilage, where the cell density is lowest and the large chondrocytes form radial columns and produce a matrix rich in proteoglycans, especially aggrecan. The content of collagen is low and the collagen fibrils are vertically oriented. The tidemark is the zone separating the cartilage from the underlying bone and below the tidemark is the calcified cartilage layer, with chondrocytes demonstrating hypertrophic phenotype and a matrix rich in type X collagen but without proteoglycans (Poole et al., 2001; Bhosale et al., 2008). Beneath the calcified zone the subchondral bone can be found.

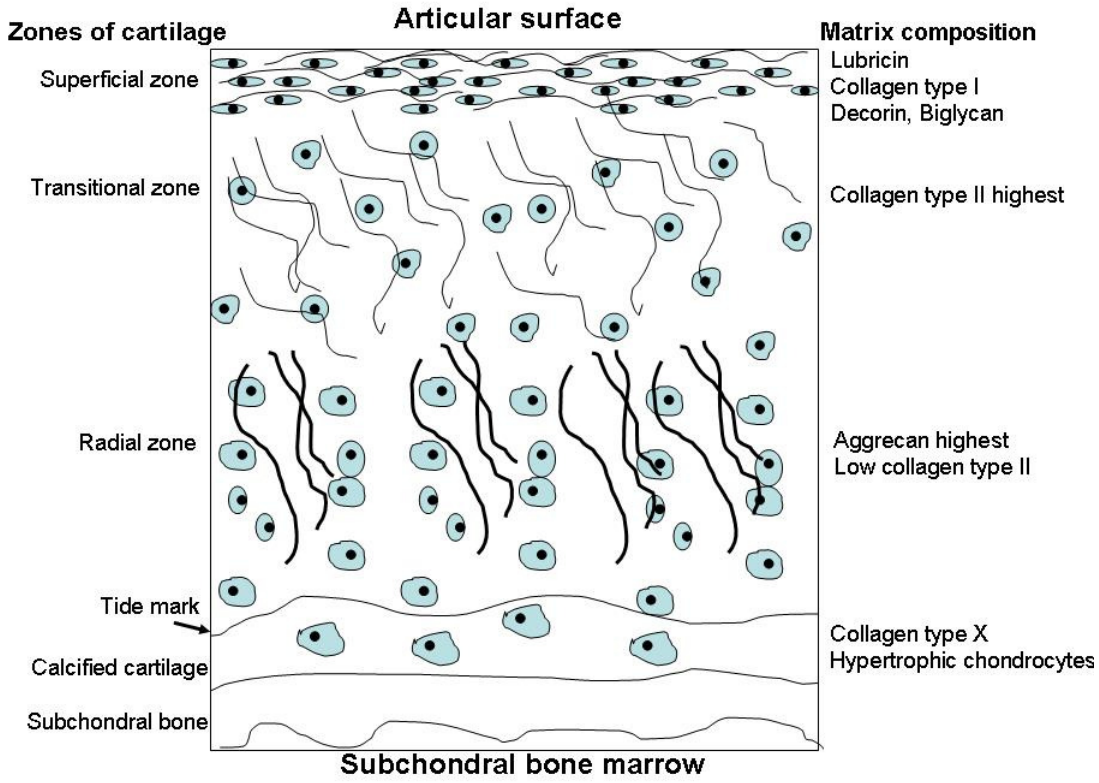


Figure 4. Zonal organization of articular cartilage. The orientation of the collagen fibres are shown in the figure.

Transcriptional control of proliferation and differentiation

One definition of proliferation is an increase in cell number by division. One characteristic of articular cartilage is low cell turnover and it is assumed that the chondrocytes do not divide *in vivo*, at least not in the adult, although decades ago it was shown in the literature that proliferation may occur in healthy cartilage (Crelin, 1957). Cell differentiation on the other hand, is a decrease in cell proliferation and structural and functional cells changes, with the development of a more mature phenotype. The links between these two events in normal tissue and especially in cartilage have not been well explored.

Transcription factors i.e. proteins that regulate transmission from DNA to RNA, are known to be involved in these processes. A transcription factor either stimulates or represses transcription of a specific gene and thereby regulates proliferation and differentiation. There are several families of transcription factors. Below two important groups are described in greater detail.

The HLH and Notch family of transcription factors

The transcription factor family of helix-loop-helix (HLH) proteins has been found to play an important role in cellular differentiation and thus to be involved in the

developmental control of gene expression. They have been extensively studied in muscle tissue. HLH proteins have been held responsible for muscle-specific differentiation from mesenchymal stem cells (Weintraub et al., 1991). HLH proteins are divided into different classes: class A consists of the ubiquitously expressed E-proteins E12 and E47 (Murre et al., 1989). These proteins form heterodimers with the tissue restricted class B proteins, e.g. MyoD in muscle (Weintraub et al., 1991). The heterodimer is formed through the HLH domain of the proteins and the complex is then capable of binding to a specific enhancer element in the DNA, known as the E-box consensus sequence (CANNTG). The basic amino acid region next to the HLH motif mediates the DNA binding. Recently the HLH proteins have been divided into more detailed classes depending on the DNA binding sequence: six classes and 44 families (Ruzinova and Benezra, 2003).

In the intricate regulation of proliferation and differentiation there is a balance between factors with reciprocal functions. Here another member of the HLH family, a group of proteins called Id (inhibitor of differentiation/ inhibitor of DNA binding) plays a role. These proteins lack the basic amino acid domain and are therefore not capable of DNA binding. Thus, heterodimers formed by Id and bHLH proteins will be inactive. No transcriptional activation will appear and no further differentiation will take place (Benezra et al., 1990; Benezra et al., 2001) (figure 5). Today, there are four known human Id proteins: Id1, Id2, Id3 and Id4 (reviewed in Ruzinova and Benezra, 2003). These Id proteins have highly homologous HLH domains, but homology is low outside this region. An alternative splicing form has been demonstrated in both mouse and human for the Id1 gene, called Id1.25 because the “coding intron” is an insert of about 250 bp (Tamura et al., 1998). The functional role of this non-spliced gene product is still unknown. The wide expression especially of Id1 and Id3 in different cell types suggests an important role, and it has been proposed that Id proteins function as general inhibitors of terminal differentiation and thus are in control of cell growth in many tissues. Id proteins have also been demonstrated to be closely involved in cell cycle regulation and are of great importance in the progress from the G0 to G1 phase (Hara et al., 1994; Peverali et al., 1994; Wong et al., 2004). The role of Id proteins and their involvement in tumorigenesis and prognosis of tumours has recently been described (Norton, 2000; Ruzinova and Benezra, 2003).

Other HLH proteins acting as negative transcription factors are the HES proteins, a family of proteins homologous to the Drosophila genes Hairy and Enhancer of split (HES) (Massari and Murre, 2000). HES proteins, like Id proteins, form non-functional heterodimers with other bHLH proteins such as the E-proteins, and they also act by binding directly to the N-box sequence, CACNAG, thus repressing genes inducing differentiation (Akazawa et al., 1992).

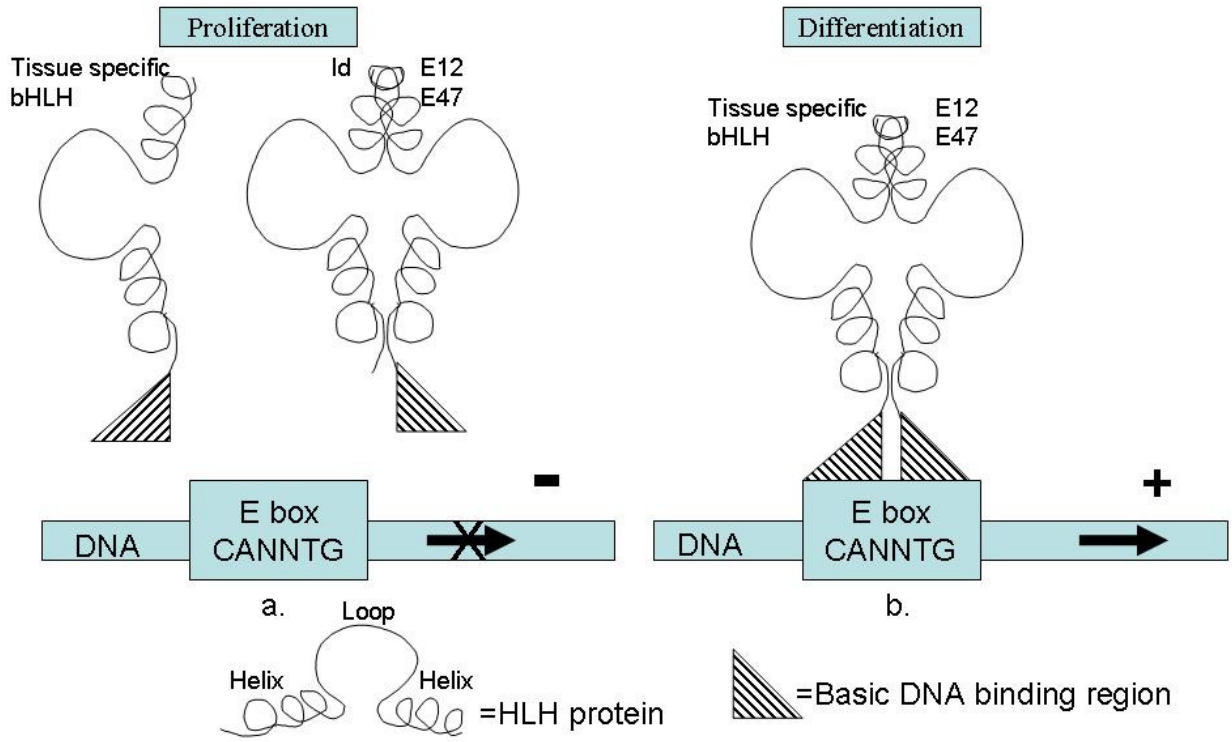


Figure 5. Dimerisation of HLH proteins. a) The Id proteins lacking the basic region form heterodimers with preferably the generally expressed bHLH proteins, E12 and E47, and thereby omitting DNA binding and gene activation. There is an inhibition of differentiation. b) The bHLH proteins form heterodimers and bind to the E-Box consensus sequence CANNTG, and start gene transcription important for differentiation of the cells.

The HES proteins are activated by the Notch signalling pathway. Notch is a transmembrane cell surface protein receptor and four Notch genes have been identified. The ligands for the Notch receptors in mammals are Jagged 1 and 2 and Delta 1 and 2 presented on adjoining cells (Artavanis-Tsakonas et al., 1999). By cleavage of the receptor an intracellular domain is released and translocated to the nucleus. In the nucleus it forms heterodimers with the transcriptional repressor RBP-J (recombinant recognition sequence binding protein at the J kappa site), also known as CSL (CBF1/Su(H)/Lag-1) which it activates to initiate transcription of for instance the HES genes (Tamura et al., 1995; Gho et al., 1996; Honjo, 1996). Notch signalling is an evolutionary conserved mechanism involved in proliferation, differentiation and apoptosis in a variety of cell types and organs including the peripheral and central nervous system, pancreas, hematopoietic cells, and muscle tissue (Bray, 1998; Artavanis-Tsakonas et al., 1999). Notch has been demonstrated in the developing cartilage and it has been suggested to be one of the important factors controlling the early stage of chondrocyte proliferation and differentiation.

It is also suggested to function as a gatekeeper and cell fate controller (Watanabe et al., 2003; Dowthwaite et al., 2004). In articular chondrocytes, blockage of Notch signalling has been demonstrated to cause a decrease in proliferation and down regulation of HES5 (Karlsson et al., 2007b). Karlsson et al. have also demonstrated that Notch1, Jagged1 and HES5 are abundantly expressed in OA cartilage as compared with healthy cartilage (Karlsson et al., 2008).

As described above, the HES proteins achieve their effects in similar way as the Id-proteins. It has also been demonstrated that Id proteins and HES1 can form complexes both *in vivo* and *in vitro* in neuroblastoma (Jögi et al., 2002). Id proteins have also been demonstrated to act as upstream regulators of HES1 in neural stem cells (Bai et al., 2007). In Drosophila it has been shown that Notch control of differentiation and proliferation may involve activation of the Id3 transcription (Reynaud-Deonauth et al., 2002). This provides evidence for an additional level of regulation of the HLH proteins.

Stem Cells

General background

Stem cells are defined to be undifferentiated, show unlimited potential to divide and be able to differentiate into more than one functional cell type. Stem cells can be divided into embryonic stem cells (ESCs), fetal stem cells (FSCs) and adult stem cells (ASCs) (Lensch et al., 2006, Alison and Islam, 2009). The pluripotent ESC is derived from the inner cell mass of the blastocyst and has the ability to give rise to all three embryonic germ layers; ectoderm, endoderm, and mesoderm (Chambers and Smith A, 2004). FSCs are more tissue-specific than ESCs and generate a more limited number of progenitor type of cells. Below I focus on ACSs.

ASCs are involved in tissue homeostasis, tissue regeneration and cell replacement owing to injury or natural death (Potten and Loeffler, 1990; Pittenger et al., 1999; Beltrami et al., 2007; Caplan, 2007). The origin of adult stem cells in mature tissues is still under discussion. The question of whether there are universal stem cells or stem cells resting in the individual tissue has not yet been answered. There might be adult stem cells circulating in the blood stream or located in the blood vessels and able to populate different tissues. Several researchers have noted that dividing cells in adult tissue often appear near a blood vessel, such as candidate stem cells in the hippocampus and pericytes in the blood vessels (Palmer et al., 2000; Caplan, 2007; da Silva Meirelles et al., 2008). Another hypothesis is that the stem cells reside in the tissue from the embryonic development. In some tissues it is clear that these stem cells are located in a special microenvironment known as the “niche”. The location and nature of this niche can vary depending on the type of tissue. Niches well studied in mammals include the bulge area of hair follicles where

epithelial stem cells are located, and the intestinal stem cell location near the crypt base (Fuchs et al., 2004; Cotsarelis, 2006; Marshman et al., 2002; Mitsiadis et al., 2007). The niche is assumed to be a dynamic structure keeping the stem cells in quiescence and contributing to the activation of stem cells when required. Two families important to the signalling and regulation in the niche are the Wnts and the Notchs, discussed above in the sections *on synovial joint formation and transcriptional control of proliferation and differentiation* (Jan and Jan, 1998; Watt and Hogan, 2000; Mitsiadis et al., 2007).

When stem cells divide they can hypothetically divide either asymmetrically or symmetrically. Asymmetric division means that the stem cell divides into two daughter cells one of which is a stem cell and one a cell with a specific destiny, while symmetric division takes place when the stem cell divides into two identical daughter cells, both stem cells. The daughter cells from a stem cell are often known as progenitor cells or precursor cells. The term progenitor cell is often used in the literature. Although the definitions vary between scientists and publications, it can be summarised as follows: a progenitor cell can give rise to a daughter cell that is more specialized than itself, but cannot renew itself. In other words a progenitor cell is more differentiated than a true stem cell but can still got multi or oligopotent properties. Steindler described the word progenitor: "Although a progenitor cell is more committed, the word also applies to stem cells (i.e., stem/progenitor cells) when the degree of "stemness" is not certain" (Steindler, 2007). Plasticity is a characteristic demonstrated by stem cells or progenitor cells, which describes the ability for a cell in one tissue to generate a differentiated cell in another tissue (Weissman, 2000; Watt and Hogan, 2000; Oswald et al., 2004).

Some tissues have been known for decades to contain adult stem cells, including hematopoietic stem cells and bone marrow stromal cells (mesenchymal stem cells) (Lajtha, 1975; Caplan, 1991), epithelial stem cells in the deep crypts of the digestive tract (Potten and Loeffler, 1990), and epidermal stem cells (Alonso and Fuchs, 2003). In contrast, the heart, the brain and articular cartilage, have been proposed to be terminally differentiated organs, lacking stem cells and having very little if any capacity for self-repair. Recently, however it has been demonstrated that adult nerve and heart tissue contains stem cells supporting their regeneration (Gage, 2000; Beltrami et al., 2003). Furthermore, monolayer-cultured articular chondrocytes isolated from human adult articular cartilage have shown phenotypic plasticity with chondrogenic, adipogenic and osteogenic potential (Barbero et al., 2003; Dell'Accio et al., 2003; Tallheden et al., 2003).

In immature articular cartilage *in vivo* it has been demonstrated that potential stem cells or progenitor cells are localised in the superficial layer. Hayes et al. suggested an appositional growth hypothesis after studies with BrdU and thymidine labelled marsupial Monodelphis domestica, where progenitor cells were localised to the surface of the articular cartilage (Hayes et al., 2001). Additional data has been

published describing progenitor cells in the superficial layer of bovine articular cartilage (Dowthwaite et al., 2004; Hattori et al., 2007).

It is important to study and learn more about if and where adult stem cells exist, especially in a tissue engineering perspective, because to use adult stem cells instead of ESCs or FSCs would be of advantageous. ASCs can be isolated from the patient, which would solve the immunological problems, and there is a smaller risk of tumour formation and fewer ethical problems as compared with using ESCs or FSCs (Gaissmaier et al., 2008).

Stem cell associated markers

Stem cells/progenitor cells can be identified and characterized by their expression of specific proteins, although no unique marker for these types of cells exists today. Markers associated with and suggested to define possible stem cells or progenitor cells in mesenchymal tissue and also, in some cases, in adult cartilage are CD105 (Endoglin), CD166 (Alcam) and FGFR3 (Fibroblast Growth Factor receptor 3) (Alsalameh et al., 2004; Fickert et al., 2004; Robinson et al., 1999).

It is well known that members of the TGF-β super-family are essential mediators of cell proliferation and differentiation during cartilage and bone formation (Massagu´e, 1998; Jakob et al., 2001). CD105 is an accessory receptor of the TGF-β receptor complex and has recently been found on chondrocytes. The antibody was initially raised against human marrow-derived mesenchyma stem cells (MSCs) (Parker et al., 2003). CD166 (ALCAM) is an activated leukocyte cell adhesion molecule and a member of the immunoglobulin super-family mediating both heterophilic and homophilic cell-cell interactions (Swart, 2002). CD166 has been reported to be expressed on human articular chondrocytes together with CD105 and to function as markers for a progenitor population (Alsalameh et al., 2004). A MSC population from periosteal tissue was demonstrating CD105 as well as CD166 expression and also showed multipotency demonstrating chondrogenic, osteogenic and adipogenic phenotypes (De Bari et al., 2006; Choi et al., 2008). FGFR3 is a growth factor receptor belonging to the FGF family. FGFs are broad-spectrum mitogens and stimulate e.g. limb outgrowth, proliferation and angiogenesis during limb development. FGFR3 has been identified in rat perichondrium located at the ring of La Croix and in resting zone chondrocytes (Robinson et al., 1999). It has also been shown that monolayer cultured human chondrocytes used for ACI consists of a heterogeneous population of cells with some cells expressing FGFR3. These cells are suggested to represent a prechondrogenic population (Robinson and Nevo, 2001). Nevertheless, none of these markers have been demonstrated *in vivo* in adult human articular cartilage.

Markers previously not studied in human articular cartilage are the Stro-1 and Bcrp1. Stro-1 is a widely accepted marker for mesenchymal stem cells and is also present on stem cells in the native bone niche (Simmons and Torok-Storb, 1991; Song et al., 2005). Stro-1 was originally identified as a cell surface glycoprotein on colony-forming osteogenic precursor cells isolated from bone marrow (stromal) cells. The selected Stro-1+ cells were multipotent and gave rise to adipocytes, osteocytes, smooth myocytes, fibroblasts, chondrocytes, and blood cells (Gronthos et al., 1994; Stewart et al., 1999; Dennis et al., 2002). Selected Stro-1 expressing cells within the dental pulp have also shown multipotent properties (Jo et al., 2007). In the hematopoietic system within the stromal bone marrow a side population (SP) of cells positive for Bcrp1 (Breast cancer resistance protein) was identified as stem cells (Zhou et al., 2002). This SP has the capacity to strongly efflux Hoechst 33342 fluorescence dye in a process mediated by the ATP-binding cassette transporter Bcrp1, this high level of dye efflux activity is a characteristic of adult stem cells (Goodell et al., 1996). A progenitor population of cells from the superficial zone of bovine articular cartilage was identified by the Hoechst 33342 dye, and they differentiated exclusively to superficial zone cells identified by expression of the superficial zone protein, lubricin (Hattori et al., 2007). Cells with MSC properties have also been identified in synovial tissue by Bcrp1 (Teramura et al., 2008).

Cartilage injuries

Articular cartilage serves as a low friction surface, and acts as a shock absorber, indicating that injuries of the joint surface have detrimental effects. Several studies have demonstrated high incidence of articular cartilage pathologies during consecutive arthroscopic procedures: one study reported a 66% (Aroen et al., 2004) and another a 63% incidence (Curl et al., 1997).

Injuries to articular cartilage can occur as results of either traumatic mechanical destruction or progressive mechanical or inflammatory degeneration, sooner or later leading to osteoarthritis (OA). The patient often has problems with joint pain, disability and disturbed function. At the end stage, when OA is visible, the patient often needs total knee arthroplasty (Bhosale and Richardson, 2008). The suffering for the patient and the cost to society are both enormous. In Sweden the cost in 2001 for the osteoarthritis and spondylosis group of diseases was calculated to about $12.5x10^9$ Sek (Schmidt et al., 2003).

Cartilage repair

There is as yet no golden standard for the treatment of cartilage defects in the joint. Depending on the type of lesion and the depth and size of the defect, age of the patient and type of trauma, articular cartilage has shown different abilities to heal (Brittberg, 1996, Jackson et al., 2001). The types of lesions often referred to are

chondral lesion i.e. a lesion limited to the articular cartilage, a full-thickness chondral lesion extending to the subchondral bone plate, and an osteochondral lesion passing through the bone plate (Khan et al., 2008) (figure 6). Once the lesion has been identified, different cartilage repair techniques may be chosen to treat the articular cartilage defect. Methods used include microfracturing, mosaicplasty, subchondral drilling, and arthroscopic abrasion. The mechanism for repair in these methods involves opening of the subchondral vascular area to stimulate fibrocartilage ingrowth and resurfacing with tissue lacking the characteristics of hyaline cartilage (Bhosale and Richardson, 2008; Khan et al., 2008). Although there are different surgical methods to repair chondral defects, they basically all lead only to repair of the cartilage. The optimal treat of course would be regeneration of the cartilage. Usually repair refers to formation of neo-tissue but not necessarily with the same qualities as the original tissue, while regeneration refers to formation of tissue indistinguishable from the original tissue (Bhosale and Richardson, 2008).

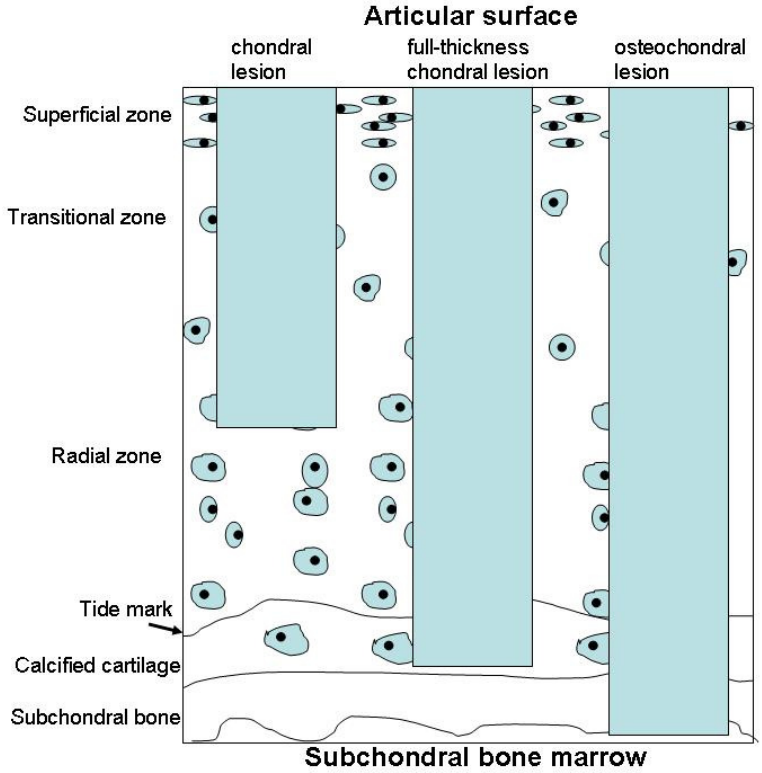


Figure 6. Different types of lesions: chondral lesion i.e. a lesion limited to the articular cartilage, a full-thickness chondral lesion extending to the subchondral bone plate, and an osteochondral lesion passing through the bone plate.

One cell based method for treatment first described in 1994 by Brittberg et al. was autologous chondrocyte transplantation or implantation (ACT or ACI). In ACT the patient's own chondrocytes that have been expanded *in vitro* are implanted into the cartilage defect in combination with a covering membrane, the patients own periosteum (Brittberg et al., 1994). The first generation of autologous chondrocyte

transplantation was initially termed ACT, out of which both second and third generation ACT developed, using membranes instead of periosteum and carriers for the cells, now considered as tissue engineering techniques (Lindahl et al., 2003; Lindahl, 2008; Brittberg, 2008). The cell based theories focus on the fact that chondrocytes are normally entrapped in their matrix and unable to migrate, but when administered as single cells directly in the lesion they can take part in the regeneration of the tissue. In the traditional ACI there is no penetration of the subchondral bone and bleeding is avoided (Brittberg, 1996).

However, although good clinical results have been presented using ACI method it has also been criticised, because there are still questions about the function of the cells and it is not clear whether seeded cell technology is better than bone marrow stimulation techniques (Brittberg, 1999; Peterson et al., 2000; Brittberg et al., 2001; Peterson et al., 2002; Mithöfer et al., 2005). Although, six clinical randomised trials have been published, still no clear answers can be given regarding the effectiveness of ACI versus other techniques (Brittberg, 2008 and references therein). Follow-up routines after ACI vary and involve evaluation of clinical symptoms, direct visualisation during arthroscopy or indirect visualisation with magnetic resonance imaging (MR). The difficulty in studying the true regenerated tissue on a molecular level makes these evaluations even more problematic (Henderson et al., 2003; Roberts et al., 2003; Tins et al., 2005).

All existing cartilage repair methods seem to work acceptably, but efforts are now being focused on a modern regenerative tissue engineering approach. Both the Swedish government and the Europe Union are funding these research projects.

Knowing that the articular cartilage tissue is characterized by low cell turnover, lack of vascularisation and innervation and lack of stem cells, it is clear that articular cartilage does not have the basic prerequisites to regenerate. However, in recent decades tissue engineering, stem cell research and molecular signalling have all made great progress. This might help us to understand the mechanisms for adult tissue repair and regeneration. There seem to be a promising future for cartilage tissue engineering.

Studying chondrocytes *in vitro*

Articular cartilage is difficult to study *in vivo*, and so *in vitro* cultures are usually used in primary studies. It is well-known fact that chondrocytes dedifferentiate when transferred to an adherent monolayer culture. Exposure to serum leads to a diminished chondrogenic phenotype, and the genetic expression profile is also altered. This is not regression to an earlier multipotent state but the cells lack differentiating functions (Benya and Shaffer, 1982). The cells have the ability to redifferentiate when replaced into a 3D (three-dimensional) environment without anchorage and under the right culture conditions (Benya and Shaffer, 1982;

Bonaventure et al., 1994; Tallheden et al., 2004). Different culture systems have been developed to enhance the chondrogenic phenotype *in vitro*: alginate culture, collagen matrixes culture, agarose suspension culture and pellet mass culture (Bonaventure et al., 1994; Schulze-Tanzil et al., 2002; van Susante et al., 1995; Grigolo et al., 2002; Benya and Shaffer, 1982; Kimura et al., 1984).

The latter two culture models are presented here.

Agarose suspension culture

The 3D agarose suspension culture allows chondrocytes to grow in a hydrogel that stimulates cell differentiation and matrix formation in an environment resembling the *in vivo* conditions in cartilage (Benya and Shaffer, 1982). In the agarose suspension culture system, chondrocytes are the only cell type able to survive, apart from tumour cells (Wittelsberger et al., 1981; Benya and Shaffer, 1982). Previously, the agarose system has been used to study the phenotype of chondrogenic cells and the effects of compressive strain on the morphology, metabolism and proliferation of chondrocytes from the different zones of articular cartilage (Buschmann et al., 1992; Lee et al., 1998).

Pellet mass culture

Using high-density pellet mass cultures makes it possible to study cartilage differentiation and regeneration *in vitro* (Manning and Bonner, 1967; Tallheden et al., 2004). Pellet mass culture is a well-established method for chondrogenesis and offers great advantages for research purposes. The cells are first expanded in monolayer and thereafter cultured at high density in pellets, mimicking the condensation phase of chondrogenesis. Subsequently, there is differentiation into mature chondrocytes. Tallheden et al. have shown that the different phases of chondrogenesis observed *in vivo* during embryonic formation of the cartilage also occur in pellet mass cultures (Tallheden et al., 2004). Furthermore, the differentiation to a hyaline phenotype during culture time in the pellet mass system has been demonstrated using real-time PCR for collagen types I, IIA, and IIB and immunolocalization of collagen types I and II, Safranin O staining, and biochemical analyses of GAG and hydroxyproline (Tallheden et al., 2004; Karlsson et al., 2007a). One of the great advantages as compared with agarose suspension culture is that the pellets can easily be used for both RNA analysis and protein analysis (Tallheden et al., 2004).

AIMS OF THE THESIS

The overall aim of this thesis was to search for chondrocytes with progenitor properties and to study associated signalling pathways in articular cartilage used for autologous chondrocyte implantation.

The specific aims were:

- To study the proliferation of adult human chondrocytes and investigate the functional role of the helix-loop-helix proteins Id1 and Id3 in the transcriptional regulation of chondrocytes.
- To study the growth of human chondrocytes in agarose suspension culture and the biological effects of the periosteum on the chondrocytes.
- To identify, select and characterize a subpopulation of adult human chondrocytes with progenitor cell properties, using agarose suspension culture.
- To locate stem cells/progenitor cells in the joint by using a rabbit model and BrdU labelled cells.
- To identify and locate a progenitor population in human articular cartilage using stem cell associated markers.
- To study the expression of Wnt and Notch signalling pathways in repaired and regenerated human articular cartilage.

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in the Material and Methods section of the individual papers. A more general discussion of the methods is presented below.

Ethical approvals

All studies were approved by the local ethical committee at the Medical Faculty of the University of Gothenburg. In paper V, approval from the Ethics Commission in Warsaw, Poland was given.

Subjects and samples

Normal cartilage

Cells and tissues from normal human articular cartilage used in papers I-III were isolated from surplus biopsies harvested from patients undergoing ACI or patellar groove reconstruction. The articular cartilage was taken from macroscopically unaffected areas. To ensure the anatomical orientation including all zones of articular cartilage as well as the quality of normal cartilage in paper V, articular cartilage was taken from the medial and lateral femur condyles from diseased donors with macroscopically intact cartilage and no clinical history of pathology affecting cartilage. The biopsies were cylindrical, full thickness, and taken with a 5-mm diameter punch biopsy perpendicular to the surface.

Chondrosarcoma

Chondrosarcoma cells in paper I were obtained from 9 cases of chondrosarcoma of different histological grades. The grading of chondrosarcomas is a three-step grading system based on histology. Tumours resembling normal cartilage are regarded as low-grade tumours (grade I) and the ones with most abnormal appearance are regarded as high-grade (grade III) (Asp, 2002). There were one grade I-II (K12), four grade II (K5, K6, K8, K9), two grade II-III (K1, K2) and two grade III (K7, K11) cell cultures, generated from tumours surgically excised at the Department of Orthopaedics, Sahlgrenska University Hospital, Gothenburg, Sweden. The biopsies were graded at the Department of Pathology Sahlgrenska University Hospital. K5 and K6 correspond to the cell lines FS090 and 105KC, respectively, both kindly donated by Dr. J. Block, Section of Rheumatology, Dept. of Medicine, Rush-Presbyterian-St. Luke's Medical Center, Chicago.

Osteoarthritic cartilage

Osteoarthritic cartilage (OA cartilage) used in paper V was taken from patients undergoing total knee replacement either from areas macroscopically affected by OA or from unaffected areas in order to be able to make comparisons. The biopsies were scored according to Mankin score, by one examiner. Originally the Mankin score system was based on a 14 point score including cellular changes, histochemical staining and architecture of the cartilage (Mankin et al., 1971). Since not all the biopsies obtained were full-depth biopsies, a modified Mankin score was used, not including the tidemark integrity. The maximum score was therefore 13 instead of 14 in paper V.

Debrided cartilage from injury

In connection with arthroscopic evaluation of patients with joint pain but without signs of OA debridement of affected articular cartilage is often performed. Depending on patient, location, depth and size of the injury there are large variations in these biopsies in hyaline character. Therefore the biopsies used in the study in paper V were evaluated using the modified Mankin score described above. The scoring was performed by one observer familiar with the Mankin score. The biopsies were scored only to be able to classify them as more or less hyaline/fibrous like cartilage.

Cartilage from the ACI area

Cartilage from the ACI area was taken from 15 patients during a follow up time between 2 and 6 years after ACI. Arthroscopy was performed for new pain situations needing an arthroscopic evaluation. After permission from the patient, a biopsy was taken during arthroscopy from the previously transplanted area. In an attempt to make a more practical evaluation score for biopsies taken from ACI cartilage, the International Cartilage Repair Society (ICRS) committee established a score 2001 based on visual patterns (Mainil-Varlet et al., 2003). Surface, matrix, cell distribution, cell viability, subchondral bone and mineralization are all evaluated. The highest score indicates ideal repair results (truly regenerated tissue) and the lowest score the poorest repair results. The problem, as with all evaluation scores, is to ensure the quality of the biopsy, depending on orientation and full depth. In paper V we had to modify the ICRS score depending on inadequate biopsies and the subchondral bone was excluded to be able to get enough of material. The maximum score was therefore 15 instead of 18. The biopsies were evaluated by three different observers familiar with the scoring system.

It is recommended that the scores from the separate criteria not should be summed, although in paper V we wanted to divide the biopsies in two groups, low scored and high scored respectively therefore we summed the scores.

New Zealand White rabbits

The New Zealand White rabbit joints have previously been extensively studied between the ages of embryonic limb bud and skeletal maturity (Masoud et al., 1986; Rivas and Shapiro, 2002). New Zealand White rabbits at age 3 months were used in paper IV. They had reached sexual maturity but were still not fully skeletal mature (they reach skeletal maturity at about 8 months). In animals of this age it is possible to use the epiphyseal growth plate as an internal control for labelling slow cycling cells in the resting zone. Another advantage is that there is still high proliferation in joint tissue at this age and it is therefore easier to distinguish proliferating cells at early time points from slow cycling cells at later time points.

Isolation and culture of chondrocytes

Cultured cells isolated from articular cartilage biopsies were used in papers I-III and V. The donors' ages ranged from 16-82 years in the different experiments and papers, for detailed information see each respective paper. The cartilage biopsies followed the ordinary handling for cartilage biopsies used for transplantation (Brittberg et al., 1994). The harvested biopsies were transported to the laboratory in sterile saline solution supplemented with antibiotics and fungicide. The chondrocytes can be preserved for up to 48 hours in this transport medium, as shown in validation studies (Cell Matrix, Gothenburg, Sweden). Bone and soft tissue were removed from the biopsies, after which they were minced and digested overnight (16 to 20 h) using clostridial collagenase in culture incubators with air containing 7% CO_2 at 37° C. This treatment digests the cartilage tissue into single cells, as identified using a microscope.

Independently of the culture system used, further culturing of chondrocytes includes antibiotics such as gentamicin sulphate (Gibco, Paisley, Scotland) as well as antifungoral (Amphotericin B, Gibco) additives in the culture medium to prevent infectious agents from becoming established in the culture. Supplementation of ascorbic acid to chondrocyte cultures is of importance for collagen production as a cofactor of the proline and lysine hydroxylase, and is required for the proper assembly and stabilization of the collagen fibrils (Giannoni and Cancedda, 2006). Ascorbic acid also functions as an antioxidant while L-glutamine, also added, is an essential amino acid. Usually human articular chondrocytes are cultured in autologous serum, as in papers I-III, but for the standardization of the agarose suspension assay in papers II and III FCS was used, in order to be able to use the same batch of serum.

Monolayer culture

In monolayer culture the cells grew adherent to the plastic in culture bottles. Single cells of chondrocytes were seeded at a density about 3000-8000 cells/cm^2 (Brittberg, 2008). This has been determined to be adequate for chondrocytes used in ACI to initiate proliferation and to reach confluence in 8 days with no more than 8 cell doublings. Medium was changed twice a week. Cells were expanded by passage to new culture bottles when they reached 80% confluence, if necessary. The cells were released from the culture bottles using trypsin-EDTA solution diluted in PBS.

Chondrosarcoma cells were isolated from tumour tissue in paper I. To isolate cells from the chondrosarcoma, pieces of tissue were placed in culture bottles and cells were allowed to grow out from the piece, after which they were cultured in monolayer. In the culture medium for chondrosarcoma cells 1% Ultroser® (Invitrogen, Paisley, UK) was added as a serum substitute in addition to FCS. Ultroser® is a serum substitute with unknown content. In paper I the normal chondrocytes also had this addition to be able to compare the cultures. In separate experiments in paper I the influence of serum on gene expression was studied, and both the normal chondrocytes and the chondrosarcoma cells were therefore subjected to complete serum withdrawal for 24 hours before harvesting (sometimes this is called "starvation" of the cells).

Agarose suspension culture

In papers II and III agarose suspension culture was used. In paper II, both directly isolated (primary) chondrocytes as well as chondrocytes previously monolayer cultured were studied in different experiments. In paper III, only surplus cells from ACI were used. The agarose cultures were made using a modification of the protocol published by Benya and Schaffer, 1982. Culture dishes with grids (60 mm in diameter in paper II, 50 mm in diameter in paper III) were precoated with 1% standard low agarose (SLG, Bio-Rad, Hercules, CA, USA), after which a mixture of 0.75 ml 2% low gel temperature agarose (LGT, Bio-Rad) and 0.75 ml DMEM/F12 (Invitrogen) and 1.5 ml cell suspension containing $5x10^4$ cells were added to the culture dishes. This gave a final concentration of cells of $5x10^4$ cells/50-mm culture dish, i.e. $16.7x10^3$ cells/ml agarose (in paper II the final concentration was $25x10^3$ cells/ml agarose). FCS was used instead of HS in the culture medium as mentioned above, but the medium contained the same supplements as in monolayer cultures, i.e. antibiotics and fungicide, which is of special importance in these cultures owing to the risk of infection during the long culture times.

To be able to study different concentrations of cells and the effects on cluster formation, separate experiments were carried out in which different final concentrations of cells were used.

A Nikon inverted microscope was used to examine the cell clusters. To ensure that a cell cluster consisted of more than 4 cells, the minimum size of a cell cluster was set to 50 μM. To determine that a cluster initially consisted of one single cell which underwent clonal division, separate cells were followed by photography for 10 weeks of culture time. To study the proliferative capacity of individual cell clusters, individual cell clusters were isolated by using a sterile Pasteur pipette and subcultured in monolayer in 24-well plates, 12-well plates, 6-well plates and finally in 25-cm^2 culture bottles.

To gain further support for the findings of others that only chondrocytes and tumour cells can grow in agarose suspension culture, we cultured mesenchymal stem cells, fibroblasts, and osteogenic cells, and found no evidence of growth in the agarose suspension culture.

Co-culture experiments

To study the effects of periosteal tissue on chondrocyte cluster formation, a modified protocol by Lindahl et al. was used in paper II (Lindahl et al., 1986). Primary isolated chondrocytes were suspended in DMEM/F12 with 5% FCS and 0.5% LGT at a concentration of 20 000 cells/ml. The cell suspension was added to 25 cm^2 culture bottles with or without presence of pieces of autologous periosteum taken from the upper medial side of the tibia. The cells were cultured for 21 days and thereafter evaluated depending on number and types of cell clusters formed.

Cultures to study the effects of conditioned medium on chondrocyte cluster growth

To study the effects of dermal fibroblasts, periosteum and periosteal cells on cluster formation in paper II cells were isolated from dermal tissue and periosteum. The dermal tissue and periosteal biopsy were cut into pieces and put into culture bottles. When cells had migrated out from the pieces the supplementation of FCS was changed from 10% FCS to 5% FCS in the medium. Culture media was collected weekly after the cultures have reached confluence (the collected medium was called conditioned medium). Chondrocytes cultured in agarose suspension cultures were incubated with the conditioned medium, and the medium was changed once a week. After 7 weeks the number and type of clusters were evaluated.

Cell cultures to measure cytokines and growth factors

To evaluate potential cytokines and growth factors secreted by articular chondrocytes, periosteum and co-cultures of articular chondrocytes and periosteum, culture medium from these cultures were collected and studied in paper II.

Human articular chondrocytes were seeded into 24-well culture plates in medium supplemented with 10% FCS. Pieces of periosteum were minced and placed in co-culture with the chondrocytes. Periosteum was also placed alone in cultures. After 48 h, 400 ml supernatants from the chondrocyte cultures and periosteal tissue cultures as well as the co-cultures were collected, centrifuged and stored at -20C°, until they were tested for the presence of Interleukin-6 (IL-6), Interleukin-8 (IL-8), granulocyte-monocyte colony-stimulating factor (GM-CSF) and TGF-β1.

Pellet mass culture

It has previously been shown that the 3D pellet mass culture systems act as differentiation systems for chondrocytes (Xu et al., 1996; Yoo et al., 1998; Tallheden et al., 2004). A number of $2x10^5$ cells were placed in polypropylene conical tubes. The medium used was a small volume of DMEM-HG (0.5 ml [PAA Laboratories, Linz, Austria]) supplemented with ascorbic acid, ITS (insulin, transferrin and selenium [Life Technologies, Sweden]), human serum albumin, TGF-β1 and dexamethasone to stimulate matrix production. Serum was not used since it is assumed to stimulate chondrocyte de-differentiation. An initial centrifugation was carried out before starting the culturing to allow the cells to be in high cell density condition. Medium was changed twice weekly. Detailed studies have previously been made to confirm the differentiation of the chondrocytes during culture time from days 7 to 14 and on to day 21 (Tallheden et al., 2004).

Methods for RNA studies

RNA preparation and RT-PCR

In order to study expression of a specific gene, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) can be used. First RNA has to be prepared from the cells of interest.

In paper I, total cell RNA was prepared from cultured human articular chondrocytes and chondrosarcoma cells using the RNeasy total RNA kit (Qiagen, Hilden, Germany). In paper III total RNA was extracted from 5 pooled cell clusters from the separate types of clusters. The cell clusters were picked from the agarose culture using a sterile Pasteur pipette. A buffer containing NaCl, Tris, $MgCl_2$, DDT,

RNase inhibitor and NP40 with vigorous shaking for 10 min, made the cell clusters lyse. This lysate was used for further RT-PCR. The above mentioned method for preparing RNA was chosen because the small amounts of starting material made it impossible to use conventional phenol-chloroform extraction or other commercially available methods.

In RT-PCR the mRNA is first converted to complementary DNA (cDNA) in the RT step, before amplification in the PCR. The RT-PCR method is non-quantitative. If quantitative expression analysis is needed real-time PCR should be used instead. RT-PCR is a sensitive method requiring a small amount of starting material. Owing to the sensitivity of the method it is important to use negative controls, such as omitting cDNA in control samples, and to perform the laboratory experiments in restricted laboratory areas to ensure that the sample not is contaminated. To ensure that the amplification product is not genomic DNA, the primers selected usually cover exon-intron boundaries. Dnase treatment of the sample is often done to digest any persistent genomic DNA. This is of special importance when the DNA sequence lacks introns.

In paper I the RT-PCR method was used to amplify the Id1 and Id3 genes, in order to be able to clone their cDNA into suitable cloning vectors. The vector used was PCRII (Invitrogen, Leek, The Netherlands). The cloned cDNA was then used for synthesis of radiolabelled antisense RNA probes of 298 bp, 245 bp and 354 bp for Id1, Id1, 25 and Id3, respectively. Using sequencing, it was confirmed that the right sequences were amplified.

In paper III, the RT-PCR was used to amplify FGFR3, collagen type IIA, collagen type IIB and collagen type X, the products were visualised on an ethidium bromide stained agarose gel to confirm their presence or absence. Ethidium bromide is incorporated into DNA and makes it visible under UV-light.

RNase protection assay

Using Ribonuclease protection assay (RPA), a semi quantitative analysis of a specific RNA can be made. We used a commercially available RPAII-kit from (Ambion, Austin, TX, USA) in paper I.

γ^{33}P labelled ribo-probes for Id1, Id3 and 18S were hybridised with total RNA isolated from chondrocytes and chondrosarcoma cells. Thereafter RNase was used to digest single stranded RNA in the sample. The protected double stranded fragments in the sample were separated using gel electrophoresis on a denaturing polyacrylamide gel and visualised by phosphoimage analysis (Molecular Dynamics, USA). To be able to estimate the levels of Id1 and Id3 expression, Id1 and Id3 were normalised to the 18S probe. 18S has been demonstrated to function as an internal standard in chondrocytes (Matyas et al., 1999; Thellin et al., 1999). In

this assay negative controls were probes hybridised with yeast tRNA. The size of the fragments was compared with a ^{33}P labelled HaeIII DNA size marker (Promega, Madison, WI, USA). The labelling of probes was done with ^{33}P. Usually ^{32}P is used for probe labelling but the advantages of using ^{33}P instead of ^{32}P are that ^{33}P is less energetic and gives better resolution, and is easier to handle. However it is more expensive.
Northern blot can be used to analyse RNA expression, but owing to that RPA is more sensitive and more tolerant of partially degraded RNA, RPA was chosen in paper I.

Methods for protein studies

ELISA (Enzyme-Linked Immuno Sorbant Assay)

ELISA is used to determine whether a protein is present in a sample, if so, to be able to quantify the protein. In paper II, the supernatants from the articular chondrocyte culture, periosteum culture and co-cultures of articular chondrocytes and periosteum as described above, were studied.

To be able to measure TGF-β1 an ELISA kit from Genzyme Diagnostics, USA was used. The detection limit of the assay was 50 pg/ml. IL-8 and GM-CSF were quantified with an in house sandwich ELISA using specific monoclonal antibodies for the respective cytokine. The detection limit for IL-8 was > 40 pg/ml and for GM-CSF > 60 pg/ml.

IL-6 bioassay

In paper II IL-6 concentration in media from articular chondrocyte culture, periosteum culture and co-culture of articular chondrocytes and periosteum was measured. IL-6 concentration was measured with the murine hybridoma B cell line B9. The specificity of the assay has been demonstrated by Helle et al., 1988. Human IL-6 induces the proliferation of the B9 cells. To quantify the proliferation, thymidine incorporation was measured in a beta-counter. A standard curve was made of serial dilution of recombinant human IL-6 of known concentrations.

Western blot

To study the expression of the Id1, Id3 and E12 proteins, Western blot analysis was performed in paper I. Protein extracts were prepared from cultured cells of interest by sonication in dithiothreitol and the amount of protein was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Thereafter 20 μg of protein from each sample was size fractionated on tris-glycin gels and electrophoretically

transferred onto nitro-cellulose membranes. To identify the separate proteins, polyclonal antibodies for IdI, Id3 and E12 were used. They were visualised using chemiluminescence and exposure of the membranes to autoradiography films.

To ensure the specificity of the antibodies used for Id1 and Id3, experiments were performed where the antibodies were pre-incubated with an excess of the recombinant protein for Id1 and Id3 respectively, before the antibodies were added to the membranes. This abolished the positive signal.

Immunohistochemistry

Immunohistochemistry was used to visualise the location of separate proteins both in cultured cells and sections of tissues in papers I and III-V, but with different approaches.

Cultured chondrocytes and chondrosarcoma cells can easily be studied with immunohistochemistry by culturing them in chamber slides, fixate them with e.g. cold acetone, and thereafter performing the experiment in each separate chamber, which was done in paper I. The agarose gel in paper III had to be handled in a different way. Pieces were cut from the agarose gel (5 mm^3), fixated with Histofix (10% formalin, buffered with PBS [0.01 M and pH 7.4], Histolab, Göteborg, Sweden) and thereafter imbedded with paraffin. This resulted in relatively good histological sections suitable for immunohistochemistry. All other material such as biopsies and pellet mass cultures, were fixated with Histofix (Histolab) before paraffin imbedding.

Cartilage is a tissue where immunohistochemistry in paraffin sections can be problematic, owing to the presence of highly negatively-charged proteoglycans, and usually an enzyme such as hyaluronidase is used to digest the matrix before the immunohistochemistry is begun, especially when collagen epitopes are studied.

Independently of whether chamber slides or sections are used, unspecific binding sites always have to be blocked, to prevent background staining. FCS, goat serum, and bovine serum albumin (BSA) are, for example, useful.

For detection of the specific proteins, polyclonal or monoclonal antibodies are used. In this thesis all antibodies except anti-collagen type I, II, aggrecan, Stro-1, beta-catenin, β1-integrin, Bcrp1, BrdU were polyclonal. The advantages of polyclonal antibodies are that they are easily available and their sensitivity is high, while monoclonal antibodies are relatively more expensive but have higher specificity.

The specificity of all antibodies used has to be tested, with positive and negative control tissues or with specific blocking antibodies. The use of negative tissues can be problematic for studying proteins known to be expressed in almost all cells and

tissues. Therefore, a positive tissue where the antibody and the distribution are well described can be used to ensure the specificity of the antibody. To test the specificity of the secondary antibody, the primary antibody was omitted and replaced with normal immunoglobulin from the respective species the primary antibody was made in. When secondary horseradish peroxidase (HRP) antibodies were used as in papers I and IV, the endogenous peroxidase activity has to be irreversibly inactivated by exposing the sections with H_2O_2.

Different technologies can be used to visualise the antibodies. In paper I, a biotinylated goat-antirabbit secondary antibody and diaminobenzidine chromogen were used to visualise the immunoreactive proteins. In paper III peroxidase conjugated secondary antibody with the addition of a substrate kit Vector VIP (Vector Laboratories, Burlingame, Calif, USA) was used for visualisation. In papers IV-V, HRP labelled secondary antibody was visualised using a TSA-Direct Cy3 kit (tyramide signal amplification-direct cyanin3, [Perkin Elmer, Boston, Mass., USA]) where the TSA-Direct Cy3 also functioned as an enhancement step. Results were visualised using a Nikon light microscope in paper I, a Nikon Optiphot2-pol microscope in paper III and in papers IV and V a Nikon fluorescence microscope; Eclipse90i with NIS-elements software.

Transmission Electron Microscopy, TEM

To be able to study the morphology of the separate cell clusters in paper III, small pieces (5 mm^3) were cut out from the agarose gels. A specific fixation for TEM, with glutaraldehyde and potassium ferrocyanide, combined with post-fixation in an OsO4 solution, was used. To be able to first study the sections and appropriate areas, semi-thin sections (0.7 μm) were cut for light microscopy. Ultrathin sections (50-60 nm) were then sectioned for TEM. All sections were contrasted with lead citrate and uranyl acetate before being examined in a Zeiss 912AB digitized electron microscope. Digital images were taken with a Megaview III camera (SIS, Munster, Germany).

Histological staining

In studying cartilage tissues different types of histological staining are of great importance. Dyes often used include Alcian Blue van Gieson and Safranin O. Alcian Blue van Gieson and Safranin O were used in all papers in this thesis. Both stain negatively charged proteoglycans (GAGs) characteristic of hyaline mature cartilage. PH and electrolyte concentration are important to the outcome of the staining (Hyllested et al., 2002, Kavanagh et al., 2002).

Alcian Blue is a cationic dye carrying up to four cationic groups that binds to GAGs. Alcian Blue can be combined with van Gieson dye, where Van Gieson stains collagens. Alcian Blue van Gieson stains cartilage blue and connective tissue red, muscle and cytoplasm yellow. Alcian Blue van Gieson sections are often counterstained with Weigerts hematoxylin to stain cell nuclei black-brownish (Hyllested et al., 2002) (Figure 7).

Safranin O is a monovalent cationic dye and is thus likely to bind weaker to GAGs. Safranin O stains the proteoglycan rich cartilage orange to red, cytoplasm blue-greenish and nuclei black (Hyllested et al., 2002) (Figure 7).

As a complement, polarized light microscopy of the cartilage to examine the collagen organisation is usually also done. Evaluation with a polarized microscope is even easier if the sections are pre-stained with picro Sirius red that stains the collagen fibrils bright yellow or orange (Figure 7).

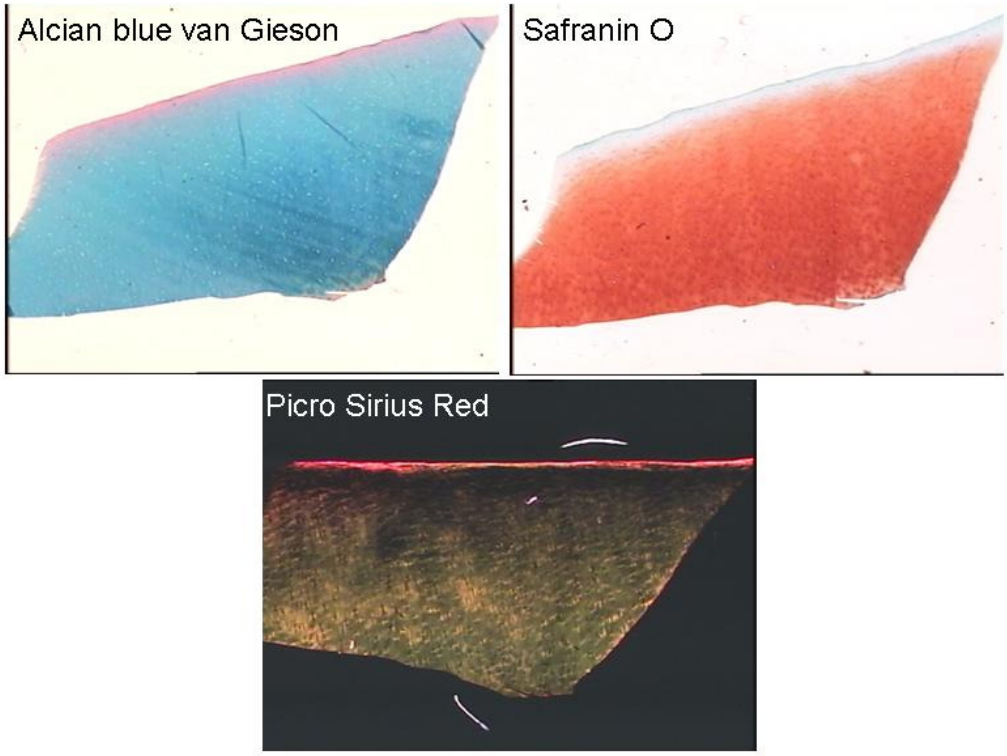


Figure 7. Biopsy from normal human hyaline articular cartilage stained with Alcian Blue van Gieson, Safranin O, and Picro Sirius Red. The Picro Sirius Red stained biopsy is photographed in polarized light. Magnification 20x.

Functional studies

BrdU-labelling *in vitro*

To study the functional role of Id1 and Id3 in paper I, an antisense experiment was performed. According to a protocol by Barone et al., antisense phosphorothioate oligonucleotides covering the translation starts of Id1 and Id3 mRNAs were added to non-confluent cultures of chondrocytes and chondrosarcoma cells in chamber slides to study their effect in blocking DNA synthesis and proliferation (Barone et al., 1994). 5-bromo-2-deoxy-uridine (BrdU) is a thymidine analogue that is incorporated into DNA of dividing cells and was therefore added to be able to study the effects of the antisense treatment on proliferation. Briefly, to exclude the growth factors in normal culture media the cells were exposed to serum withdrawal for a period of 23 hours, after which the cells were cultured for 1 hour in the presence of the antisense phosphorothioate oligonucleotides and then further stimulated with serum addition for 24 hours to initiate proliferation. The number of cells that incorporated BrdU was then counted to calculate the effects of Id1 and Id3 antisense in treated and non-treated cultures. In our study a minimum of about 150 cells were counted in each experiment (cells in 10 visual areas of a chamber).

The Id1 and Id3 antisense and sense oligonucleotides were modified with phosphorothioate to make them stable, water-soluble and increase their penetration into the cell. As a control, a random oligonucleotide is appropriate to confirm that addition of oligonucleotides is non-toxic to the cells.

Methods to study multilineage differentiation

Phenotypic plasticity assay can be used to study a specific population of cells and their ability to show multilineage differentiation, for example in forming the three different mesenchymal phenotypes cartilage, fat and bone. In paper III the phenotypic differentiation capacity of an isolated subpopulation of chondrocytes was studied using this assay (figure 8). The differentiation into cartilage was done in a pellet model where the cells are allowed to differentiate into a 3D high-density pellet as described above in the pellet mass culture section.

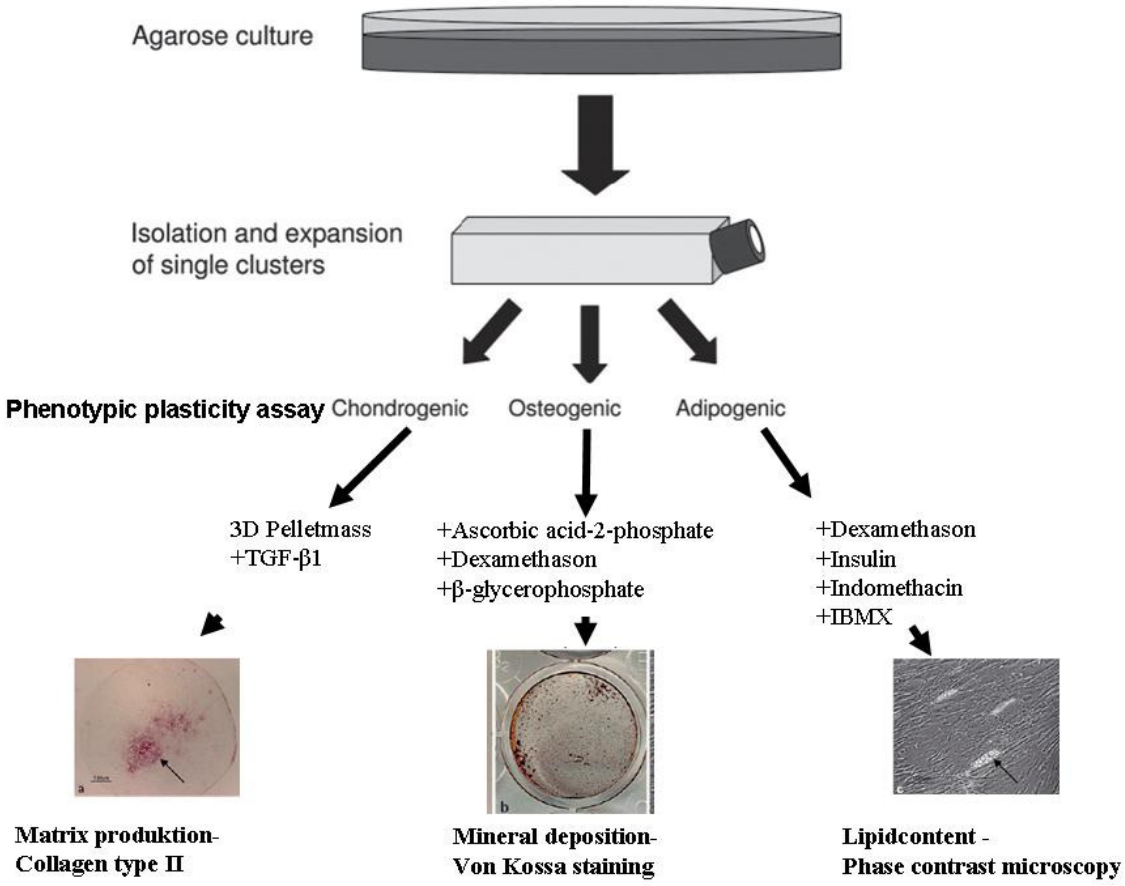


Figure 8. Schematic overview demonstrating the phenotypic plasticity assay and multilineage differentiation of cell clusters.

To analyse the fat differentiation capacity, formation of cytoplasmic lipid droplets typically seen in pre-adipocytes were detected with phase contrast microscopy as described by Pittenger et al., 1999. The control medium including DMEM-LG, L-glutamine, and FCS was supplemented with adipogenic inductive components as insulin, dexamethasone, isobutylmethylxanthine (IBMX) and indomethacin to facilitate adipogenic lineage.

Von Kossa staining, i.e. staining of the deposition of calcium that appears during mineralization, was used to study ability of the cells to differentiate towards the osteogenic lineage. For the osteogenic assay specific osteogenic additives were added to the control medium, such as dexamethasone supplemented with beta-glycerophosphate (Lennon et al., 1995; Jaiswal et al., 1997).

Methods for identifying slow cycling cells

BrdU labelling *in vivo*

BrdU is usually used as a marker of proliferation as described in *the BrdU-labelling in vitro* section above. In paper IV, BrdU was also used for detection of slow cycling cells. Stem cells are usually defined as cells with very low cell turnover. Using BrdU it is possible to discriminate between rapidly proliferating cells and cells with slow turnover, such as stem cells (Cotsarelis et al., 1990; Potten et al., 2002). To be able to identify the pool of cells with a very slow rate of proliferation, i.e. the stem cell population in the joint, labelling with BrdU was used in a rabbit experiment according to Hunziker et al., 2006. Since BrdU has a short half-life of a few hours duration in both synovial fluid and serum (Bicknell et al., 1994), it has to be administered continuously on a daily basis for a period that covers the estimated cycling time of the stem cells, i.e. 12 days according to Hunziker et al., 2006. To be able to discriminate proliferating cells from slow cycling cells animals were sacrificed 4, 6, 10, 14, 28, and 56 days after the first BrdU administration. The BrdU was administered by adding it to the rabbit's drinking water in a concentration of 25 mg/kg of body weight daily. During administration of BrdU the animals were kept in separate cages in order to monitor their BrdU intake.

Control animals not exposed to BrdU were kept simultaneously during the experiments. Positive controls were sections from skin obtained from BrdU exposed rabbits. BrdU was detected by immunohistochemistry on sections from the selected parts of the rabbit knee.

Statistical Analysis

Standard statistical methods were used to calculate means, standard deviations and standard errors.

To determine the effects of Id1 and Id3 antisense oligonucleotides on cultured cells in paper I, the statistical significance of differences between means of labelled cells was calculated using Student's t-test for dependent samples. The samples were considered to be normally distributed with equal variance.

Student's t-test for dependent samples was also used in paper II, where the effect on colony stimulation with conditioned medium was studied. Wilcoxon´s non parametric rank sign test was used for paired observations and to compare different groups in paper II, where we considered the distribution to be unknown.

In paper III the degree of association between age, sex and cluster formation was calculated using the correlation coefficient Pearson r. If there had been a strong correlation, a value around r=0.8 would have been expected.

SUMMARY OF RESULTS

The helix-loop-helix proteins Id1 and Id3 play a role in the proliferation of adult articular chondrocytes (I)

To study the expression of the transcription factors Id1 and Id3 RNase protection assay and Western blot were used. Both Id1 and Id3 mRNA were expressed in normal chondrocytes and chondrosarcoma cells cultured in monolayer. However, the expression of Id1 mRNA was higher in chondrosarcoma cells than in normal chondrocytes. In normal chondrocytes the Id1 expression was regulated by the presence of serum, with almost abolished expression after serum withdrawal, while the expression in chondrosarcoma cells was not affected by serum withdrawal. Id3 mRNA expression was not dependent on the presence of serum in either normal cells or chondrosarcoma cells.

When protein expression was studied, Id1 protein correlated well with the Id1 mRNA expression in most cases. After serum withdrawal Id1 protein was only detected in chondrosarcoma cells. Id3 varied greatly at the protein level in cells cultured in the presence of serum but was totally absent after serum withdrawal in both chondrocyte and chondrosarcoma cell cultures.

Immunohistochemistry was carried out on both cultured chondrocytes and chondrosarcoma cells to study the cellular location of Id1 and Id3. Examination of E12 proteins was also added to this study, since they are known to function as heterodimerization-partners to both Id proteins and tissue specific bHLH proteins. Id1 showed mainly cytoplasmic expression, while Id3 could be detected both in cytoplasm and nucleus. E12, on the other hand, showed nuclear staining in normal chondrocytes and pre-confluent chondrosarcoma cells, while the nuclear staining was absent in areas of confluent chondrosarcoma cells.

To study the function of the Id proteins, antisense experiments were carried out. Antisense oligonucleotides against Id1 and Id3 mRNA were added to proliferating non-confluent cultures and proliferation (DNA synthesis) was determined using BrdU labelling. A significant decrease in DNA synthesis was noticed when antisense nucleotides against Id1 and Id3 were added, both in normal chondrocytes and chondrosarcoma cells. BrdU incorporation in chondrosarcoma cells was reduced from 43% to 30% with Id1 antisense oligonucleotide and to 26% with Id3 antisense oligonucleotide. In chondrocytes the reduction was from 39% to 33% for Id1. The disruption of cell boundaries after addition of Id3 to normal chondrocytes and low-grade chondrosarcoma cells was noteworthy. Additional BrdU

experiments showed that chondrosarcoma cells were able to undergo cell division even without serum.

Chondrocytes proliferate and show clonal growth in agarose suspension culture (II)

Chondrocytes have been cultured in agarose suspension after monolayer culture to verify the chondrogenic phenotype of cells used in ACI. In paper II a more thorough study was performed on the agarose suspension cultured chondrocytes. Using phase contrast microscopy it was possible to study different morphological cell growth of the chondrocytes. To identify the matrix formed in the culture the gels were stained with Alcian Blue and Safranin O. Most of the cells remained as single cells with more or less surrounding matrix. Some cells started to divide and formed cell clusters (same as cell clones). When a cluster was larger than 50μM it was verified as a true cluster, i.e. containing more than four cells. Depending on their morphological appearance, the clusters containing few cells with matrix between the cells and surrounded by matrix were called differentiated clusters (D). Some of the cell clusters formed showed a dense homogenous cell cluster with surrounding matrix, named matrix clusters (M) and, finally, cell clusters consisting of only a spherical mass of cells without surrounding matrix were called homogenous cell clusters (H). The number and types of cell clusters were independent of the age or sex of the patients. There was no significant difference in number of cluster formed if the chondrocytes were primary isolated (direct from cartilage to agarose suspension culture) or passaged in monolayer culture before agarose suspension culture. However, there was a great difference between individuals in the distribution and relative numbers of clusters formed.

The periosteum demonstrates biological effects on the chondrocytes (II)

To study the influence on the chondrocytes of the periosteum used in ACI, different *in vitro* experiments were carried out: co-cultures in agarose of chondrocytes with or without the presence of periosteum and chondrocytes cultured in presence of either conditioned medium from fibroblast cells, periosteal tissue, periosteal cells or chondrocytes.

The presence of periosteal tissue or conditioned medium from periosteal tissue or periosteal cells showed a stimulatory effect on cluster growth, especially an increased formation of D cell clusters was noticed. Further study of the culture media from periosteum, as well as chondrocytes and co-cultures of both demonstrated that periosteum produced significant amounts of IL-6, IL-8 and TGF-β. Chondrocytes produced IL-6, IL-8, GM-CSF and TGF-β and, finally, co-cultures

of chondrocytes and periosteum demonstrated a significant increase of only IL-6 and IL-8.

A subpopulation of adult chondrocytes with progenitor properties can be identified using agarose suspension culture (III)

As described in paper II, agarose suspension culture can be used as a method for identifying the chondrogenic phenotype of cells. In paper II it was also demonstrated that chondrocytes showed different proliferation and growth capacities in agarose suspension cultures. In paper III further studies of the cell clusters in agarose suspension was performed. While cell division began about 5 days after culture start, different types of cell clusters were ultimately formed after 6 weeks with about 3.6% (range 1.5–6.1%, n = 18) of the cells seeded forming cell clusters > 50 μm in diameter. Further culture time did not increase the number of clusters formed. Similar as in paper II no correlation between sex, age and cluster formation could be found, nevertheless a great difference between individuals was noticed. By studying the combination of the morphological appearance and ability to grow in paper III, the different clusters formed were named as given in paper II, with the addition that M clusters were divided into homogenous matrix (HM) and the differentiated matrix (DM) clusters, because they displayed different morphology and growth properties (figure 9). The matrix produced from all clusters expressed collagen type II as well as aggrecan and COMP. Using RT-PCR, expression of collagen type IIA and IIB as well as FGFR3 was demonstrated in all the clusters, while none expressed collagen type X, a marker of hypertrophic chondrocytes. TEM also morphologically demonstrated two types of cells in the H cell clusters, whereas the HM cell clusters only contained one type of cell. The matrix proteins shown using TEM also differed in composition between the H and HM cell clusters. Fine fibrillar proteins were found within the H cell clusters while the matrix demonstrated a ramified structure within the HM cell clusters.

The number of cell clusters formed was inversely related to the seeding density in the agarose suspension. Chondrocytes seeded at a higher density $45x10^4$ cells/50 mm culture dish compared to $5x10^4$ cells/50 mm culture dish showed both a lower percentage of cluster formation 0.27% instead of 3.1% and a different distribution of cell clusters, with only DM and D clusters present. The majority of cells in these cultures with high density were single cells or pairs of cells.

To study the proliferative properties of the different cell clusters, they were examined on the basis of their ability to further subculture and to expand in monolayer. All of the H and HM cell clusters, 0% of the DM and 11% of the D cell clusters could attach after subculture, and further expansion was possible for about 67% (H), 56% (HM), 0% (DM) and 11% (D) of the cell clusters.

To demonstrate progenitor properties and phenotypic plasticity of the cells from the different cell clusters, the cells were cultured in cartilage, fat and bone multilineage assay after subculturing. Only cells from the H and HM cell clusters could be studied in the assay, while neither DM nor D cell cluster could be studied because of lack of growth in monolayer. When cells from H and HM cell clusters were studied in the assay both lipid droplets, mineralization, and pellets containing type II collagen were demonstrated, indicating mesenchymal stem cell/progenitor properties of cells from these two types of clusters.

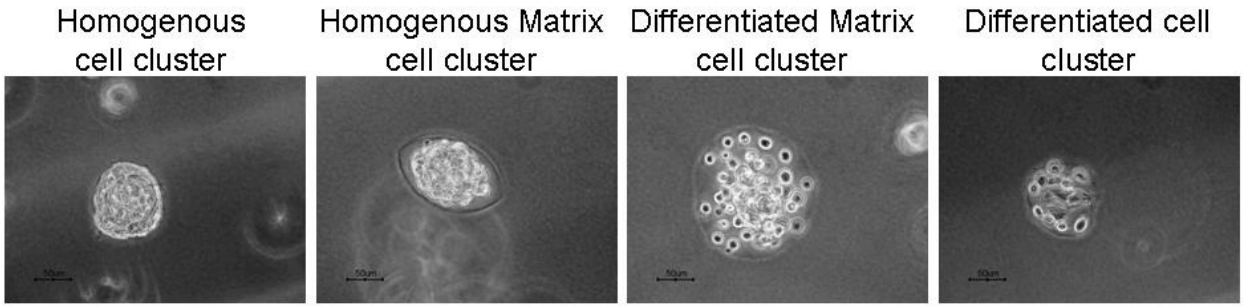


Figure 9. The four different types of cell clusters identified in agarose suspension culture.

Progenitor cells exist in the perichondrial groove of Ranvier and in the articular cartilage of rabbits (IV)

To be able to identify stem cells/progenitor cells in the knee joint, a rabbit model was used. In order to discern between rapidly and slowly proliferating cells, BrdU labelling was done. The animals were studied at different time points. Rapidly proliferating cells or slowly proliferating cells incorporating BrdU into their DNA were detected after 10 to 12 days from the start of BrdU exposure. Cells were detected mainly in the epiphyseal plate, the zone of Ranvier and all zones of articular cartilage (although in smaller amount). At the early time points the BrdU positive cells were either rapidly or slowly proliferating cells, but it was not possible to separate these populations. There was a marked decrease in positive BrdU cells at late time points such as 28 and 56 days after the start of BrdU exposure. Positive BrdU cells at these time points were supposed to be slowly proliferating cells. Slowly proliferating cells were found in the germinal zone of the growth plate as expected, and in the apical area of the perichondrial groove of Ranvier. Although there was almost no expression of BrdU positive cells in the proliferative zone of epiphyseal cartilage, some slow cycling cells were still noticed in the growth plate near the perichondrial groove of Ranvier. At late time points a few slow cycling cells were still detectable throughout the articular cartilage, and these cells often had a columnar appearance.

Stem cell niche associated markers are expressed in the rabbit joint (IV)

The markers associated with stem cells/progenitor cells and stem cell niches: Stro-1, Notch1, Patched, Jagged1, BMPr1a, β1-Integrin and N-cadherin were used to study the locations where slow cycling cells were found. The most abundantly expressed markers were Jagged1 and Stro-1, they were detected on almost all cells in the perichondrial groove of Ranvier, while cells in the growth plate directly adjacent to the perichondrial groove of Ranvier did not express these markers. Jagged1 positive cells formed a distinct boundary between cells in the surrounding tissue and the perichondrial groove of Ranvier. N-cadherin, Patched, and BMPr1a were expressed on small numbers of cells in the perichondrial groove of Ranvier, but they were more abundant than BrdU positive cells at late time points. The Notch ligand Delta4 was expressed on cells just above the perichondrial groove of Ranvier.

In the articular cartilage Notch1, Stro-1, and N-cadherin were exclusively expressed on cells in the superficial zone. Stro-1 positive cells were detected in the uppermost 2 cell layers of the articular cartilage, while Notch1 and N-cadherin were detected in cells in the deeper layers of the superficial zone. BMRr1a and β1-Integrin were located in the superficial cells but with positive cells also detected deeper in the cartilage than Notch1, Stro-1, and N-cadherin. Delta4 was expressed in the transitional and deeper zones of the articular cartilage. None of the markers studied seemed to be exclusively expressed by the slow cycling cells.

Stem cell associated markers Stro-1 and Bcrp1 are expressed in human articular cartilage (V)

To further study the proliferation, differentiation and potential progenitor cells in human articular cartilage we used an in vitro 3D pellet mass culture model demonstrating cartilage regeneration, previously presented by Tallheden et al., 2004. Stro-1 was detected all over the day 7 pellet in almost all cells, while after 14 days a decrease was noticed and most of the positive cells showed a zonal expression in the outer part of the pellet mass. Bcrp1 demonstrated similar expression on day 7 as day 14 concerning both the amount of positive signal and location, and no expression in the outer zone of the pellet could be detected.

To be able to study these markers *in vivo*, normal articular cartilage and biopsies from regenerated and repaired articular cartilage were used. In normal hyaline cartilage there was a zonal expression of Stro-1 and Bcrp1. Stro-1 was expressed in the upper 2-4 cell layers of the superficial zone whereas Bcrp1 was demonstrated in the deeper part of the superficial zone, although Bcrp1 showed lower expression. In the high ICRS scored ACI biopsies demonstrating normal histological architecture,

Stro-1 was expressed in cells in the superficial layer of the biopsy similar to in normal cartilage. In repaired cartilage containing cell rich areas and cell foci, Stro-1 expression was increased throughout the biopsies. This was noticed in the OA biopsies as well. Bcrp1 was expressed in a sparse population of cells in all cartilage studied.

Regenerated and repaired human articular cartilage show different expression of the Wnt and Notch signalling pathways (V)

The same 3D regeneration model as above was used to be able to study the expression and activity of the Wnt and Notch signalling pathways during regeneration of cartilage. Frizzled showed decreased expression from day 7 to day 14 where it was almost absent. The marker for the Wnt canonical pathway, beta-catenin, could still be detected at day 14 in the elongated cells in the outer zone of the pellet, at least at low levels. In the proliferating day 7 pellet, HES5 was positive in some cells but decreased significantly on day 14, where it was almost absent, although a few positive cells were located in the outer zone of the pellet.

The activity of these pathways was also studied in biopsies from normal human adult articular cartilage as well as in biopsies from various types of regenerated and repaired cartilage. In normal articular cartilage weak expression of beta-catenin was found in the same cell layers that expressed Stro-1 and Brcp1. Frizzled was found at low expression in the deep superficial and upper transitional zones. Hes5 was identified in the superficial zone in the same area as Stro-1. In the high scored ACI biopsies with almost normal histological appearance, beta-catenin, Frizzled and HES5 all showed zonal distributions as in normal articular cartilage. Expression was also found in cell foci (usually found in these biopsies close to bone). In the low scored ACI biopsies with a disorganized morphology and fibrocartilage formation, beta-catenin and Frizzled both showed increased expression throughout the biopsies. HES5 was virtually absent in the low scored biopsies, while an increase could be noticed in more hyaline like parts of the biopsies. In all biopsies from debrided injured cartilage beta-catenin and Frizzled demonstrated general expression throughout the fibrotic parts, with a slight increase in cell foci. HES5 showed regular expression in the part of the biopsy with hyaline like cartilage, while almost no expression was demonstrated in the fibrotic part. Frizzled and beta-catenin expression were both increased throughout the OA lesion biopsies as compared with normal cartilage from the same OA patient. HES5 showed low expression in the OA lesion biopsy with the exception of cell foci, cells close to bone, and columnar cells. Interestingly, the expression of HES5 seemed to be higher in the “normal” biopsy of cartilage from the same OA patient.

DISCUSSION

The overall aim of this thesis was to search for chondrocytes with progenitor properties, and to study associated signalling pathways. This was done by performing studies at molecular and cellular levels. Today tissue engineering for treatment of articular cartilage lesions is a field of growing interest. The results from this thesis will hopefully add some important facts to the understanding of articular cartilage and improvements of regenerative medicine and tissue engineering methods, as discussed below.

Proliferation of chondrocytes *in vitro* and a role for the HLH transcription factor Id1

Proliferation of cells in the articular cartilage is assumed to be low. To be able to study proliferation of chondrocytes, their behaviour and genetic activity, cell cultures in monolayer are usually used. In paper I, we wanted to study the transcriptional regulation of proliferation and differentiation in chondrocytes. For this reason, cultures of surplus chondrocytes from ACI as well as chondrosarcoma cells were used. The chondrosarcoma cells were chosen to represent the less differentiated stage of chondrocytes.

Cells in monolayer culture need culture medium. Normally, culture medium is supplemented with human serum or FCS that contains growth factors supporting proliferation of the cell culture (Jakob et al., 2001). In cell cultures for ACI, autologous serum is used (Brittberg et al., 1994, Tallheden et al., 2005a). When serum is withdrawn from a cell culture proliferation of the chondrocytes stops and the cells are growth arrested, because the components in the culture media that are the prerequisite for cell division disappear. This understanding was used in paper I to establish a proliferation-non proliferation model. Neither Id1 mRNA nor protein was found when chondrocytes were subjected to serum withdrawal. This finding is consistent with the link between Ids and the proliferation/differentiation process. Antisense experiments also showed significantly lower cell proliferation when antisense for Id1 was added to either chondrocytes or chondrosarcoma cells. This supported the hypothesis that Id1 was associated with proliferation of cells of cartilaginous origin and directly or indirectly stimulated cell division in the same way as in other tissues e.g. mammary epithelium (Desprez et al., 1995) and NIH 3T3 cells (Barone et al., 1994). The tumour cells from chondrosarcoma, on the other hand, expressed both Id1 mRNA and protein, irrespective of serum conditions. The strong Id1 protein expression even after serum withdrawal indicates another regulatory mechanism which induces a less differentiated state of the tumour and indirectly stimulates

proliferation. Furthermore, the BrdU-labelling experiments showed that chondrosarcoma cells were able to undergo cell division even without serum, indicating a non-serum dependent regulation of Id1 in chondrosarcoma cells. Recent studies have demonstrated Id1 stimulated but serum independent growth of prostate cancer cells through inactivation of the $p16^{ink4a}$/pRB pathway by Id1 (Ouyang et al., 2002). It has recently been shown in tumour tissues that Id1 plays an intricate role and is involved in the promotion of cancer progression through regulation of multiple pathways (Wong et al., 2004; Ling et al., 2006).

In any case, the role of Id proteins as key proteins in signalling in normal cells as well as during cancer genesis is unknown, and the regulation upstream and downstream of the Id genes is far from clear, as has been reviewed in Ruzinova and Benezra, 2003. In paper I, we presented the involvement of Id proteins in proliferation and differentiation of human chondrocytes. Using Id1 as a tool in our study we found for the first time that one or more HLH factors may control the growth and differentiation of chondrocytes. The E12 protein studied in paper I demonstrated a nuclear staining, indicating that there might exist a bHLH dimerization partner in the nucleus. Although we have not yet seen confirming results regarding the existence of a tissue specific bHLH protein in cartilage like MyoD and Myf5 in muscle tissue (Weintraub et al., 1991; Braun et al., 1989), it seems realistic to believe that Id1 has a direct role in regeneration and different cartilage pathologies. In e.g. OA there is increased proliferation of cells, increased activity of matrix metalloproteases (MMPs) and increased Wnt signalling (Aigner et al., 2006; Blom et al., 2009; Luyten et al., 2009). It has been demonstrated that Id1 up-regulates MMPs during tumour invasion and cancer progression (Fong et al., 2003; Ling et al., 2006). MMPs have a given role in OA development and the linkage between Id proteins and MMPs in cartilage would be of interest to study. Furthermore, Id signalling has been coupled to the Wnt-beta-catenin and BMP pathways in both myoblasts, and during neurogenesis and osteogenesis, which also suggests cross-talk between these signalling pathways in normal cartilage regeneration and in OA, although this requires further investigations (Nakashima et al., 2001; Katagiri et al., 2002; Nakashima et al., 2005).

In paper V we demonstrated that the negative acting HLH protein HES5 is assumed to be involved in the regeneration of human articular cartilage. The HLH genes Dec1, HES1 and HES5 have previously been suggested to play a role in chondrogenesis *in vivo* and *in vitro*, (Shen et al., 2002; Watanabe et al., 2003; Karlsson et al., 2007b). Some studies have shown direct interaction between the Ids and the HES proteins, as during the development of the neural tubes of chicks where the Id proteins participate in neuronal stem cell maintenance by sustaining HES1 expression (Bai et al., 2007). It has also been demonstrated that Id proteins interact with HES1 in neuroblastoma (Jögi et al., 2002). Accumulating data adds further evidence of a role for the HLH-family of transcription factors in human articular cartilage.

Subpopulations of chondrocytes demonstrates different growth potential

The chondrocytes used for ACI have been phenotyped by culturing in agarose suspension after monolayer expansion. We were able to separate potential contaminators from the chondrocytes with the agarose suspension culture in that we were unable to establish cultures of either fibroblasts, periosteal cells or MSC, as proposed by Benya and Shaffer (Benya and Shaffer, 1982). In our studies in papers II and III more than 90% of the chondrocytes remained single cells in the agarose culture, with a small amount of surrounding matrix indicating differentiated cells. Some of the single-cell seeded chondrocytes began to divide after 5 days in culture and after 1 week cell clusters appeared (i.e. one single cell formed a clone). When the separate cells were followed during culture time the clusters formed after one week did not change in morphology over the next 10 weeks, except for an increase in size. After 6 weeks of culture a small population of the initial number of cells seeded, 3.6% had formed cell clusters defined as > 50 μm. We therefore postulated in paper II that articular chondrocytes consist of subpopulations of cells with different growth properties in agarose suspension regarding proliferation and differentiation. This theory has not been described previously with the same culture technique (Benya and Shaffer, 1982; Buschmann et al., 1992). Higher seeding density could possibly inhibit cell populations with proliferative properties through contact inhibition, which might explain the lack of cluster formation in these studies where a cell density of $2x10^6$ to $2x10^7$ cells/ml agarose medium was used (in our study in paper III $16.7x10^3$ cells/ml was used). The results in paper III further support the idea that initial seeding density could be of importance. If the cell density affects the ability of the chondrocytes to proliferate and favour certain populations of progenitor cells and/or favour matrix producing cells still has to be evaluated. Do we want proliferation or differentiation or both in the tissue engineering process? This is of particular interest when using scaffolds or carriers for the transplanted cells. In traditional ACI high cell densities are used, $30x10^6$ cells/ml cell suspension administrated, but the optimal cell density to use in scaffolds or carriers are under evaluation in a lot of studies (LeBaron and Athanasiou, 2000; Brittberg et al., 2003; Concaro et al., 2008; Freyria et al., 2008).

Subpopulations of chondrocytes with progenitor properties

In recent years many papers have described the existence of stem cells in specific tissues previously considered to lack them (Gage, 2000 and references therein, Beltrami et al., 2003). A great deal of data points to the existence of progenitor cells in adult cartilage, but it remains to be seen whether any of these cells are true stem cells. Results to date, both in papers III and IV in this thesis and in the work of others indicate that there are subpopulations of chondrocytes with potential to take part in possible repair or regeneration in the adult cartilage, although no definitive

marker has yet been identified for these progenitors (Barbero et al., 2003; DellAccio et al., 2003; Tallheden et al., 2003). In all studies searching for stem cells we have to be aware of the fact that stem cells are difficult to define and recognize morphologically in their *in vivo* location. Their stemness may vary according to their physiological status and the environment, which makes it difficult study possible stem cells *in vitro* in other surroundings than their original locations (Kindler, 2005).

Nevertheless, in paper II we did demonstrate that chondrocytes used for ACI showed subpopulations with different growth capacities when cultured in agarose. Depending on both morphological properties and growth potential the cell clusters were further divided into H, HM, DM and D cell clusters in paper III. This is in analogy with dermal tissue, much studied for many years and known to form various cell clones representing different grades of differentiation in culture (Barrandon and Green, 1987). The DM and D cell clusters in our study were assumed to represent more differentiated chondrocytes, while the H and HM cluster types represented more primitive clusters of chondrocytes. All cell clusters demonstrated chondrogenic phenotypes in that they produced typical extracellular matrix proteins in the agarose and expressing genes, such as collagen type II, aggrecan and COMP. None of these cell clusters demonstrated collagen type X, indicating that they did not represent a hypertrophic phenotype of cartilage. This is consistent with the hypothesis that the chondrogenic potential of dedifferentiated articular chondrocytes from former monolayer culture has distinct features from cells from bone marrow stroma, epiphyseal chondrocytes and periosteal cells, which choose the path through hypertrophic chondrocytes and endochondral ossification (Binette et al., 1998).

To further elucidate the properties of the different clusters except for their growth potential in agarose suspension, they were studied regarding their mesenchymal stem cell (MSC) properties. Because only the H and HM clusters demonstrated high proliferative potential after subculturing they were the ones further studied. Both clusters did show MSC properties and demonstrated osteogenic and adipocytic lineage differentiation apart from their chondrogenic lineage.

Although the H and HM cell clusters demonstrated similar progenitor properties: good proliferative potential and ability to form other mesenchymal tissues, they showed morphological differences. The H cell cluster contained a flat layer of endothelial like cells surrounding the dense central cell population of chondrocytes. The flat cells seemed to restrain matrix proteins to be secreted out into the gel, while the HM cell clusters lacked the surrounding cells and only consisted of the dense population of chondrocytes surrounded by secreted matrix. The H clusters might have additional progenitor properties, i.e. the ability to differentiate into two distinct cell types, since one of the cell-type must have given rise to the other. This finding that one single cell forming the H cell cluster, might differentiate into similar chondrocytes as them found in the surface zone of the articular cartilage the

flat endothelial like cells and the matrix-producing chondrocytes found in the transitional zone, is in line with the appositional growth hypothesis and hierarchical model described by Hayes et al., 2001. Therefore, the question of which layer the cells and clusters formed in the agarose culture origin from is of importance. It has previously been demonstrated in articular cartilage that there exist subpopulations with morphological heterogeneity when human articular cartilage was separated into surface cells and deep zone cells and cultured in agarose suspension cultures (Archer et al., 1990). Archer et al. demonstrated that cells from the superficial zone formed a type of cell cluster with morphological similarities to our H cell clusters in papers II and III. In their study, cell clusters from the superficial zone showed similar outer whorl of flattened cells enclosing central cells with little extracellular matrix as our H clusters (Archer et al., 1990). Our cultures are from full thickness articular cartilage biopsies used for ACI, and therefore we can not assume that the H cluster is from a specific layer. The difficulties associated with working with articular cartilage biopsies, especially from humans, and trying to isolate a specific layer for further culture has been a limitation for us in these studies (data not published). Further support for a separate origin of the clusters can be seen in our findings in paper III, which showed based on TEM studies that matrix produced by the two cell clusters also seemed to be different. H cell clusters only formed a fibrilar matrix, but HM cell clusters formed matrix of both the fibrillar structure and aligning granules. Similar findings have been shown by others, where the cells or clusters from the superficial zone formed a fibrillar matrix, presumably collagen, while the cells or clusters from the deep zone formed a matrix fibrillar network rich in proteoglycan granules aligning along the collagen network (Aydelotte and Kuettner, 1988; Archer et al., 1990). Nevertheless, which of the clusters represent true stem cells and which transient amplifying stem cells remains to be elucidated.

It is still debatable whether articular cartilage tissue contains progenitor cells or whether the phenotypic plasticity detected *in vitro* is due to dedifferentiation of the cells during monolayer expansion. In an attempt to describe the properties of primary isolated chondrocytes as compared with chondrocytes that have been passaged in monolayer, we cultured primary isolated chondrocytes and chondrocytes passaged in monolayer, in agarose suspension cultures in paper II. Interestingly, there was no difference in clonal capacity, indicating that the assumed de-differentiation in monolayer cultures did not change the composition of cell clusters or number of cluster formed. The number of progenitor cells remained constant.

The agarose suspension culture was used to study the effects of periosteum on chondrocytes in paper II. We demonstrated that the periosteum used in ACI secreted significant amounts of IL-6, IL-8, GM-CSF and TGF- β. The paracrine effect from the periosteum increased chondrocyte cloning and the number of D cell clusters. Nevertheless, the functional role of the periosteum and the studied cytokines and growth factors in cartilage repair remains to be elucidated. In an

experiment not previously published we were able to decrease the number of cell clusters formed. After adding IL-1 to the agarose suspension cultures almost no cell cluster formation was noticed. If IL-1 was added together with TGF-β1 or if TGF-β1 was added after withdrawal of IL-1, the inhibitory effect disappeared and TGF-β seemed to "rescue" the cells, which started to form clusters again, and exclusively H cell clusters. This indicated that the IL-1 inhibiting effect on chondrocytes could act on cell populations with progenitor potential. It remains to be seen whether this is applicable *in vivo* in development of OA. These results support previous findings demonstrating an intricate interplay between the IL-1 and the TGF-β systems in cartilage and particular in OA (Rédini et al., 1993; Pujol et al., 2008).

Progenitor cells detected in small numbers dispersed in the articular cartilage *in vivo* in rabbits

In papers II and III the studies were performed *in vitro*, and indicated that there are subpopulations of chondrocytes with stem cells/progenitor properties. To be able to prove the existence of possible stem cells *in vivo*, such experiments have to be performed. Models using BrdU for this purpose have previously been used in mouse (Zhang et al., 2006), rat (Naylor et al., 2005), and rabbit (Hunziker et al., 2006), as well as in human beings (Eriksson et al., 1998). The BrdU positive cells represent the label-retaining cells or the slow cycling cells, assumed to be the stem cells.

In articular cartilage it has been suggested that there are different locations of potential stem cells. The focus has been on a superficial location (Hayes et al., 2001; Dowthwaite et al., 2004; Hunziker et al., 2006; Hattori et al., 2007). It has been demonstrated, according to the theory of appositional growth, in 3-months-old monodelphis domestica that there are slow cycling cells on the surface of the articular cartilage. In that study, BrdU labelling of the animals was used to identify the cells (Hayes et al., 2001). In our study in paper IV we were also able to demonstrate label-retaining cells in the articular cartilage using BrdU, but there was no exact zonal location, although some cells were identified within the superficial zone. Explanations for the discrepancy found could be the different maturity grades of the animals used, or different arrangements and thickness of cartilage. The *monodelphis domestica* reach puberty at the age of 6 to 8 months while the rabbit reaches puberty at the age of 3 months. The animals in that study were younger and showed more isotropical cartilage than our animals, which had structurally more mature cartilage. An explanation is that as the articular cartilage grows, the progenitor cells are not only found in the superficial zone instead they are more dispersed throughout the articular cartilage as the tissue becomes less isotropic. This may also explain why young individuals show better repair capacity of superficial lesions than older individuals (Messner and Maletius, 1996; Wei et al.,

1997; Namba et al., 1998). It remains to be evaluated whether this is the case in adult human articular cartilage.

A panel of markers known to be associated to stem cells and their environment were used for further investigation of the rabbit articular cartilage. Neither Stro-1 nor Notch1 were exclusively expressed on the label-retaining cells, and thus are not specific markers for early progenitor cells but it remains to be further explored if the Stro-1 and Notch1 expressing cells are less mature chondrocytes. Stro-1 was expressed in a zone above the Notch1 expressing cells, indicating that these cells represent another population. The Delta4 expression found deeper in the cartilage than Notch1 suggests that the signalling in the Notch pathway passes through zones of the cartilage and, as previously suggested, Notch1 may have several different functional roles in cartilage homeostasis (Dowthwaite et al., 2004). The zonal expression of β1-integrin slightly beneath the N-cadherin positive zone, might be explained by signalling between different cartilage zones and more or less differentiated cells and their matrix. In summary, the strict zonal expression of all the markers further strengthens the involvement of these important proteins in the tissue, although their function and importance *in vivo* has to be further explored.

Identification of a potential stem cell niche in the joint

In paper IV we could demonstrate, except in the articular cartilage, the expression of slow cycling cells in the germinal zone of growth plate, as concluded on the basis of previous studies (Ohlsson et al., 1992; Abad et al., 2002). The finding of slow cycling cells in the perichondrial groove of Ranvier was of particular interest. The perichondrial groove of Ranvier has previously been demonstrated to contain proliferating cells (Shapiro et al., 1977). Shapiro et al. also showed that this area contained densely packed cells the morphology of which was similar to mesenchymal stem cells (Shapiro et al., 1977).

Stem cells are in some tissues located in a special microenvironment, the "niche". Examples of niches are described by Marshman et al., 2002; Zhang et al., 2003 Fuchs et al., 2004; Cotsarelis, 2006 and Yin and Li, 2006. It is tempting to think that the joint also contains niche-like structures, or at least a micro environment with similar signalling pathways as a niche. A panel of previous studied markers known to be associated with the niche was therefore used. The distinct expression of Jagged1 and Delta4, markers of Notch signalling within the groove of Ranvier supports the idea of a micro environment regulating cell commitment (reviewed in Watt and Hogan, 2000; Fuchs et al., 2004; Song et al., 2007; Mitsiadis et al., 2007). Furthermore the presence of markers known to control the number of progenitor cells BMPr1 (Zhang et al., 2006) and to regulate stem cell renewal as Patched (Moshiri and Reh, 2004) and presence of the critical cell-to-cell adhesion molecule N-cadherin known from niche structures (Hayashi et al., 2007) all suggest a

delicate balance in this micro environment, further supporting the idea that the groove of Ranvier behaves like a niche. The fact that all these markers were more abundantly expressed than the slow cycling cells demonstrated that none of these markers per se was a specific progenitor marker. Nevertheless, preliminary results have demonstrated expression of the stem cell marker Bcrp1 on a few cells in the groove of Ranvier in rabbit (data not published).

We were able to detect a larger number of BrdU positive cells in the epiphyseal plate near the perichondrial groove of Ranvier than in the central area of the epiphyseal plate at late time points. This might be explained by the fact that cells from the perichondrial groove of Ranvier migrate into the epiphysis. The cells in the perichondrial groove of Ranvier might thus function as a reservoir of mesenchymal stem cells, and the cells migrating from the perichondrial groove of Ranvier might be the label-retaining progenitor cells. This has also been suggested by Robinson et al., who demonstrated that injection of LacZ transfected cells from the perichondrial groove of Ranvier migrate deep into the epiphysis (Robinson et al., 1999). It is tempting to assume that the cells in the stem cell niche in the perichondrial groove of Ranvier can also migrate to the surface of the articular cartilage, but this remains to be proved in further studies, such as lineage tracing to demonstrate that the proposed stem cells have multipotency, and tracing to follow the cells *in vivo* and their involvement in tissue regeneration, (da Silva Meirelles et al., 2008; Alison and Islam, 2009).

Progenitor cells and niche signalling in normal human adult articular cartilage

Although the rabbit is an acceptable animal model for location of potential stem cells and niches *in vivo*, it is still an animal model and differs from human tissue (Hunziker et al., 2002). The results from the study in paper IV, together with the *in vitro* data from agarose suspension cultures of human articular chondrocytes in papers II and III, increased our interest in how the human articular cartilage tissue is constituted with stem cells, progenitor cells and niche signalling molecules. Although, the adult articular cartilage is not supposed to contain a traditionally niche we were interested to study if the chondrocyte micro environment expressed primitive signalling pathways as known from the stem cell niche.

Owing to ethical considerations in studying normal human articular cartilage *in vivo*, we chose to use immunohistochemical studies of biopsies from normal articular cartilage from femur condyles of diseased donors in paper V. The biopsies were taken from macroscopically intact articular cartilage, and there was no known cartilage defect in the patients´ history. We used stem cell and stem cell niche associated markers already studied in other tissues such as Stro-1 (Simmons and Torok-Storb, 1991; Jo et al., 2007), Bcrp1 (Goodell et al., 1997) and the Wnt (Watt

and Hogan, 2000; Mitsiadis et al., 2007) and Notch pathways (Lai, 2004; Mitsiadis et al., 2007; Song et al., 2007). The results showed the locations of potential progenitor cells in the superficial layer of articular cartilage, if we assume that Stro-1 and/or Bcrp1 represent progenitor cells. However, Stro-1 and Bcrp1 seemed not to be expressed by the same cells, and Stro-1 was positive in a larger population than Bcrp1. The results were nevertheless in agreement with previously locations discussed for stem cells in articular cartilage (Hayes et al., 2001; Dowthwaite et al., 2004; Hunziker et al., 2006; Hattori et al., 2007). The results from our rabbit study together with these results, indicate that Stro-1 does not represent a progenitor population, but a subpopulation of primitive cells. It is more likely that the small number of Bcrp1 positive cells could represent a true progenitor population. Hattori et al. have also demonstrated a small SP of cells in the superficial layer of bovine articular cartilage with stem cell properties, supporting our data. Anyhow, the role of Bcrp1 as a stem cell/progenitor marker has to be further proven. The Notch signalling pathway showed similar activity of HES5 in the superficial layer as previously shown by Karlsson et al., 2008. The Wnt activity, and particularly the canonical pathway, has not been previously demonstrated in biopsies from normal human articular cartilage, but showed strict zonal expression in the superficial and upper transitional zones. The role of this strict zonal expression of the markers is further discussed below.

Repair versus regeneration of articular cartilage

Articular cartilage is a tissue with almost no capability of repair, if by repair we mean the formation of new tissue with properties as the original tissue (Brittberg, 1996). Usually formation of new tissue with the properties of the original hyaline cartilage in terms of strength, smooth surface and matrix composition is known as regeneration. Regeneration has been explained as recapitulating the embryonic skeletal development processes, while repair is an attempt of the body to heal an injury, not always with adequate results as defined by Yannas, 2007 “Regeneration restores the normal structure and function of the organ, repair does not”. If we summarize the minimal therapeutic goals we would like achieve by cartilage tissue engineering as: a) symptom control b) long term preservation of the joint and c) restoring the functional properties of the cartilage tissue, repair can fulfil criterion *a* but probably not *b* or *c*.

The Wnt and Notch pathways in repair and regeneration of articular cartilage

Studies in animal models, cell cultures and in pathological conditions such as OA have increased our knowledge of early embryonic skeletal signalling and its close involvement in regeneration and in pathological processes (Shapiro et al., 1993; Ferguson et al., 1999; Pfander et al., 2001; Tallheden et al., 2004).

We wanted to further study regeneration and repair of human articular cartilage based on our findings from the normal human articular cartilage and focused on the same markers as discussed above since these markers and pathways are not only involved in stem cell niche homeostasis but also in early embryonic signalling.

Using a 3D pellet mass *in vitro* model of cartilage regeneration we were able to demonstrate expression of Stro-1, Frizzled, beta-catenin and HES5. They all showed down-regulation during differentiation/regeneration. The novel finding of the Bcrp1 gene expression in normal articular chondrocytes showed constant low expression independent of the differentiation grade of the pellet mass.

The involvement of these pathways and distribution of the stem cell markers needed further investigation, and studies had to be undertaken on regenerated and repaired articular cartilage tissue. Therefore, apart from normal articular cartilage we used biopsies from ACI areas with good or bad outcome according to ICRS score, injured articular cartilage with different grades of regenerated or repaired tissue, and finally OA cartilage from macroscopically normal area and from OA lesions. This was a great opportunity to be able to study biopsies from ACI areas and to compare regenerated transplanted articular cartilage with other forms of regenerated and repaired cartilage tissue.

The regenerated tissue formed after ACI could be divided into two main groups depending not only on morphology but also depending on the expression pattern of the selected markers. The first group of biopsies with almost reconstituted morphological articular cartilage tissue demonstrated zonal expression similar to that of normal articular cartilage of the Wnt and Notch pathways as well as the Stro-1 and Bcrp1 markers, together with a matrix of hyaline character. The other group contained mostly fibrocartilage with dispersed expression of the markers studied throughout the tissue. When the biopsies showed a mixed tissue of hyaline and fibrocartilage a variation in expression of the studied markers was noticed. In summary, the results from the biopsies showed that when there was adequate regeneration of the original architecture of the articular cartilage tissue the zonal expression of progenitor markers and signalling pathways were distributed as in normal articular cartilage. In contrast, the repaired articular cartilage and low scored ACI biopsies showed irregular expression patterns of the markers with no signs of zonal distribution. The results were similar to the results from the pathological tissue from OA. The expression of Bcrp1 was low, independent of cartilage studied, further suggesting a specific role for the cells expressing this marker (for a summary of expression of the studied markers in articular cartilage, see figure 10).

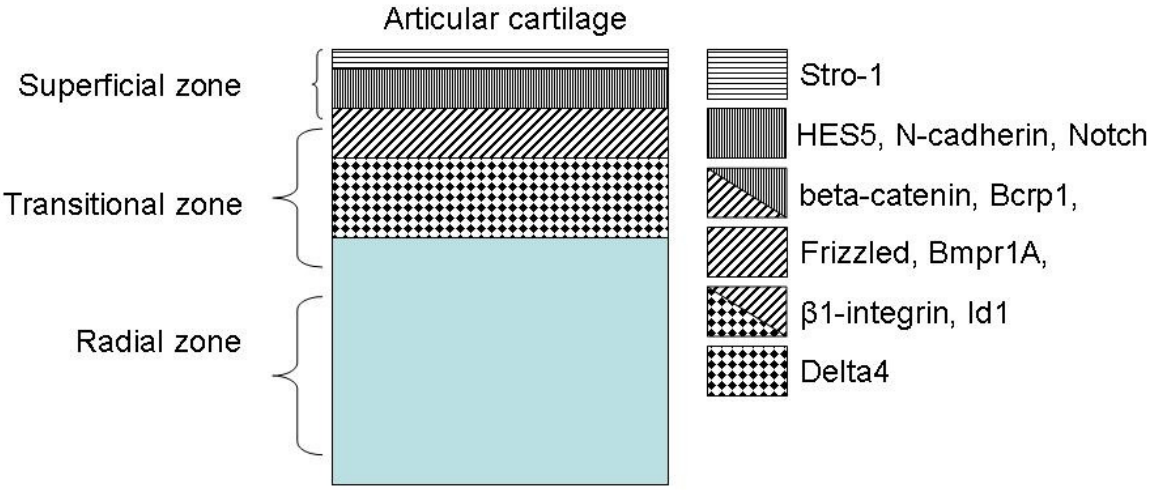
The explanation of the differences between the repaired and regenerated cartilage has not yet been found. The micro environment in these different cartilages might have some common feature of importance. These questions remain: Why does the articular cartilage with apparently good quality express the signalling and

progenitor markers in a zonal pattern. Is that a requirement for an adequate tissue formation?

The Wnt and Notch signalling pathways have both demonstrated an essential role in postnatal tissue repair in other types of tissues, and it has recently been demonstrated that both Notch and Wnt have a major impact on maintenance and repair of adult tissue in skeletal muscle (Conboy et al., 2003; Brack et al., 2007). Furthermore studies of silencing beta-catenin by RNA interference or by repressing its transcriptional targets using dominant-negative beta-catenin have shown promoted differentiation and reduced self-renewal of muscle satellite cells (Perez-Ruiz et al., 2008). Glycogen synthase kinase 3β (GSK3β) has been demonstrated to play a pivotal role in muscle tissue repair. Notch pathway signals via GSK3β (Brack et al., 2008), which is maintained in an active form by Notch, while GSK3β is inhibited by Wnt in the canonical Wnt signalling cascade in other words Notch and Wnt act with divergent regulation of GSK3β in muscle tissue. This converts the proliferative muscle tissue through lineage progression, and muscle regeneration (Brack et al., 2008). GSK3β has also been suggested to play a role in cartilage pathologies, for example in arthritis in mice where GSK3β inhibition exerts an anti-inflammatory effect (Cuzzocrea et al., 2006). Yuasa et al., have also demonstrated the possible role of Wnt-beta-catenin signalling in stimulating matrix catabolic genes in articular chondrocytes as well as their possible role in joint degeneration (Yuasa et al., 2008). Recent studies further support the data in the involvement of increased Wnt signalling in human OA (Blom et al., 2009, Luyten et al., 2009) and following mechanical injury of cartilage (Dell'Accio et al., 2006, Dell'Accio et al., 2008). Notch pathways have also been suggested to be involved in OA (Karlsson et al., 2008). General crosstalk between the Wnt and Notch pathways has been proposed by Hayward et al. in what they call the “wntch” signalling concept (Hayward et al., 2008). In spite of the fact that a great deal of attention has been paid to the Wnt and Notch families in the bone-cartilage field in recent years the “wntch” communication has not yet been studied and a great deal remains to be done in terms of understanding this inscrutable signalling system (Loughlin et al., 2004; Yates et al., 2005; Karlsson, 2007b).

In paper V we suggest that the balance in these signalling systems is a key to the ability to regenerate articular cartilage tissue of a hyaline nature. These pathways have previously mainly been studied in pathological conditions of cartilage, while we focus on normal regeneration in a tissue engineering perspective.

A



B

Marker	Function
Id1	Transcription factor, inhibitor of differentiation, regulates cell cycle, mediates BMP effects
Stro-1	Marker for mesenchymal stem cells, expressed on cells in the stem cell niche
Bcrp1	Expressed on stem cells
HES5	Mediator of Notch signalling
Notch1	Regulates cell commitment and cell fate, maintains an undifferentiated progenitor population
Delta4	Ligand to Notch1
Bmpr1a	Receptor for BMP2 and BMP4.
Frizzled	Receptor in the WNT signalling pathway
Beta-catenin	Down stream frizzled in canonical WNT pathway, subunit of cadherin protein complex
N-cadherin	Important for cell-cell adhesion, cell migration, maintains tissue specific stem cells
β1-integrin	Important for cell-cell and cell-matrix adhesion, cell migration

Figure 10. A. Schematic drawing of the articular cartilage and the zonal expression of different markers studied in this thesis. B. The different markers studied in this thesis and their function.

The role of the micro environmental compartment

As discussed above, chondrocytes have been demonstrated to redifferentiate when placed in 3D cultures such as pellet mass cultures (Tallheden et al., 2004). In parallel, when cells are prepared for ACI, it is necessary to expand the chondrocytes *in vitro* to increase the number of cells isolated from the biopsy in order to get enough cells for ACI. When these cells are replaced in the patient during ACI they are applied in a 3D environment and redifferentiate, sometimes resulting in a more fibrocartilaginous than hyaline character of cartilage, as demonstrated in paper V and previously in other studies (Roberts et al., 2003; Tins et al., 2005). In paper II we discuss the dual chondrogenic model describing the ACI as a small bio-chamber with internal influence of the periosteum, adjacent cartilage and the transplanted cells. It is likely that the high density of cells placed in such a closed chamber during ACI initiates a process of cell condensation and neocartilage formation like that seen during embryonal chondrogenesis. In paper V

this is demonstrated, to our knowledge, for the first time at molecular level in biopsies from ACI areas.

The importance of the adjacent cartilage is illustrated by the vertical and lateral integration. The importance of both vertical and lateral integration for the outcome of the ACI treatment has previously been discussed (Hunziker et al., 2002; Khan et al., 2008) where lateral integration seems most problematic. The findings of the strict zonal expression of the markers studied in articular cartilage in papers IV and V supports the idea of the importance of good lateral integration in achieving good repair results. It can be assumed to function in a similar way as within a niche, with cross-talk between the cells and numerous other mediators involved, all strictly organized and creating an appropriate microenvironment to secure the integrity of cartilage.

Age, sex and individual cell properties

Other factors discussed in the literature that may be involved in the outcome of regeneration of articular cartilage are age, sex and individual cell properties. We were not able to display any significant differences in cluster formation depending on age or sex of the patients in papers II or III. It should be added that in paper III the mean age of the patients was only 31.2 years. It would be of interest to study the cluster formation capacity in older individuals to find out whether the numbers of progenitor cells decline with the age, as suggested for MSC in bone marrow (Stolzing et al., 2008).

Some researchers have also pointed out the monolayer culture of the chondrocytes as important, and an eventual aging of the chondrocytes during the *in vitro* culture. It is estimated that *in vitro* expansion ages the chondrocytes approximately 30 years when cultured in monolayer before ACI (Parsch et al., 2002; Martin et al., 2002; Khan et al., 2008). In paper II we were not able to demonstrate any differences for separate patients in types or number of clusters formed before and after monolayer culture, however the maximum cell doublings in monolayer was about 8. An individual pattern in growth in vitro might be what determines an individual age of the chondrocytes and this is further demonstrated with differences in outcome in forming hyaline articular cartilage *in vivo* in ACI. The tissue aging in cartilage is a field for future research. One interesting aspect is that with age the systemic environment is less effective in maintaining the fate of stem cells, as suggested in muscle tissue by Brack et al., 2007. They suggest that in older animals there are an increasing numbers of Wnt signalling molecules in the serum, resulting in impaired muscle regeneration and enhanced fibrotic response. This was assumed in the Klotho mouse model (genetic model of accelerated aging) where Wnt proteins demonstrated an unexpected role in tissue aging (Liu et al., 2007). It remains to be further explored whether this is transferable to articular cartilage tissue.

To fulfil the minimal therapeutic goals in tissue engineering stated at the beginning of this section we must not forget that cartilage forms a biomechanical unit with the whole joint. If it is possible to heal an articular cartilage defect at an early time point by interfering with the signalling systems and the cells on site, this might affect the long-term prognosis and prevent the development of secondary arthritis and full joint engagement.

CLINICAL AND FUTURE ASPECTS

I conclude this discussion with some personal comments on what is of importance in a clinical and future perspective regarding tissue engineering of human articular cartilage.

Why improve articular cartilage repair?

Since the 1978, ACI has been performed around the world and more than 35,000 patients have now been treated (Brittberg et al 1994, Lindahl, 2008).

Why do we need to understand more about cartilage and why do we have to improve the methods for treatment of articular cartilage lesions? Because still there is no optimal treatment for one of the world's biggest medical problems.

No treatment strategy in use today is perfect, although they are all acceptable in terms of symptomatic relief to the patient. Irrespective of the method used we want to simplify the surgical procedure and preferably use arthroscopic surgery. We want to develop biomaterials to be able to apply factors, signalling molecules and cells we are not able to administer otherwise, and last but most importantly improve the outcome of the formed cartilage to restore long-term joint function.

Cell source

One question in recent years has been which cell source to use: chondrocytes, MSC or other cells, such as synovial cells. Conflicting studies have been presented. In some studies MSC have been successfully used in animal models of tissue regeneration, in e.g. myocardial infarctions, stroke, muscle and bone repair. However, MSC are not fully clinically available (Shake et al., 2002; Lee et al., 2000; Li et al., 2005). In an *in vitro* study it was demonstrated that MSC and articular chondrocytes used in ACI differentiate along separate pathways (Karlsson et al., 2007a) but once we know how to guide MSCs and prevent bone formation, it is tempting to use MSC for tissue engineering of hyaline articular cartilage. Regarding MSC, Caplan suggested an interesting point in 2007: the important role of MSC not in replacing cells but in establishing a microenvironment at the site of the injury to increase the local trophic activity. This gives a new perspective on using MSC in transplantation, but if this is applicable in articular cartilage remains to find out (Caplan, 2007). The discussion of using MSC is far from over.

In recent years the use of synovial cells has been discussed, i.e. MSC isolated from the synovial tissue. These cells have demonstrated high multi-potency and ability of chondrogenesis (Sakaguchi et al., 2005; Teramura et al., 2008). Nevertheless, there

still is no convincing evidence for using other cell sources than true committed articular chondrocytes for clinical transplantation. This thesis and additional recently published data (Khan et al., 2009), however, shows evidence that articular cartilage contains cells with properties to form new hyaline cartilage. Isolation and use of those cells will be the focus of future cartilage tissue engineering research.

Molecular targets

In this thesis we have reported that there are potential stem cells or progenitor populations in articular cartilage and groove of Ranvier, and pointed out the importance of the signalling pathways HLH, Wnt and Notch. These progenitor cells and signalling pathways may represent potential targets or valuable tools in joint tissue engineering. For example, the stem cells residing in the perichondrial groove of Ranvier or articular cartilage identified could be recruited and guided to the site where it is needed, by administering the right attractants. The attractants and other molecules interacting with the signalling pathways could be administered by scaffold or carrier structures.

We have also demonstrated that the signalling proteins in the HLH-, Wnt- and Notch pathways are closely involved in the repair, regeneration and pathogenesis of cartilage. For this reason, future studies to understand tissue engineering of cartilage have to include pathologies, such as OA and tumours, in addition to repair and regeneration (Asp, 2002; Tallheden et al., 2005b; Dell'Accio et al., 2006; Karlsson et al., 2008). This is of special importance since epidemiological studies have demonstrated a genetic component to primary OA (Spector and MacGregor, 2004; Valdes et al., 2004). In a regenerative tissue perspective it is possible that any of the genes studied will be identified as novel genes for use as drug targets.

The extracellular matrix

Although ECM and its role is beyond the scope of this thesis, it must not be forgotten. The crucial role of this part of the articular cartilage is that it is the chondrocytes that produce the matrix and enzymes, while the matrix modulates chondrocyte differentiation and activity. Under normal circumstances there is homeostasis in the tissue, but under pathological conditions it is disturbed. More knowledge of the ECM and its interaction with chondrocytes and molecular signalling in the chondrocyte will clearly be important in future tissue engineering (Kirn-Safran et al., 2004; Lefebvre et al., 2005; Lories, 2008).

SUMMARY AND CONCLUSIONS

The primary aim of cartilage tissue engineering is to regenerate a tissue engineered product with the specific signs of hyaline articular cartilage to meet individual demands. This thesis adds additional information to the large field of regenerative medicine and tissue engineering of articular cartilage. Adult articular cartilage consists of populations of cells with progenitor properties. HLH proteins, Wnt and Notch signalling are important transcriptional and signalling pathways in cartilage. Using these findings, it becomes possible to select the proper population and influence the important signalling pathways for tissue engineering.

- Members of the helix-loop-helix transcription factor family are important elements in the balance between proliferation and differentiation in cartilaginous tissue.

- Chondrocytes demonstrate subpopulations of cells with mesenchymal stem cell properties when cultured in 3D agarose suspension culture.

- Progenitor cells exist in the knee of sexually mature rabbits and are mainly located to the perichondrial groove of Ranvier. Progenitor cells have also been detected in small numbers dispersed throughout the articular cartilage.

- The groove of Ranvier in the joint is a potential stem cell niche.

- The stem cell associated markers Stro-1 and Bcrp1 are expressed in adult human articular cartilage. However, they do not represent the same population.

- The normal balance between the Wnt- and Notch pathway seems to be impaired in repaired cartilage tissue as compared with regenerated cartilage tissue.

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