The secretome of cartilage, chondrocytes and chondroprogenitors:

Implications for cell transplantation strategies.

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“If one does not know to which port is sailing, no wind is favourable”

Seneca

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2 List of papers

Paper I Polacek M, Bruun JA, Johansen O, Martinez I. Differences in the secretome of cartilage explants and cultured chondrocytes unveiled by SILAC technology. J. Orthop. Res. 28:1040-1049; 2010

Paper II Polacek M, Bruun JA, Johansen O, Martinez I. Comparative analyses of the secretome from de-differentiated and re-differentiated adult articular chondrocytes. Cartilage 2010; doi:10.1177/1947603510383856.

Paper III Polacek M, Bruun JA, Elvenes J, Figenschau Y, Martinez I. The secretory profiles of cultured human articular chondrocytes and mesenchymal stem cells: implications for autologous cell transplantation strategies. Cell Transplantation 2010; doi: 10.3727/096368910X550215.

3 Abbreviations

ACI	Autologous chondrocyte implantation
ACs	Articular chondrocytes
ADAMTs	A disintegrin and metalloproteinase with thrombospondin motifs
AMIC	Autologous matrix induced chondrogenesis
bFGF	Basic fibroblast growth factor
BMP1	Bone morphogenetic protein 1
CDMP	Cartilage-derived morphogenetic protein
CHI3L2	Chitinase 3-like protein 2
COMP	Cartilage oligomeric matrix protein
COX2	Cyclooxygenase-2
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
ER	Endoplasmatic reticulum
GA	Golgi apparatus
GAGs	Glycosaminoglycans
GAS6	Growth arrest-specific protein 6
HA	Hyaluronic acid
IL-1	Interleukin 1
iNOS	Inducible nitrit oxide synthase
ISCT	International Society for Cellular Therapy
LC-MS/MS	Liquid chromatography, double mass spectrometry
LIF	Leukaemia inhibitory factor
MACI	Matrix-induced autologous chondrocyte implantation

MMP3	Stromelysin
MMPs	Matrix metalloproteinases
MS	Mass spectrometry
MSCs	Mesenchymal stem cells
OA	Osteoarthritis
OMD	Osteomodulin
OSM	Oncostatin M
PDGF	Platelet-derived growth factor
PGs	Proteoglycans
ROS	Reactive oxygen species
SILAC	Stable isotope labeling of amino acids in cell culture
SLRPs	Small leucine-rich proteoglycans
SPARC	Secreted protein acidic and rich in cysteine
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor alpha
t-PA	Tissue plasminogen activator
WB	Western blott

4 Definitions of key concepts

Biological repair

Regeneration of tissues, organs or body parts using the body own fluids, cells and components.

Cell de-differentiation

Regression of a specialized cell to a simpler unspecialized form.

Cell re-differentiation

Process by which a group of once de-differentiated cells return to their original specialized form.

Cell senescence

State or process of aging where isolated cells demonstrate a limited ability to divide in culture.

Secretome

Entirety of product types released by cells or tissues to the extracellular environment.

Extracellular matrix

Network of proteins and carbohydrates that surround a cell or fill the intercellular spaces.

Mascot score

Sum of the unique ions scores representing the significance of the protein identification.

Mass spectrometry

Analytical technique for the determination of the elemental composition of a sample or molecule.

Metabolic labelling of proteins

Process in which the cells are grown in the presence of metabolically labelled precursors of macromolecule synthesis.

Monolayer cultures

Sheet of cells one cell thick, such as may be formed on the surface of a culture vessel.

Primary cultures

Tissue cultures started from cells, tissues, or organs taken directly from the organism.

Spheroid cultures

Spherical aggregates of cells in culture that retain three-dimensional architecture and tissue-specific functions.

Tissue explants

Culturing of living tissue in an artificial medium.

5 Background

Articular cartilage is a few millimeters thick, avascular, alymphatic and aneural connective tissue covering joint surfaces. Its unique structural composition provides frictionless movement, shock absorption and wear resistance to the joints, while bearing large repetitive loads throughout a person's lifetime [1]. Chondrocytes maintain cartilage tissue by constant synthesis and degradation of matrix components. Any imbalance in these processes might lead to degradation and destruction of the cartilage surface with serious consequences for patients over time [2-4]. Standard treatment for advanced cases of cartilage destruction, also called osteoarthritis, is a total joint replacement, which has been an immense achievement for elderly patients. However, complications of the procedure such as loosening of the implants and infections raised concerns for using this type of surgery in younger, active individuals. Therefore, in recent decades, important efforts have been aimed at achieving biological repair of cartilage defects. An array of different methods had been developed including several bone marrow stimulating procedures, transplantation of *ex vivo* engineered tissue implants, implantation of expanded autologous cells, or the injection of novel matrices embedding cells along with tailored cocktails of growth factors [5-9]. Though strategies using potential of stem cells residing in subchondral bone do not require *ex vivo* culturing of cells, many other approaches require *in vitro* cell expansion prior to implantation. Despite the serious efforts and high number of patients treated with biological cartilage repair techniques, the combined scientific and clinical efforts have not definitely succeeded in providing hyaline cartilage repair tissue in a controlled and predictable way in adults. The tissue is in many cases of fibrocartilage morphology with inferior mechanical properties compared to native cartilage, and problematic integration into surrounding cartilage and subchondral bone [10-12]. I addition most of the studies are of empirical character and our understanding of fundamental cell

biology in *ex vivo* cultures is still very limited. That is why in order to improve the culture techniques or be able to decide which cell type is better suited for repair; basic studies focusing on gene and protein expression have gained popularity in recent years. Studies of gene expression might uncover gene activation and consequent protein synthesis [13]. However, while the organism's genome is more or less constant, protein secretion differs from cell to cell and from time to time as the cell reacts to different stimuli and signals. Thus studies of secreted proteins might provide more complex and accurate information about cells phenotype and functional status [4]. In this thesis, I aimed to explore phenotypical changes the cells undergo in ex vivo cultures by studying the secretory profiles of cartilage tissue explants, de- and re-differentiated cultured chondrocytes and un-differentiated mesenchymal stem cells.

6 Introduction

6.1 *Articular cartilage*

Articular or hyaline cartilage is an aneural and avascular connective tissue of mesodermic origin composed predominantly of extracellular matrix (ECM), water and cells. Chondrocytes which represent only around 5% of total cartilage mass are located in small so called **"lacunas"**. Surrounding ECM is organized into several layers: i) **pericellular matrix** – directly adjacent to the cells; ii) **the basket** or **pericellular capsule** – encloses the pericelular matrix; and iii) **territorial matrix**, which forms the transition towards adjacent interterritorial matrix. The cells surrounded by pericellular matrix and capsule form the **chondron**, the basic functional unit of cartilage (Fig. 1) [14-17].

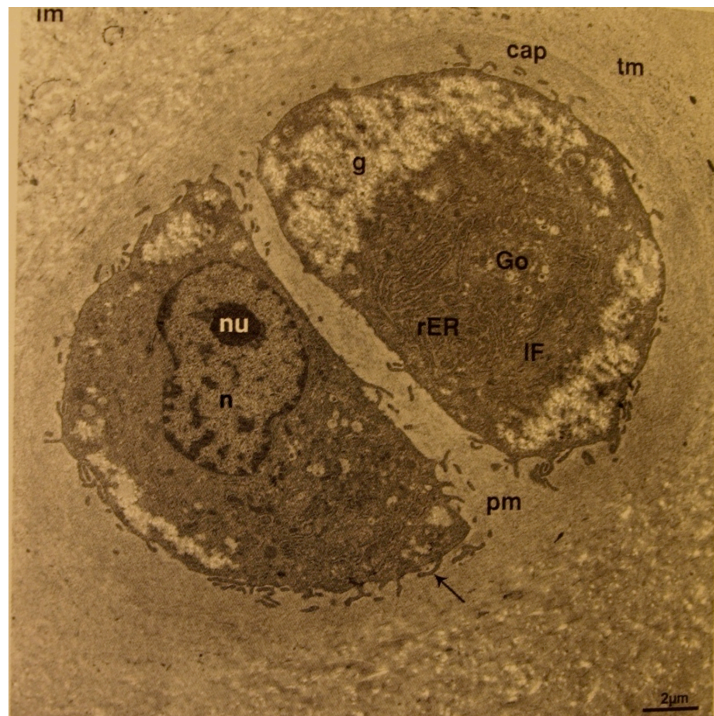


Fig. 1: Chondrocytes of a double chondron in the middle zone with Endoplasmatic reticulum (rER), Golgi-apparatus (GA), glycogen accumulations (g), and intermediate filaments (IF). Microvilli extend into the pericellular matrix (pm), but do not transverse the capsule (cap), and do not penetrate into the territorial matrix (tm) and the interterritorial matrix (im). Nucleus (n), nucleolus (nu); femoral condyle; glutaraldehyd-osmium tetroxide fixation. (*Reprinted with permission from Nurnberger S, Marlovits S. Electron microscopy of human articular chondrocytes. In: Zanasi S, Brittberg M, Marcacci M, editors. Basic Science, Clinical Repair and Reconstruction of Articular Cartilage Defects. Current Status and Prospects. Rastignano, Bologna, Italy: Timeo Editore; 2006 p. 59-68)*

ECM of cartilage is explicitly organized to resist high mechanical loads and could be divided into four zones (Fig. 2, 3). The **superficial zone**, or the joint gliding surface, is the thinnest cartilage layer (10-20% of full thickness). It contains elongated chondrocytes (Fig. 2a) and thin collagen fibrils running parallel to the surface. The concentration of proteoglycans (PGs) is low and the concentration of collagen fibrils is high (Fig. 3a). The zone is covered by a thin membrane called **lamina splendens** composed mostly of collagen type I and responsible for frictionless surface characteristics of cartilage. Lubrication of the surface is mediated by a superficial zone protein called **lubricin** [18]. Underneath the superficial zone is a **transitional zone** which fills 40-60% of the tissue and is characterized by rounder, synthetically active chondrocytes (Fig. 2b). The matrix is rich in PGs and thicker but less organized collagen fibers (Fig. 3b). The **deep zone** occupies around 30% of the tissue depth and contains large synthetically active chondrocytes (Fig. 2c) with stack-like arrangement of the cells in chondrons and thick collagen fibers (Fig. 3c, d), both perpendicularly oriented to the joint surface. The water content in the matrix is low and the concentration of PGs is high. The **tidemark** separates the deep zone from the **calcified zone** which anchors cartilage to the subchondral bone. Chodrocytes in this zone are small (Fig. 2d) and randomly distributed in the matrix rich in hydroxyl-apatite crystals [1, 15, 19-24].

The biochemical composition of ECM is based on a framework of macromolecules (collagens, PGs) and water (75-80%). A variety of collagens is known to be synthesized by chondrocytes, including collagen II, III, V, VI, IX, X, XI, and XII [14]. Collagen type II is the most abundant one standing for around 90-95% of all collagens in hyaline cartilage. The mean diameter of collagen II fibers in cartilage varies from 10 to 100nm making them considerably thinner compared to other tissues like bone or tendons.

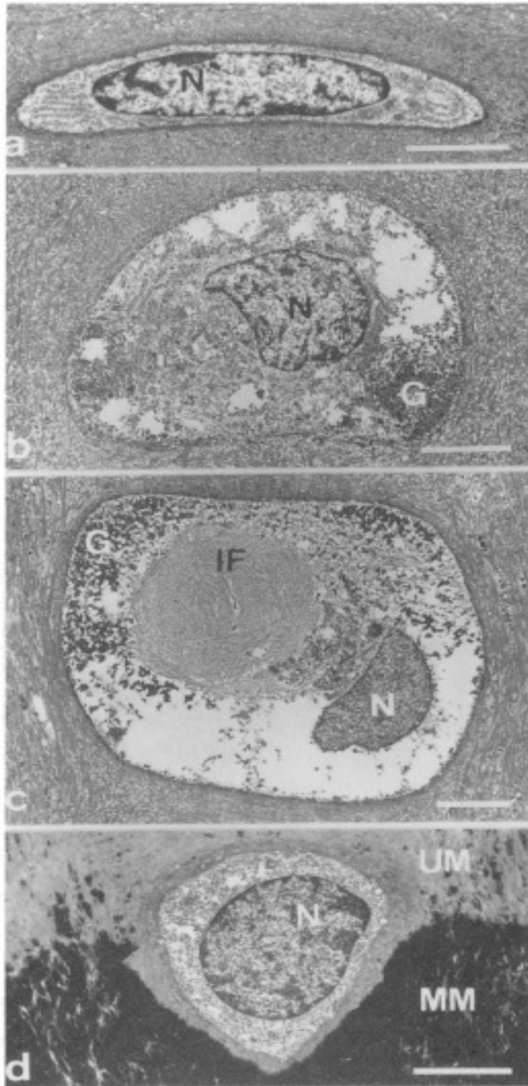


Fig.2: Electron micrographs of chondrocytes from articular cartilage from the medial femoral condyle of a skeletally mature rabbit, **a:** superficial zone; **b:** transitional zone; **c:** middle (radial), or deep, zone; and **d:** calcified cartilage zone. N = nucleus, G = glycogen, IF = intermediate filaments, UM = unmineralized matrix, and MM =mineralized matrix (bar = 3µm). *(Reprinted with permission from Woo SL-Y, Buckwalter, JA (eds): Injury and Repair of the Musculoskeletal Soft Tissues. Rosemont, IL, American Academy of Orthopaedic Surgeons, 1988.)*

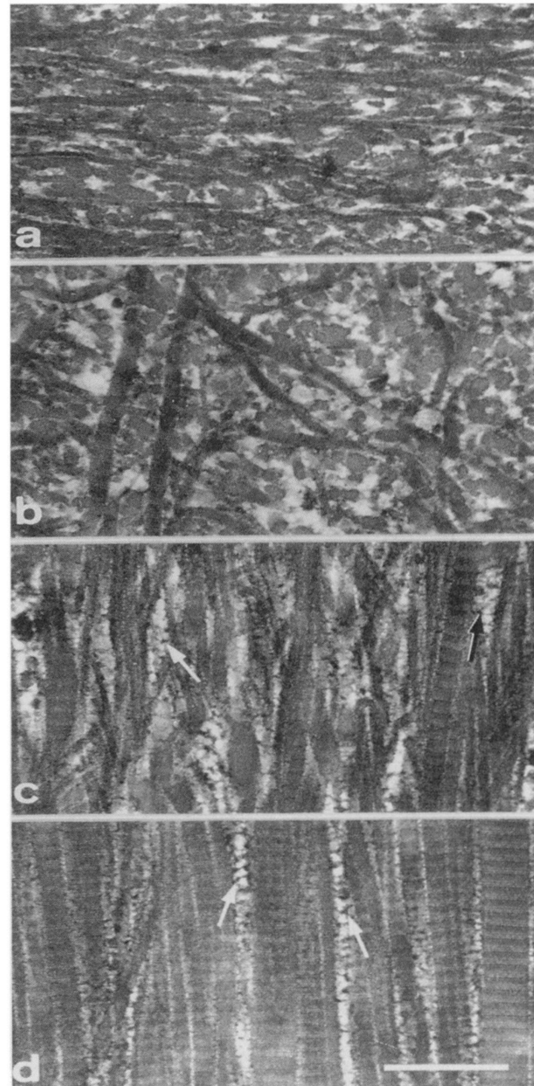


Fig. 3: Electron micrographs of the interterritorial matrix of articular cartilage from the medial femoral condyle of an eight-month-old rabbit, *a:* superficial zone; *b:* transitional zone; *c:* upper portion of the middle (radial), or deep, zone; and *d:* lower portion of the middle (radial), or deep, zone. The arrows indicate proteoglycans precipitated with ruthenium hexamine trichloride (bar = 0.5µm). *(Reprinted with permission from Woo SL-Y, Buckwalter, JA (eds): Injury and Repair of the Musculoskeletal Soft Tissues. Rosemont, IL, American Academy of Orthopaedic Surgeons, 1988.)*

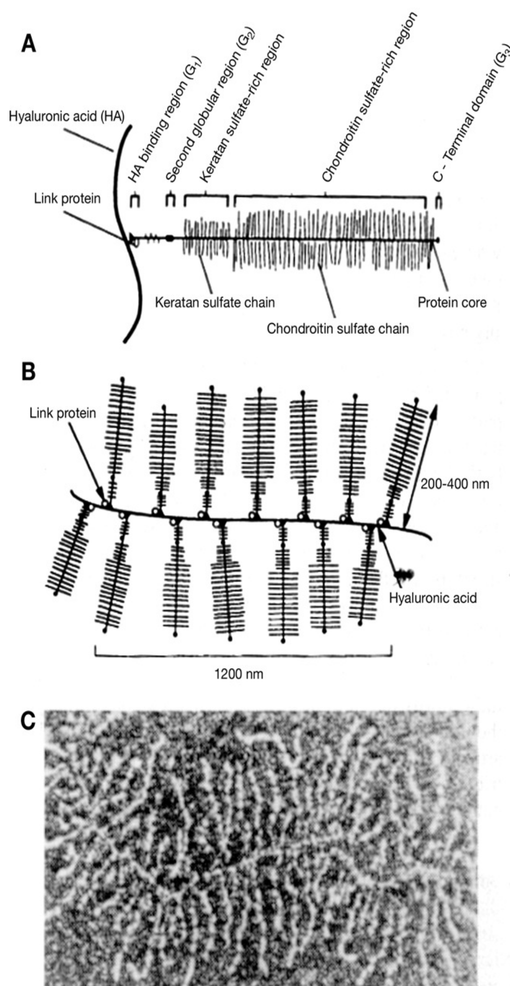


Fig. 4: The structure of proteoglycan. **(A)** Details of proteoglycan monomer structure showing chondroitin sulfate and keratan sulfate chains and the interaction of the monomer with hyaluronate chain and link protein. **(B)** Molecular conformation of a typical proteoglycan aggregate showing size of the molecule. **(C)** An electron micrograph of a proteoglycan aggregate. *(Reprinted with permission from Brinker MR, O´Connor DP, Almekinders LC, Best TM, Buckwalter JA, Garret WE, Kirkendall DT, Van Mow C, Woo SLY. Articular Cartilage Injury. In: DeLee JC, Drez D, editor. Orthopaedic Sports Medicine. Principles and Practice. Philadelphia: Saunders Elsvier; 2009 p. 40-56.)*

Table 1: Collagen types identified in human articular cartilage [19, 25-32].

Collagen type	Function
Collagen II	Main coll type in cartilage (90-95%), responsible for tensile strength.
Collagen III	Most prominent in chondron capsules, minor component, copolymerised to coll II, increased in OA cartilage.
Collagen V	Copolymerised to coll XI.
Collagen VI	<1% of coll, most in pericellular matrix, interact with SLRPs, have function in attachment of cells to matrix.
Collagen IX	~1% of coll, highest proportion in peri-cellular basket, cross-link coll II to proteoglycans.
Collagen X	Produced by hypertrophic chondrocytes, most concentrated in calcified layer of cartilage, have function in cartilage mineralization.
Collagen XI	~3% of coll, highest in peri-cellular ECM, cros-link coll II, heparan, heparin sulfate, nucleates fibril formation.
Collagen XII	Member of FACIT coll. subfamily, most probably bind SLRPs.
Collagen XIV	Member of FACIT coll. subfamily, most probably bind SLRPs.

A main structural characteristics of collagens is their triple helix organization, formed by a union and folding of three single alpha chains [20]. Functions of other collagen types identified in ECM of articular cartilage are described in table 1. The collagen framework is embedded in a gel-like substance formed by PGs. Structure of PGs is based on the protein core **aggrecan,** with **glycosaminoglycans (GAGs)** covalently bound to the core as side chains. Additionally, the structure is bound to **hyaluronic acid (HA)** via link-protein forming large aggregates (Fig. 4) [1, 33, 34]. Because GAGs are highly negatively charged, they bind positively charged ions and entrap water, thus providing mechanical support to ECM. The combination of solid organic matrix and high amount of interstitial fluid moving through the collagen-

proteoglycan network under pressure load gives the cartilage its unique compressive strength [21, 23].

6.2 *Cartilage defects*

Because of its very low self-repair potential, cartilage defects in adults are usually irreversible and might lead to destruction of the joint surfaces. It was reported that the incidence of cartilage lesions in the knees of patients having arthroscopic surgery varied from 11% to 63% making cartilage lesions a serious issue in developed countries [35, 36]. Cartilage tissue in the adult organism is sensitively balanced by constant synthesis and degradation of ECM in order to sustain its unique mechanical properties. Disturbances of this balance might increase the catabolic activity and lead to cartilage destruction. It is generally believed that different genetic, mechanical, age-related factors and soluble mediators might up-regulate the catabolic processes in cartilage matrix [37]. In spite of chondrocytes isolation in lacunas, they are able to respond to different cell-cell and cell-matrix signalling through numerous surface receptors and adhesion molecules. It is also known that cartilage tissue requires a certain amount of biomechanical load to hold anabolic and catabolic processes in balance. Both immobilization with too low load and overuse with high cyclical mechanical stress might lead to cartilage degradation. Moreover, it has been shown that static compression of cartilage decreases the synthesis of ECM molecules and increases pro-inflammatory and catabolic events [38, 39]. On the other hand, intermittent dynamic compression and tension were showed to increase synthesis of matrix components and reduce the activity of inflammatory and catabolic substances [38, 40, 41]. Mechanical changes in cartilage are most probably sensed by short micro-cilia extending from the cells into surrounding matrix [42].

There are also numbers of other active substances like cytokines playing an important role in the process of cartilage degradation. They might induce pro-inflammatory changes and

stimulate the cells to produce higher amounts of proteolytic enzymes. The best characterized cytokine known to modulate the degradation of cartilage matrix is **IL-1**. IL-1 inhibits synthesis of collagen type II and induces expression of **collagenases**, **stromelysin (MMP3)** and **tissue pasminogen activator (t-PA)** which is essential for transforming pro-proteases into active substances [43]. **Tumor necrosis factor alpha (TNF-α)** has also well documented effect on cartilage degradation similar to that of IL-1, and together they might act in synergistic manner [44]. IL-1 may also activate **IL-18,** which enhances the catabolic response of ACs by inducing the expression of **COX2 (cyclooxygenase-2)**, **iNOS (inducible nitrit oxide synthase)**, **IL-6** and MMP3 genes [45]. **IL-17** is known to stimulate secretion of other pro-inflammatory cytokines and **NO (nitric oxide)** [46]. NO together with **superoxide anion (O_2^-)** are known as **reactive oxygen species (ROS)** and might generate other derivative radicals, including **peroxynitrite (ONOO−)** and **hydrogen peroxide (H_2O_2)**. ROS may damage ECM components directly by attacking the structure of proteoglycan and collagen molecules, or indirectly by reducing matrix synthesis, activation of latent matrix metalloproteinases and down regulation of the activity and secretion of protease inhibitors. Levels of ROS produced by ACs in response to mechanical stress and different inflammatory mediators might be as high as the levels produced by immune cells. Such an abnormal amount of free radicals could not be met by the cell's antioxidant capacity thus intensifying catabolic processes and degradation of cartilage matrix [37, 47]. Cytokines and growth factors contributing to cartilage matrix degradation and repair are summarized in table 2.

Table 2: Cytokines and growth factors contributing to cartilage matrix degradation and repair.

Catabolic; pro-inflammatory	Anti-catabolic; anti-inflammatory	Modulatory	Anabolic
IL-1	IL-4	IL-6	IGF-1
TNF-α	IL-10	IL-11	TGF-β
IL-8	IL-13	LIF	BMP-2,-4,-7
IL-17	IL1-ra	OSM	CDMPs
IL-18			

(Reproduced from Grad S, Lee CR, Alini M. Biology: mechanisms of cartilage breakdown and repair. In: Zanasi S, Brittberg M, Marcacci M, editors. Basic Science, Clinical Repair and Reconstruction of Articular Cartilage Defects. Current Status and Prospects. Rastignano, Bologna, Italy: Timeo Editore; 2006 p. 69-85.)

6.3 ***Cartilage repair***

Regeneration by hyaline cartilage, complete integration of newly formed tissue into surrounding cartilage and subchondral bone and restoration of normal joint function is the ultimate goal for researchers working with biological treatment of cartilage defects. Over time cartilage defects might lead to osteoarthritis and progressive destruction of joint surfaces causing bad quality of life with excessive pain and limited joint movement. In older patient groups total artificial joint replacement has been a remarkable success. However, complications of these procedures like loosening of the implants and inferior mechanical properties compared to healthy joints, makes this type of surgery not a good option for young, active patients. That is why surgeons and researches all over the world have explored and established different biological methods trying to improve symptoms and delay the artificial joint replacement surgery. Biological repair techniques went through tremendous development during the last decades and have been successfully used on large groups of patients. In this chapter I would like to focus on clinical approaches most frequently used by practioners around the world.

6.3.1 Microfracture

Microfracture might also be described as the most used and modern technique for bone marrow stimulation. The rationale behind the procedure is the recruitment of multipotent MSCs from underlying bone marrow to cartilage defect. Throughout the history, several different techniques like Magnuson's debridement and abrasion in 1941 or Pridie's drilling in 1959 were developed to achieve the bone marrow stimulation [48, 49]. Modern arthroscopic microfracture technique was introduced by Steadman and colleagues [50-52]. Shortly, once the lesion has been identified, the area is cleared of all damaged cartilage so the surgeon creates a stable perpendicular edge of healthy cartilage. Afterwards using angled awl, the hole are made through subchondral bone approximately 3-4 mm apart in direction from periphery to the center of the defect. Marrow blood containing MSCs, fibrin and platelets forms then a sort of "superclot" which is later transformed by MSCs into repair tissue [53-55] (Fig. 5).

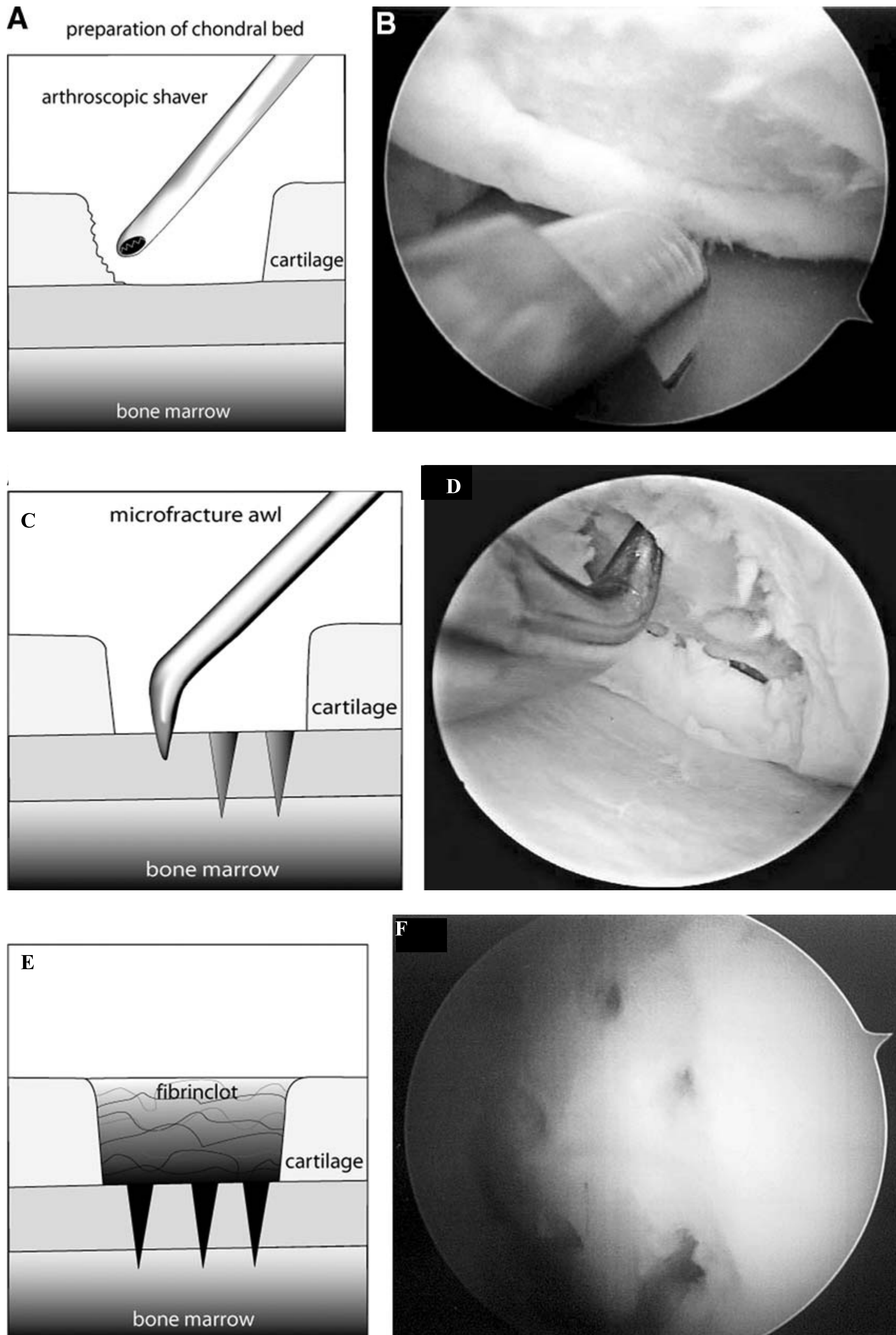


Fig. 5: Illustration of microfracture technique. **A, B:** Arthroscopic preparation of chondral lesion; **C, D:** Microfracture procedure using angled awl; **E:** Defect fills with fibrin clot contained by prepared wall of intact cartilage around lesion; **F:** Arthroscopic image of blood with marrow elements emanating from microfractures. *(Reprinted with kind permission of Springer Science+Business Media from Solomon DJ, Williams RJ, Warren RF. Marrow Stimulation and Microfracture for the Repair of Articular Cartilage Lesions. In: Williams R.J., editor. Cartilage repair strategies. Totowa, New Jersey: Humana Press; 2007 p. 69-84.)*

6.3.2 ACI (Autologous Chondrocyte Implantation)

ACI technique uses in vitro expanded autologous chondrocytes and periost (or artificial collagen membrane) cover to achieve repair of cartilage defects. The first paper presenting the first results using ACI in humans was the one of Mads Brittberg et al. in 1994 [5]. Initially, cartilage biopsies are taken from macroscopically healthy non-weight bearing area of the joint. The cells are then extracted and expanded in the laboratory in order to achieve a sufficient number for the repair (one million cells/cm^2). Secondly, open knee surgery is performed. The defect is debrided and damaged cartilage is removed until normal vertical cartilage margins are achieved. Periostal membrane is harvested predominantly from the proximal medial tibial diaphysis. In addition, commercially available artificial membranes like Chondrogide® (Geistlich biomaterials, Switzerland) and Restore® (Depuy, MA, USA) might be used. Consequently, the membrane is sutured over the defect, sealed with fibrin glue and the cells are injected under the membrane [5, 56-62] (Fig. 6).

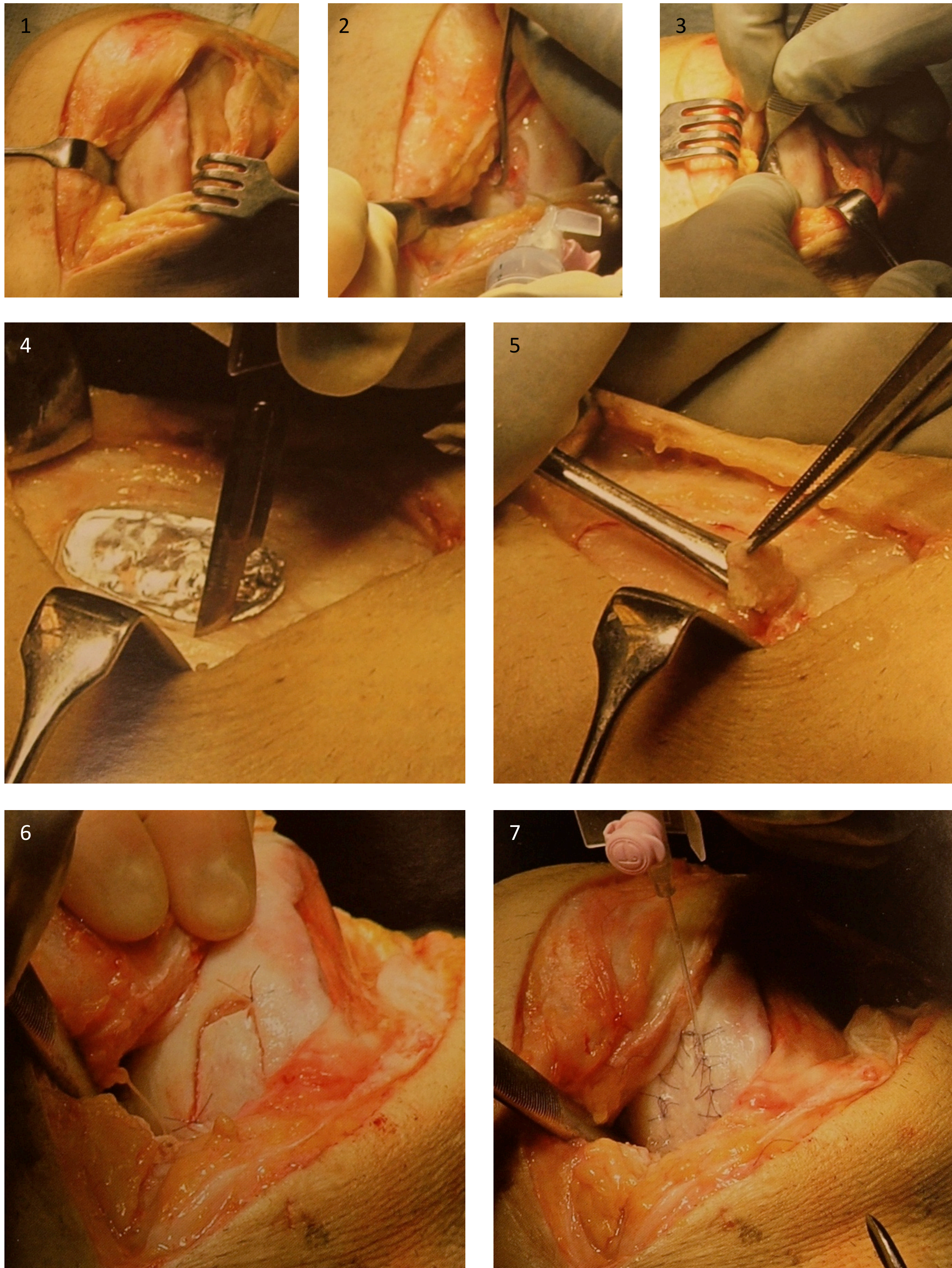
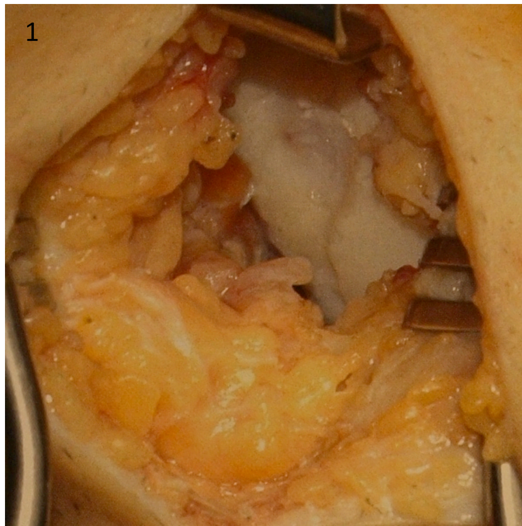
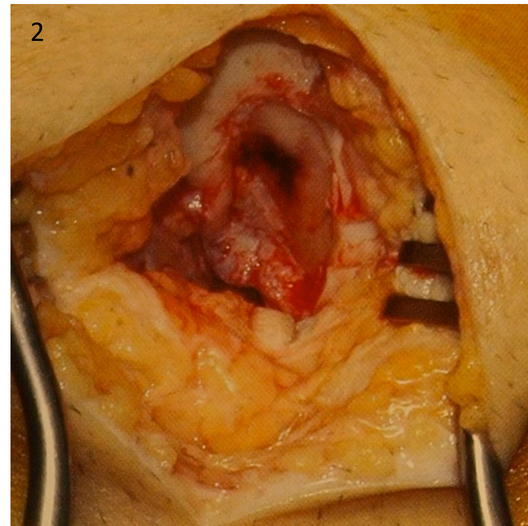


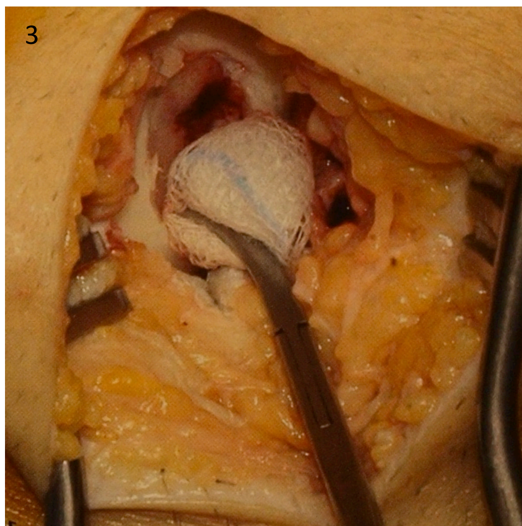
Fig. 6: ACI. Cartilage lesion (1) is debrided (2) and a template (3) is made. Using the template (4) to harvest periostal flap from tibia (5). The flap is sutured to the cartilage rim of the defect (6) and the chondrocytes are implanted (7). (*Reprinted with permission from Peterson, L. ACI surgical technique and results at 2-10 years. In: Zanasi S, Brittberg M, Marcacci M, editors. Basic Science, Clinical Repair and Reconstruction of Articular Cartilage Defects. Current Status and Prospects. Rastignano, Bologna, Italy: Timeo Editore; 2006 p. 325-332*)

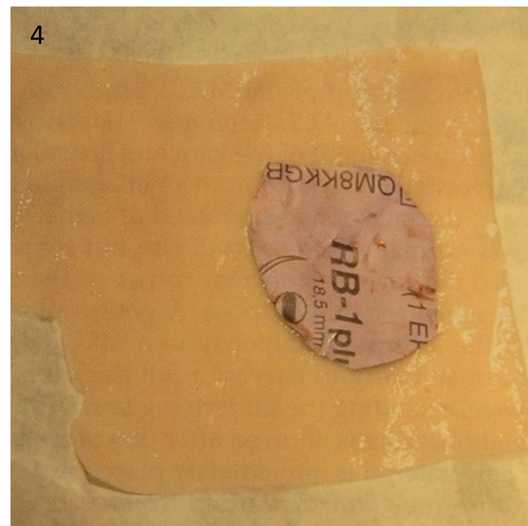
6.3.3 MACI (Matrix-induced Autologous Chondrocyte Implantation)

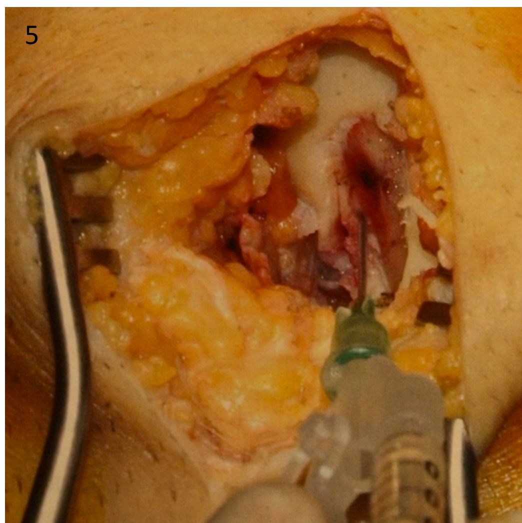
MACI can also be described as a second generation ACI using an artificial type I/III collagen membrane. The rationale behind this method is that the membrane represents a sort of scaffold which temporarily replaces the cell's ECM in order to provide them with three dimensional environment. That is why the cells are not implanted as in ACI but directly inoculated/seeded into the membrane. After debridement of the defect, the inoculated membrane is attached using fibrin glue or sutures [63-68] (Fig. 7).











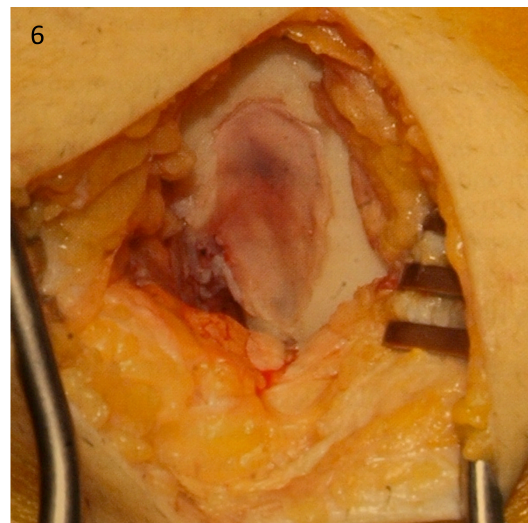
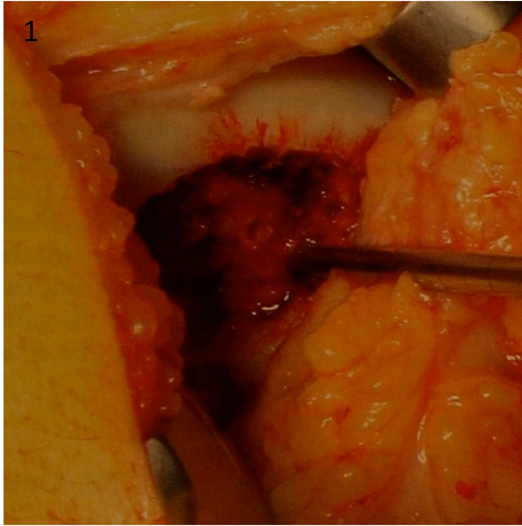
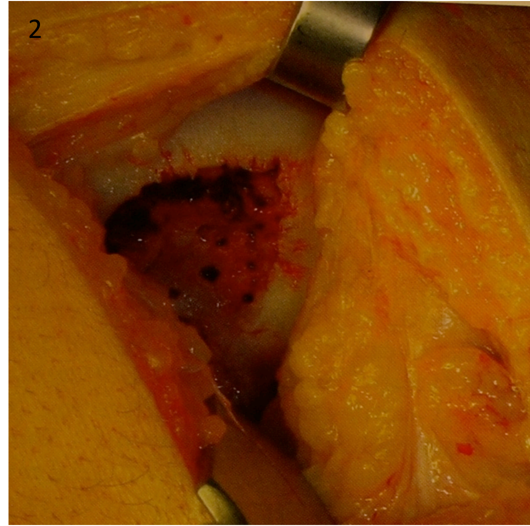


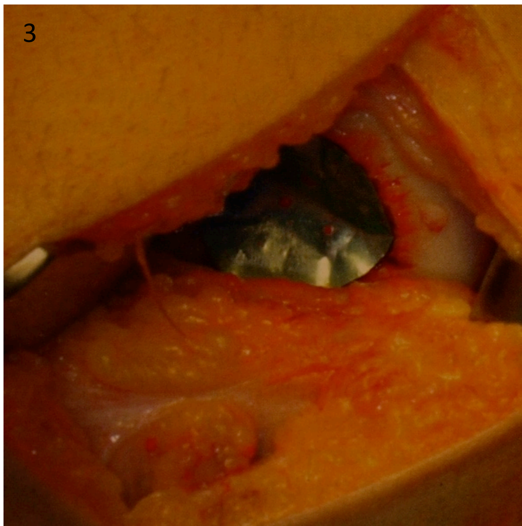
Fig. 7: MACI. Exposure of the chondral defect (1), debridement (2), haemostasis (3), cutting of the seeded membrane (4), fixation of the membrane with fibrin glue (5), final appearance (6).
(Reprinted with permission from Cherubino, P. et al. Surgical transplantation technique. In: Zanasi S, Brittberg M, Marcacci M, editors. Basic Science, Clinical Repair and Reconstruction of Articular Cartilage Defects. Current Status and Prospects. Rastignano, Bologna, Italy: Timeo Editore; 2006 p. 533-537)

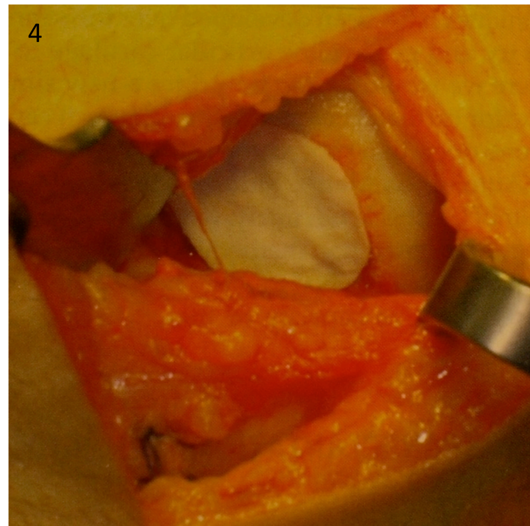
6.3.4 AMIC (Autologous Matrix Induced Chondrogenesis)

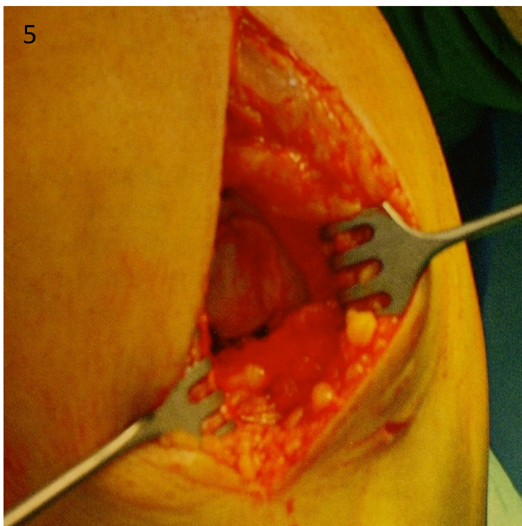
AMIC uses regenerative potential of MSCs in combination with artificial collagen membrane to heal cartilage defects. Firstly, the defect is debrided until normal vertical cartilage edges are achieved. Afterwards the microfracture holes are made with an angled awl. The defect is then covered by Chondro-Gide membrane (Gestlich Biomaterials, Switzerland) which could be attached by sutures and/or fibrin glue [69-73](Fig. 8).











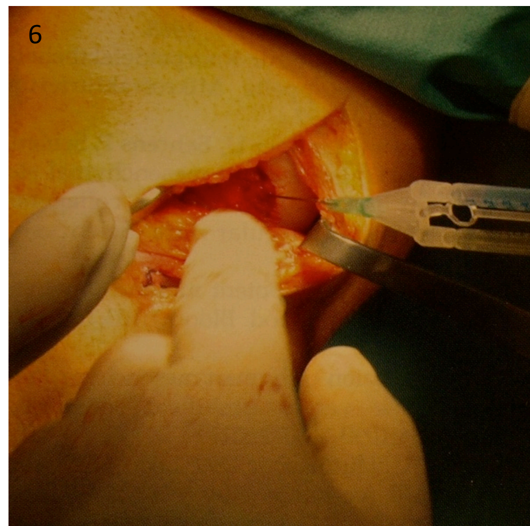


Fig. 8: AMIC. Preparing the defect area (1), performing the microfracture (2), making template (3), covering the defect with membrane (4), applying fibrin glue (5), final result (6).
(Reprinted with permission from Behrens, P., Mackenzie, R. AMIC®: Autologous Matrix Induced Chondrogenesis. In: Zanasi S, Brittberg M, Marcacci M, editors. Basic Science, Clinical Repair and Reconstruction of Articular Cartilage Defects. Current Status and Prospects. Rastignano, Bologna, Italy: Timeo Editore; 2006 p. 766-770)

6.4 *The chondrocyte*

Articular chondrocytes are around 13 μm in diameter and are the only cell type present in human articular cartilage. They most probably arise from MSCs differentiation under the growth of the organism [74-77]. In growing individuals ACs reach a high level of metabolic activity and proliferation rate. They synthesize large amounts of ECM components, organize them into new tissue and thus expand the cartilage surface. With skeletal maturation the proliferation rate, metabolic activity and matrix synthesis decline [78]. Most probably, in healthy adult cartilage the cells do not proliferate but rather maintain the cartilage matrix by synthesizing different ECM components and necessary regulatory agents [22, 79]. Because of their isolation in surrounding matrix, they usually do not form cell-to-cell contacts. However, on their surface short cilia extending from the cell into the ECM might be observed. The role of the cilia is not completely known, but it seems they might have a role in sensing the mechanical changes in ECM [22, 80, 81]. Electron microscopy of chondrocytes reveals cells of round morphology with a spherical or oval nucleus containing one or two nucleoli in the centre of the cell. Well developed **endoplasmatic reticulum (ER)** and **Golgi apparatus (GA)** might be identified in the surrounding cytoplasm. In addition, the cytoplasm might contain elongated mitochondria, lipid droplets, intermediate filaments, variable amounts of glycogen and secretory vesicles (Fig. 1) [15, 82-85]. According to their morphology, the chondrocytes could be divided into four types corresponding to the cartilage zones (Fig.2). i) **Morphotype I** ACs are situated in the superficial layer of cartilage. They have elongated morphology, contain an ovoid nucleus and are oriented parallel to the cartilage surface. They do nearly not show any metabolic and synthetic activity. ii) **Morphotype II** ACs are located in the transitional layer. They have spherical morphology with the large synthetic apparatus like GA and ER and high number of secretory vesicles in the cytoplasm. iii) **Morphotype III** ACs might be found in the deep layer of cartilage. These cells have also spherical morphology

with cytoplasm containing large amounts of glycogen, lipid droplets, and aggregations of intermediate filaments. iv) **Morphotype IV** ACs are situated in the calcified layers of cartilage. They are small, round cells with little cytoplasmatic volume and only few organelles [15].

In spite of avascularity of the cartilage tissue, glycolytic rate per cell is similar to vascularised ones revealing high metabolic activity of ACs. However, because of the low number of cells in cartilage, metabolic activity of the tissue as a whole is low [22]. It has been observed that native ACs operate at very low oxygen levels (around 6% at the synovial face and around 1% in the deep layers of cartilage) and reveal energy metabolism based on **Embden-Mayerhof-Parnas pathway** of anaerobe glycolysis [86, 80, 87]. Interestingly, it has been shown that in vitro expansion of chondrocytes in hypoxic cultures might lead to inhibition of glycolysis and low matrix production. This paradox is called negative **Pasteur effect** [88, 89]. With aging organism also the chondrocytes become old and undergo typical phenotypical and morphological changes. The nucleus becomes more lobed, and increased accumulation of lipids and glycogen might be observed in the cytoplasm. Degeneration and death of the ACs occur mostly by necrosis and disintegration, rather than by standard programmed cell death. If the neighbouring cells are closely adjoining, they might engulf the dying cells, although in most cases, the remnants remain in the lacuna of the cell [15, 90, 91].

6.5 *Mesenchymal stem cells*

Box 1: Sources and differentiation lineages of multipotential adult mesenchymal stem cells.

Tissue sources

Bone marrow
Trabecular bone
Muscle
Adipose
Periosteum
Synovial membrane
Articular cartilage
Deciduous teeth
Pericyte
Peripheral blood

Multilineage differentiation

Chondrocyte
Osteoblast
Adipocyte
Muscle
Tendon or ligament
Endothelial cells
Stromal cells
Astrocyte
Neuron
Cardiomyocyte
Hepatocyte
Mesangial cells

(Reproduced from Chen FH, Rousche KT, Tuan RS. Technology Insight: adult stem cells in cartilage regeneration and tissue engineering. Nat Clin Pract Rheumatol 2006; 2(7):373-382).

Mesenchymal stem cells are adult multipotent cells residing in bone marrow and other tissues (Box 1)[92]. In the bone marrow, they are widely dispersed in a network of extracellular matrix fibrils and represent only a minor fraction of the total nucleated cells population [93]. The morphology of MSCs is characterized by the relatively long cell body, large nucleus, prominent nucleolus, and dispersed chromatin particles. Cytoplasm contains small GA, ER, mitochondria and polyribosomes (Fig. 9) [94]. The multipotency of MSCs is characterized by their capacity to differentiate into different mesodermic and non-mesodermic tissues (Box 1, Fig. 10). They have an ability to keep this multipotent potential also *in vitro* for numerous populational doublings, which makes them an attractive source for cell transplantation techniques [9, 95]. To distinguish MSCs in culture from other nucleated cells originated in bone marrow, characterization is required [96-99]. To overcome the lack of clearly defined surface markers, Mesenchymal and Tissue Stem Cell Committee of the ISCT (International Society for Cellular Therapy) proposed a set of criteria to define MSCs in culture; i) adherence to plastic surfaces; ii) over 95% of cell population express surface antigens CD105, CD73, CD90 and under 2% of cell population express

antigens CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II; iii) multipotent differentiation potential (table 3) [100]. Following the adhesion of MSCs to plastic, the non-adherent cells are washed away, and after their initial expansion the MSCs might be differentiated into different cell-lines [101]. Addition of substances such as B-glycerol-phosphate, ascorbic acid-2-phosphate, dexamethasone and fetal bovine serum to the culture media lead to their osteogenic differentiation. The cells acquire osteoblastic morphology, up-regulate alkaline phosphatase activity and deposit calcium-rich mineralized ECM [102]. Expansion of MSCs in three dimensional serum-free cultures supplemented with growth factors such as TGF-β leads to chondrogenic differentiation of the cells. They up-regulate expression of cartilage specific genes and synthesize cartilage specific proteins [32, 103]. Differentiation of MCSs to adipocyte cell-line in culture requires an addition of isobutylmethylxanthine. The cells change their morphology, and large lipid-filled vacuolas might be observed in the cytoplasm.[102].

Table 3: Criteria for MSCs characterization.

1	Adherence to plastic in standard culture conditions		
2	Phenotype	Positive (≥ 95% +)	Negative (≤ 2% +)
		CD105	CD45
		CD73	CD34
		CD90	CD14 or CD11b
			CD79α or CD19
			HLA-DR
3	In vitro differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of in vitro cell culture)		

(Reproduced from: Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8(4):315-317.)

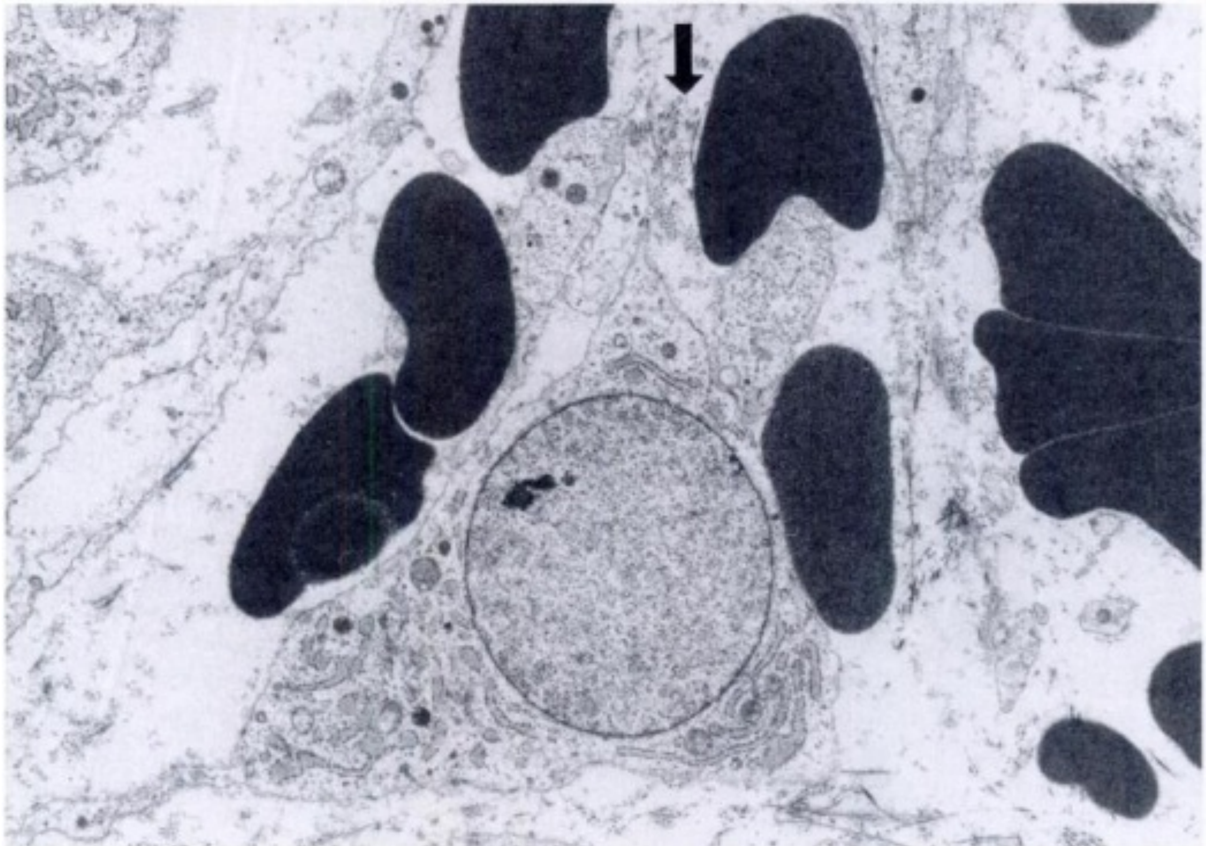


Fig. 9: Electron micrograph of the region of low cellular density, made twelve hours after experimental fracture of bone, showing a polymorphic mesenchymal cell that is touching some of the erythrocytes, and small bundles of collagen fibrils (one bundle is marked by an arrow) (X 7200). *(Reprinted with permission from Brighton CT, Hunt RM. Early histological and ultrastructural changes in medullary fracture callus. J Bone Joint Surg Am 1991; 73(6):832-847).*

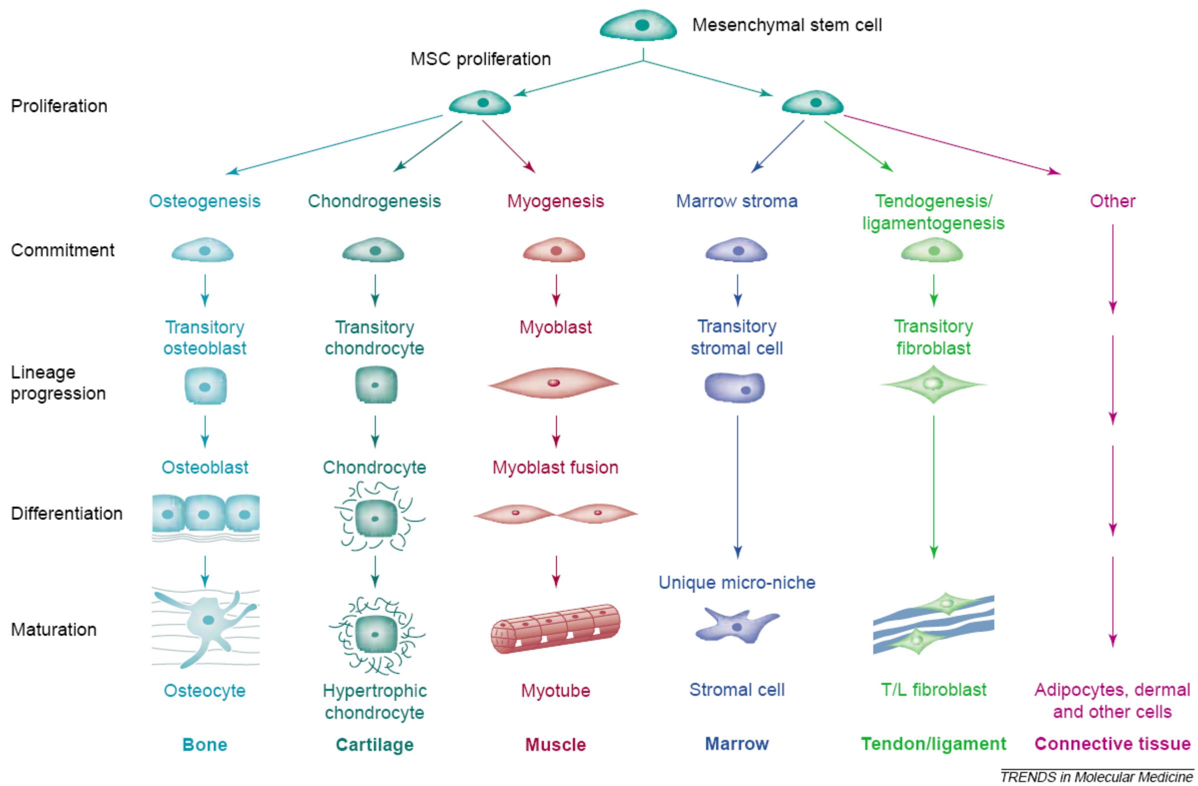


Fig. 10: The mesengenic process. The stepwise cellular transitions from the putative mesenchymal stem cell (MSC) to highly differentiated phenotypes are depicted schematically. This scheme is oversimplified and does not represent all of the transitions or all of the complexities of single lineage pathways, nor does it represent the potential interrelationships of cells moving between pathways, now commonly referred to as 'plasticity'. The individual lineage pathways are arranged from left (best understood) to right (least understood); the osteogenic and chondrogenic pathways are based on detailed experimental information. It is believed that major mitotic expansion takes place in marrow/periosteum or at sites of massive mesenchymal tissue repair, and the highly differentiated cells possess a substantially restricted proliferative potential. *(Reprinted with permission from Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med 2001; 7(6):259-264.)*

6.6 *Concepts around tissue and cell cultures*

Many approaches for biological repair of different tissues in human body require *ex vivo* culture of extracted cells in order to achieve a sufficient cell count. Therefore, understanding of the phenotypical changes that the cells undergo under culturing is crucial in order to develop new or improve existing tissue engineering techniques. Cell culture is a complex process by which the cells are grown under controlled conditions [104]. The history of cell cultures goes back to the beginning of 20th century when Ross Granville Harrison published results of his experiments establishing the methodology of tissue culture [105]. However, because of the vulnerability of mammalian cells to the contamination by bacteria, cell cultures had not become routine until 1950s when antibiotics were introduced. Cultures of cells freshly isolated from tissue are called **primary cultures**. The cells cultured for approximately 2-3 passages might also be called **short-term cultures**, those up to 15 passages **long-term cultures**. Extraction of the cells from tissues is usually achieved by **enzymatic digestion** with substances such as collagenases, trypsins or proteases breaking the ECM. The cells are then maintained in culture media in a cell incubator with appropriate temperature and gas mixture. According to the cell type, different compositions of culture media regarding pH, glucose concentrations, growth factors and nutritional supplements are used. Antibiotics and antifungal substances are crucial to avoid growth of microbes in cultures. The standard source of growth factors in the culture media is serum, which might be either autologous, fetal bovine, or calf. However, serum contains high amounts of different active substances that might exert effects on cells in an uncontrolled manner. Therefore, special serum-free media have been designed in order to standardise effect of various growth factors and other active substances on the cultured cells.

Normally, all adult human cells cultured *in vitro* have a limited life span. After a certain number of populational doublings, the cells stop proliferating while still remaining viable and

could maintain this state for months. This cell transformation is called **senescence** and is histologically characterized by cells enlargement and flat "fried egg morphology" (Fig. 11) [106].

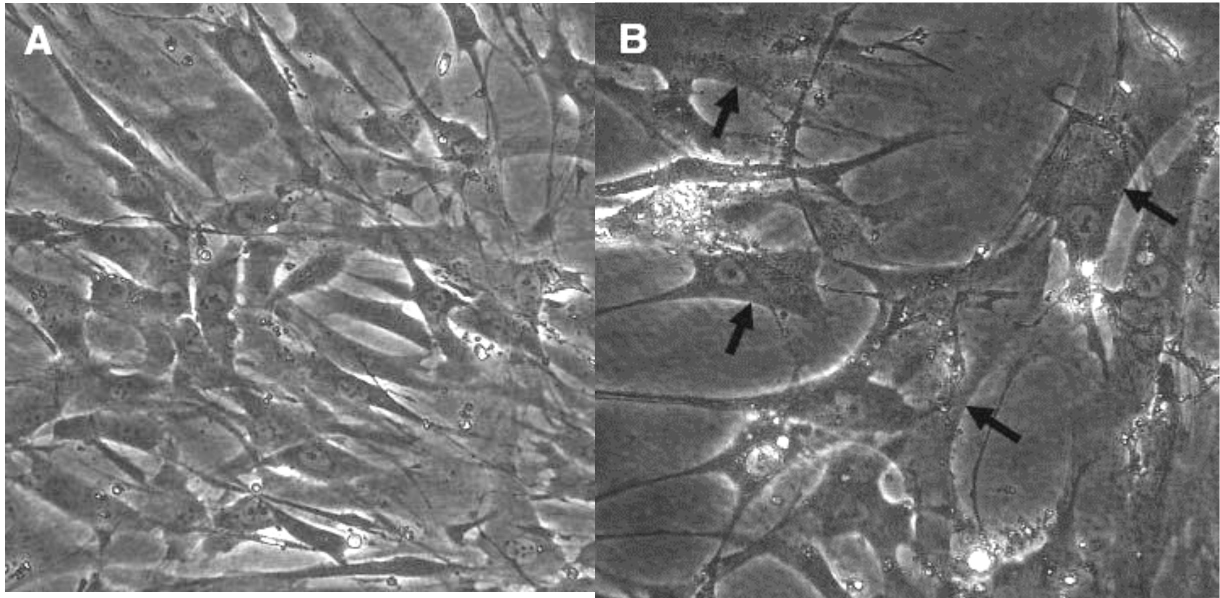


Fig. 11: Cellular morphology of mesenchymal stem cells (MSCs) in early and senescent passage. MSCs were isolated from human bone marrow and photo documented at passage 2 (A) and passage 8 (B). Upon long-term culture the majority of cells acquire a large and flat morphology (arrows). *(Reprinted with permission from Wagner W, Ho AD, Zenke M. Different Facets of Aging in Human Mesenchymal Stem Cells. Tissue Eng Part B Rev 2010; 16(4):445-453.)*

The phenomenon was for the first time described by Hayflick in 1965 [107]. He also introduced **Hayflick limit**, which is a cell-intrinsically determined limit of cell divisions in vitro [108]. Although the exact mechanisms are not completely ascertained, increasing evidence suggests that senescence is at least partially caused by telomerase shortening and that the age of the donor influences the proliferation capacity of the cells [109, 110]. Most *in vitro* studies operate with a certain number of passages (around 10-15 for MSC and 6-8 for ACs) before the cells reach senescent stage. However, because our knowledge of cell transformation in culture is very limited, the age of cells in culture measured by the number of passages is very relative. It

might depend on various factors like culture density, culture design, medium composition, age of the donor, and thus is difficult to measure in spite of standard culture conditions [106, 107, 111, 112].

Cultured cells might acquire an ability to proliferate indefinitely and not be restricted by the Hayflick limit. Such cells, also called **immortalized cell lines,** might be created by induction of different oncogenes or loss of tumor suppressor genes. The term immortalization was for the first time applied to cancer cells that expressed the telomere-lengthening enzyme, and thereby avoided apoptosis. The most famous cell line is HeLa cell line derived from cervical cancer cells from patient Henrietta Lacks, who died from her cancer in 1951 [113]. Nowadays, there are numerous well established cell lines representative of particular cell types [114] (table 4).

Table 4: Some of the commonly used cell lines [114].

Cell line	Cell type and origine
3T3	fibroblast (mouse)
BHK21	fibroblast (Syrian hamster)
MDCK	epithelial cell (dog)
HeLa	epithelial cell (human)
PtK1	epithelial cell (rat, kangaroo)
L6	myoblast (rat)
PC12	chromaffin cell (rat)
SP2	plasma cell (mouse)
COS	kidney (monkey)
293	kidney (human); transformed with adenovirus
CHO	ovary (chinese hamster)
DT40	lymphoma cell for efficient targeted recombination (chick)
R1	embryonic stem cells (mouse)
E14.1	embryonic stem cells (mouse)
H1, H9	embryonic stem cells (human)
S2	macrophage-like cells (drosophila)
BY2	undifferentiated meristematic cells (tobacco)

(Reproduced from Alberts B, Johnson A, Lewis J, Raff M, Roberts K,Walter P. Manipulating Proteins, DNA, and RNA. Molecular Biology of the Cell. 4th ed. New York: Garland Science; 2002.)

6.7 ***Proteomics***

Our increased understanding of different biological processes in cells and tissues resulted in development of advanced analytical methods in order to find substances responsible for different pathological processes. During the last decades several previously un-suspected proteins were identified as key factors in disease development. Therefore proteomics defined as study of the set of proteins expressed by tissue or cell and changes in protein expression patterns in different environments or conditions became a standard in analysing complex protein samples [115]. Proteomic analysis combines separation techniques like **polyacrylamide gel electrophoresis** (one- or two- dimensional) followed by band excision, digestion and mass spectrometric analysis revealing mass spectra or finger prints of each protein present in the sample [116]. Using bioinformatic tools and different proteins databases, the mass spectra are transformed to peptide sequences. Mass spectrometry based proteomics have undergone tremendous development in recent years and new, powerful proteomic approaches have enabled identification and precise quantification of thousands of proteins in complex tissue samples [117, 118]. Understanding of protein expression by normal and diseased tissues, as well as their interactions with other cells and tissues might increase our understanding of cells physiological and pathological processes and enable identification of new diagnostic or therapeutic agents.

6.8 ***Tissues and cells secretome***

Secretomics is a recently described scientific approach to study and describe proteins secreted by a cell, tissue or organism at any given time and under certain conditions. Secreted proteins are not only building components of ECM but also important regulatory molecules playing a crucial role in various physiological and pathophysiological processes. In the human body, secretion of proteins by cells is complex and tightly regulated. The cells continuously

react to different stimuli and signals they receive from their environment and consequently activate or down-regulate secretion of active substances. Thus misbalance or aberrant secretion pattern might indicate abnormal or pathological conditions [119]. Active substances with systemic effect on the organism are secreted not only by endocrine organs as believed in the past, but also by other organs or tissues not primarily known for their endocrine function. Like adipose tissue which is now known to secrete a variety of active substances involved in food intake, energy, glucose and lipid metabolism [120]. Muscle cells are also known to secrete amount of different cytokines with both local and systemic effects [121, 122]. The term secretome was for the first time introduced by Tjalsma et al. studying secreted proteins of Bacillus subtillis [123].

Although analysis of gene expression might provide some clues about secreted proteins, genes are only messengers and the final appearance of protein secretome might be completely different. Not only does the translation from mRNA cause differences, many proteins are also subjected to a wide variety of post-translational modifications which are often critical to the protein's function. However, studies of secretome are much more complicated than those of genes mostly because while an organism's genome is more or less constant, the proteome differs from cell to cell, from time to time and from culture to culture [124, 125]. On the other side the secretome represents a more complex and comprehensive reflection of cells functional status making secretomics a very attractive approach for discovering different diagnostic biomarkers and therapeutic agents.

6.9 ***Chondrocytes secretome***

The secretome is defined as the bulk of peptides, proteins, growth factors, cytokines and other active substances produced and secreted by cells. In cartilage, the majority of the substances

secreted by chondrocytes in vivo are ECM components especially collagen type II and aggrecan. However, there are numbers of other ECM components such as small proteoglycans and different paracrine and autocrine factors secreted in much lower amount, but with an important function in cartilage matrix regulation. **Small leucine-rich proteoglycans** (SLRPs) like decorin, biglycan, fibromodulin and lumican have roles in stabilizing the ECM. They interact with collagen fibrils, regulate their diameter during formation and moderate access of collagenases to their cleavage site on each of the collagen molecules [37, 126, 127]. A variety of other non-collagenous matrix molecules are also known to be produced by ACs. Among these, **cartilage oligomeric matrix protein (COMP)** is the main non-collagenous matrix protein, localized mostly to the territorial matrix. It binds to collagen fibrils and seems to have an important role in maintenance of cartilage's structural strength [128, 129]. **Tenascin** is another non-collagenous matrix glycoprotein. In healthy cartilage, it is most concentrated in the territorial matrix around chondrocytes. Recent studies suggest that tenascin might promote chondrogenesis, what could explain its high level appearance in degenerative cartilage [130].

ACs continuously respond to different signals and active substances like cytokines and growth factors and sense changes in their environment through a number of surface receptors. Among these active substances that might also be secreted by chondrocytes, **platelet-derived growth factor (PDGF)** appears to have mitogenic effect on chondrocytes and thus is believed to have a potential in enhancing tissue regeneration and repair [131]. **Basic fibroblast growth factor (bFGF)** has an anti catabolic effect on articular cartilage. It induces TIMPs and up-regulates **activin**, a member of TGF-β family [132]. **Transforming growth factor beta** family is a group of growth factors stimulating PGs synthesis [133]. **Insulin-like growth factors** exert also anabolic effects on ACs. Though collagen metabolism appears not to be affected, it seems that this factor balances PGs turnover by slowing their catabolism [131].

Secreted proteins playing key roles in balancing ECM turnover are proteases and protease inhibitors. Most important proteases responsible for degradation of the cartilage matrix are **matrix metalloproteinases (MMPs)** and **aggrecanases (ADAMTs - A disintegrin and metalloproteinase with thrombospondin motifs)**. MMPs are synthesized as an inactive enzyme which is extracellulary activated by cleavage of N-terminus pro-peptide domain. Both MMPs and ADAMTs might be divided into different subgroups according to their target molecules (Table 5, 6). Their proteolytic activity is balanced by **tissue inhibitors of metalloproteinases (TIMPs)** which suppress the activity of MMPs by binding to their active sites. Moreover, the TIMPs also inhibit cleaving of proteoglycans by agrecanases [1, 37].

Table 5: Selected MMPs and their representative substrates.

MMP	Substrates
MMP-1	coll I, II, III, IV, VII, VIII
MMP-2	gelatins, coll III, IV, V, VII
MMP-3	aggrecan, fibronectin, gelatin
MMP-7	aggrecan, coll I, IV
MMP-8	coll IX, X, XI, XIV, gelatin, aggrecan
MMP-9	coll IX, elastin, fibronectin, laminin, proteoglycans, pro-MMP-13
MMP-10	laminin, elastin, proteoglycans
MMP-11	coll III, IV, V, pro-MMP-13
MMP-12	elastin, fibronectin, gelatin
MMP-13	fibronectin, pro-MMP-9, coll II
MMP-14	coll I, II, III, gelatin
MMP-15	fibronectin, vitronectin, laminin
MMP-16	pro-MMP-2, pro-MMP-13
MMP-19	laminin, COMP

(Reproduced from Grad S, Lee CR, Alini M. Biology: mechanism of cartilage breakdown and repair. In: Zanasi S, Brittberg M, Marcacci M, editors. Basic Science, Clinical Repair and Reconstruction of Articular Cartilage Defects. Current Status and Prospects. Rastignano, Bologna, Italy: Timeo Editore; 2006 p. 69-85.)

Table 6: Selected ADAMTs with known aggrecan cleaving activity.

Enzyme	Substrates	Aggrecan-degrading activity
ADAMTS-1	aggrecan, versican	weak
ADAMTS-4	aggrecan, versican, COMP	high
ADAMTS-8	aggrecan	weak
ADAMTS-9	aggrecan, versican	weak
ADAMTS-15	aggrecan	

(Reproduced from Grad S, Lee CR, Alini M. Biology: mechanism of cartilage breakdown and repair. In: Zanasi S, Brittberg M, Marcacci M, editors. Basic Science, Clinical Repair and Reconstruction of Articular Cartilage Defects. Current Status and Prospects. Rastignano, Bologna, Italy: Timeo Editore; 2006 p. 69-86.)

7 Aims of the study

7.1 *Main goal*

The work included in this thesis aimed at exploring the processes of cell de- and re-differentiation experimented by chondrocytes and bone-marrow-derived progenitor cells during *ex vivo* expansion, by analyzing and comparing the protein profiles of the secretome of cells established in different configurations.

7.2 *Subgoals*

1. To study the process of cell de-differentiation undergone by adult articular chondrocytes by comparing the secretome of cartilage explants with the secretome of cells in monolayer cultures.
2. To explore the process of cell re-differentiation undergone by *in vitro* expanded adult articular chondrocytes by comparing the secretome of cells established in either monolayer or in 3D configurations.
3. To characterize the phenotype of in vitro expanded de-differentiated adult articular chondrocytes and un-differentiated bone-marrow-derived mesenchymal stem cells by studying their secretory protein profiles, and to compare them to identify the best cell type for cell based transplantation strategies.

8 Methodological considerations

Different aspects of the key methodology used in this thesis work are briefly discussed in this chapter. Detailed descriptions may be found in the papers following this introduction.

8.1 *ACs and MSCs cultures*

Many of the established techniques to attempt cartilage resurfacing require an initial *ex vivo* expansion of ACs or progenitors. Normally, after harvesting the biopsy, the cells are extracted from the tissue by enzymatic digestion and expanded for three to four weeks in monolayer cultures in autologous serum enriched culture media in order to increase the cell number. In our experiments, we have intentionally used the same method as used in standard ACI in order to reproduce the conditions used in clinics. Although ACs are intrinsically programmed to produce and maintain the cartilage matrix, in monolayer cultures they de-differentiate and acquire a more fibroblast-like phenotype. Physiologically round chondrocytes became elongated with flattened morphology and decrease secretion of cartilage specific proteins like collagen II and aggrecan. Although these changes might be irreversible if the cells reach senescense, de-differentiated chondrocytes have the ability to re-differentiate. If cultured in 3-dimensional cultures such as pellets or spheroids in hanging drops, they might regain to some content their chondrogenic potential and increase the secretion of cartilage specific proteins. However, exact mechanisms of cell de- and re-differentiation in culture are not completely understood. In order to increase the understanding of these processes, the phenotype of cultured cells needs to be contrasted with the phenotype of cells in native tissue. Tissue explants, which are small pieces of living cartilage tissues cultured in artificial media,

represent a condition where the cells keep their tissue-specific functions in natural, three-dimensional architecture.

Compared to chondrocytes, MSCs are easier to extract from patients and cause lower donor site morbidity than the harvesting of ACs. The selection of non-haematopoietic cells from initial bone marrow aspirates is based on their ability to attach to plastic surfaces. MSCs have in general terms higher proliferative potential than ACs and hold their differentiation potential for longer time periods in culture without reaching senescence. If cultured in three-dimensional culture in the presence of specific growth factors like TGF-β and dexamethasone, they might acquire chondrogenic potential. However, they might also differentiate into other tissues instead of cartilage, or in some instances the differentiation process is incomplete and thus the resulting tissue resembles immature cartilage.

8.2 ***Preparation of samples for MS and separation of proteins***

In the three studies presented in this thesis the proteomic analyses have been conducted after initial metabolic labelling of proteins in culture. Following the initial culture phase, the cells were extensively washed in order to eliminate most of the residual serum components. Thereafter, the cells were cultured in the SILAC medium for approximately 10 days in order to accomplish significant protein labelling. Pilot experiments clearly showed that small volumes of un-concentrated conditioned medium separated by electrophoresis have too low total protein concentration to show adequate band complexity. Therefore, the supernatants were concentrated by ultrafiltration in Vivaspin tubes to a smaller volume of around 600-800μl and protein content was measured. After the concentration, the average yield of protein recovery from 6 ml of the culture supernatants was about 2-3 mg/ml/10^6 cells giving nice band complexity in the gels. Measurement of protein concentration is especially important in comparative analyses where loading the equal amounts of total protein from studied samples

into the gels is crucial. To separate proteins in supernatants according to their molecular weight we used one dimensional SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) electrophoresis. Other groups have been using two-dimensional gel electrophoresis separating proteins according to their molecular weight and isoelectric point. Although this approach might provide good information about protein content in medium, several protein classes might be overlooked because of their low abundance, extreme iso-electric points or extreme masses. One-dimensional gel electrophoresis is much easier and more reproducible without compromising protein identification by MS. In addition, modern proteomic analyses combine in tandem multi-dimensional liquid chromatographic protein separation (LC) with double mass spectrometry, making it possible to separate and identify several proteins from each gel band which eventually may contain different protein mixtures. The use of "shotgun approach" where the entire gel run is excised in bands and analyzed by MS might also help to minimize the number of overlooked proteins.

8.3 ***Proteomic analysis***

Quickly emerging modern proteomic methods have eased the analysis of complex samples and allowed identification of hundreds of different proteins from a simple gel run. The technique relies on mass spectrometric analysis of peptides produced by trypsin in-gel digestion of a protein band and following matching of MS-generated mass-fingerprints to a protein database. There are two main MS techniques: i) MALDI (matrix-assisted laser desorption/ionization) which sublimates and ionizes the samples out of a dry, crystalline matrix via laser pulses and could be used to analyse simple protein samples; ii) LC-MS (liquid chromatography-mass spectrometry) which can be used to analyze complex protein mixtures [117, 119]. Because of the high complexity of cells secretome, in our experiments we used advanced Q-TOF Ultima global mass spectrometer (Waters, USA) and LC/MS-MS

(liquid chromatography/double mass spectrometry) technique providing high sensitivity, resolution and mass accuracy for the studied proteins. Although mass spectrometry is very sensitive approach, it has certain limitations. Generated results might be inaccurate due to the identification of peptides sharing very close amino acid sequences but corresponding to different proteins from the same family. Besides, the technique might not be able to identify proteins present at very low abundance accurately. Therefore, in our study only proteins identified with Mascot score higher than 50 have been considered in order to minimize the inclusion of poorly identified proteins. Mascot score represents the sum of the unique ions scores and thus significance of the protein identification.

8.4 *SILAC (Stable Isotope Labelling of Amino Acids in Cell Culture)*

The main drawback of standard proteomic studies of cell's secretome resides as the difficulty to discriminate between proteins truly secreted by cells and proteins stemmed from serum supplements or other sources. Stable isotope labelling of amino acids in cell culture (SILAC) represents an elegant solution to this problem. Regular essential $^{12}C_6$ amino acids arginine and lysine in culture medium are replaced by heavy $^{13}C_6$ labelled ones and so incorporated into all newly synthesized proteins. Heavy carbon labelled proteins are then easily identified by MS analysis. However, in order to achieve significant protein labelling, the cells need to be cultured for long periods of time. Our experiment showed that over 90% of proteins identified in supernatants are labelled after 10 days culture.

The method allows not only qualitative comparison based on "presence-absence" of the identified protein, but also quantitative comparison of individual proteins by mixing labelled groups with un-labelled ones. Shortly, because of isotope labelling the proteins including labels are heavier than non-labelled ones. Thus, after mixing labelled condition with non-labelled one in 1:1 ratio regarding the protein concentration, MS analysis reveals pairs of

labelled and non-labelled peaks (mass fingerprints) for each trypsinised peptide present in both labelled and non-labelled sample. If there is no variance in the intensity of the peak pair, there are no differences in the abundance of compared proteins. On the contrary, when peak intensity from heavy peptide is higher than peak intensity from light peptide, the protein containing labelled peptide is more abundant [119, 134]. The complexity and variation of cells' secretome makes the quantitative comparison challenging. In paper II when secretion medium of ACs in monolayers (2D) and spheroids (3D) were mixed prior to gel electrophoresis, total protein concentration was determined and used to mix equal amounts of proteins. Unfortunately, the amounts of non-labelled or weakly labelled proteins were different between the experimental groups, thus obscuring the "real" 1:1 ratio (not total protein content). That's why parallel experiments by labelling the groups also in reverse were necessary and the ratio needed to be adjusted to "real one" in order to get more precise results. At the end the "real" ratio was 2:1 in favour of monolayer proteins.

8.5 *Western blotting*

Data revealed by mass spectrometric analysis needed to be validated by other methods that specifically determine the expression of identified proteins. Because the protein determination offers a more accurate picture of the analysis when compared to other standard methods such as real time PCR, in all of our papers, we chose western blotting to validate the expression of proteins that we considered of high relevance in the context of cartilage biology. The principle behind western blotting is detection of selected proteins in samples by specific antibodies after the proteins are separated by one-dimensional gel electrophoresis and transferred to the polyvinylidene difluoride membranes. Western blots confirmed the data recorded by MS and the size of the bands (kDa) corresponded approximately to the anticipated size of the full-length proteins. However, some of the bands appeared at lower

molecular weight than expected most probably representing peptides derived from uncontrolled proteolysis of original precursors during the incubation period.

8.6 *Human cytokine microarray*

Because some of the relevant cytokines or growth factors might be expressed under the detection limit of mass spectrometric analysis, in paper II our MS analyses were complemented with protein antibody microarrays. The array membranes were manufactured to detect 79 different cytokines, growth factors or hormones after exposure to concentrated culture supernatants from ACs established in either monolayers or 3D-spheroids. Protein antigens were detected by enhanced chemiluminiscence reaction and the signals were captured on X-ray films. This approach allows an accurate comparison of two experimental groups with high sensitivity and without need for metabolic labelling of samples. However, because the affinity of proteins to their respective antibodies may differ substantially, the intensities of spots may not reflect the relative amounts of proteins. The main drawback behind this technique is that it might only be used for qualitative and not quantitative comparison of two given conditions.

8.7 *Luminex*

Luminex, or Fluorescent Bead-Based Fluorokine-Multi Analytes Profiling Assay, was used in paper III to perform a quantitative measurement of matrix metalloproteinases and tissue inhibitors of metalloproteinases in supernatants of de-differentiated ACs and un-differentiated MSCs. Specific antibodies were pre-coated onto color-coded micro particles by manufacturer. Micro particles, standards and samples were pipetted into wells and the immobilized antibodies bound the analytes of interest. Afterwards biotinylated antibodies

specific to the substances of interest were added to each well followed by addition of streptavidin-phycoerythrin conjugate binding the biotinylated detection antibodies. Consequently, the micro particles were resuspended in the buffer and read using the Luminex analyzer. The main advantage of this method is that it permits the quantification of targeted molecules in culture supernatants. In addition, it allows high sensitivity, standardization and offers the possibility to analyze many samples in parallel thus achieving reproducible results. On the weak side, the technique is limited to the number of kits commercially available, and the high cost.

8.8 *Evaluation of gene expression (real time PCR)*

Proteins are synthesized the same way in every cell of our body. Sequence of DNA corresponding to specific gene is transcribed into mRNA, which is afterwards translated into protein. In paper III we evaluated cartilage characteristic gene expression in order to explore the chondrogenicity of *ex-vivo* expanded ACs and MSCs. Real time PCR is a laboratory technique which enables both detection and quantification of one or more specific targeted sequences in a DNA sample. Firstly, total RNA is isolated from the cells, followed by reverse transcription step to produce the first strand of cDNA (complementary DNA). In the next step, many copies of the first strand DNA are generated. Repeated cycles of heating and cooling the PCR sample allow specific designed DNA primers, deoxynucleotide triphosphates (dNTPs), together with DNA polymerase to exponentially amplify the DNA sequence of interest. After about 30 cycles, millions of copies are generated and gel electrophoresis is used to size-separate the PCR products. In our experiments, we used real time PCR technique based on TaqMan chemistry using a fluorogenic probe to enable the detection of specific PCR products as they accumulate during PCR cycles. A relative quantification assay based on Comparative C_T method was used to determine the changes in gene expression in test samples

relative to a control samples [13, 135-137]. The primary data were normalized against the values measured for GADPH house keeping gene. In graphics, the expression of each analyzed gene was presented in respect to the expression found in chondrocyte monolayers. Thus up-regulated genes in the other groups appeared as positive columns and down-regulated genes as negative ones.

9 Summary of results

9.1 *Paper 1*

SILAC labelling was achieved in both expanded chondrocytes and cartilage explants. After 8-10 days in monolayer culture, the mass spectrometric analysis revealed a list of 103 proteins (or over 90% of total proteins) identified in supernatants of ACs in monolayers labelled with heavy amino acids. Non-labelled proteins were mostly contaminant plasma-associated proteins or proteins stemmed from initial culture periods in serum containing medium. Most of the labelled proteins corresponded to matrix components and matrix regulating agents including proteases, protease inhibitors, different growth factors, and anti-inflammatory proteins, though a few of the proteins identified in secretion medium of ACs were characterized as intracellular components or metabolic agents. MS analysis of supernatants from cartilage explants revealed that only 29% of identified proteins were labelled with heavy amino acids. Non-labelled ones corresponded mostly to structural matrix components considered to be a result of degradation of existing matrix, though several metabolic proteins and serum remnants were also identified. To validate the results western blotting of four relevant proteins including tissue inhibitor of metalloproteinases 1, SPARC, osteomodulin and growth arrest-specific protein 6 was performed. Validation confirmed the results acquired by MS and the size of the bands revealed in blots corresponded to expected size of the full length proteins.

9.2 *Paper 2*

Qualitative proteomic comparison using SILAC approach revealed that around 95% of all proteins identified in supernatants of ACs in monolayers were labelled by heavy amino acids. In supernatants of ACs cultured in 3D around 80% of all identified proteins were labelled.

The proteins were categorized according to their functionality and the results showed that chondrocytes in monolayer growth released higher number of extracellular matrix components into the media during monolayer growth (50% vs. 36%). However, chondrocytes in spheroids expressed lower number of matrix catabolic agents (9% vs. 10%), higher number of matrix anabolic agents (11% vs. 5%), and higher number of anti-inflammatory and anti-oxidant proteins (24% vs. 13%).

Quantitative MS analysis using SILAC approach revealed elevated expression of seven proteins including CTGF and GAS6 in monolayers along with matrix molecules characteristic for de-differentiated ACs such as collagen I and tenascin. On the other hand, the spheroid configuration favoured the expression of six proteins, among them cartilage specific matrix component aggrecan and important matrix regulators like chitinase-3-like protein 2 and stromelysin-1.

Validation of the MS results was performed by immuno-detection of eight relevant proteins (SPARC, TIMP1, OMD, GAS6, CTGF, CHI3L2, MMP3 and aggrecan). The results obtained by western blots confirmed precisely the findings acquire by quantitative SILAC analyses. The size of the bands in western blots corresponded to expected size of the full length proteins. Additionally, the intensity of the bands after immunoreaction matched up with the mascot scores in the proteomic analysis.

Because some cytokines or growth factors might be under detection limit of proteomic analysis, the data were complemented by antibody micro arrays prefabricated to detect 79 different cytokines or growth factors. Although the results showed similar pattern, ACs in monolayers revealed enhanced expression of growth-related oncogene protein (GRO), monocyte chemoattractant protein-1 (MCP-1), IL- 8, angiogenin and insulin-like growth factor binding protein-2 (IGFBP-2) compared to ACs in spheroids. On the other hand ACs in

spheroids showed over-expression of CKb 8-1, macrophage colony-stimulating factor (MCSF) and vascular endothelial growth factor (VEGF).

9.3 *Paper 3*

Initial analysis of cartilage specific gene expression showed significantly lower expression of collagen II gene and higher expression of collagen I gene in both de-differentiated ACs and un-differentiated MSCs when compared to native cartilage. However, ACs revealed higher expression of collagen II and sox9 genes than MSCs.

Qualitative proteomic analysis showed that over 92% of proteins identified in culture medium of both MSCs and ACs were labeled with $^{13}C_6$ amino acids. Similar numbers of ECM constituents (45% in ACs vs. 43% in MSCs) and matrix catabolic agents (10% in ACs vs. 9% in MSCs) were released into the media by both cell types. However, MSCs released a higher number of matrix anabolic agents (12% vs. 7%) compared to ACs. The majority of proteins were identified in supernatants of both cell types, though some proteins like clusterin, mimecan, proteoglycan 4, tenascin and sushi (SVEP1) were identified only in spent medium of ACs, and other proteins like serpins, bone morphogenic protein-1 and galectins were identified only in spent medium of MSCs.

MS results were validated by western blotting of eight specific proteins relevant in cartilage regulation (SPARC, GAS-6, CHI3L2, OMD, MMP-3, BMP-1, galectin-1 and serpin I2). Validation confirmed the findings and the size of the bands (kDa) corresponded approximately to the expected size of the full-length proteins.

Quantitative measurement of MMPs (-1,-2,-3,-7,-9,-13) by Fluorescent Bead-Based Fluorokine-Multi Analytes Profiling Assay showed significantly higher concentrations of MMP-1 ($P<0.05$), MMP-2 ($P<0.05$), MMP-3 ($P<0.001$) and MMP-7 ($P<0.05$) in supernatants of ACs compared to MSCs. Quantitative measurement of TIMPs (-1,-2,-3,-4) showed

approximately same expression of all four TIMPs in both groups, where TIMP-1 and -2 were secreted at markedly higher level then TIMP-3 and -4 in both ACs and MSCs.

10 General discussion and summary

Development of cartilage repair techniques has generally been based on empiric approaches without enough efforts spent on exploring the fundamental cell biology and consequences of in vitro cell growth on cell functions, morphology and phenotype. To move further in pursuing the ultimate goal of hyaline repair, clinical experience needs to be complemented by studies exploring ACs and MSCs at a more basic level. Studies of proteins secreted by cells might offer a unique possibility to investigate all the different matrix components, regulators, autocrine and paracrine factors responsible for tissue remodelling. In our studies, we performed proteomic profiling of chondrocytes and chondroprogenitors secretome in diverse culture settings using SILAC methodology. We showed that stable isotope labelling technology combined with advanced MS analysis represents a promising approach to explore qualitative and quantitative differences in secretory profiles of ACs and MSCs in established culture systems and to differentiate between secreted and contaminant proteins. Before us only a few groups had been studying secretome of human chondrocytes by proteomics, showing similar results [138]. Other studies using animal chondrocytes showed a comparable proportion of labelled and not-labelled proteins, although the overall protein profiles did not match with profiles found by us [139]. The difference might be explained by the fact that we used human cells for the experiments in stead of animal ones.

In paper I, we studied profiles of proteins secreted by ACs in monolayers and native cartilage (explants). Over 90 % of proteins identified in supernatants of ACs in monolayers were labelled, whereas only 26 % of proteins identified in explants were labelled. Most of the non-labelled proteins in monolayers corresponded to serum proteins, while in explants to ECM components. Moreover, the number of proteins found in explants was significantly lower than in monolayers. The reason for this discrepancy might be: i) direct incorporation of newly

synthesized matrix constituents into the pre-existing matrix in explants, ii) straightforward release of ECM components into the culture media caused by lacking tissue structure in monolayers, or iii) considerably reduced metabolic activity of chondrocytes in explants with consequent lower synthesis of proteins. To clarify these issues SILAC analysis of proteins in cell lysates extracted from the cartilage explants was performed, and the degree of metabolic labelling determined. The results showed that most of the proteins in cell lysates were labelled with heavy amino acids indicating that ACs in tissue explants are metabolically active and that most of the released matrix components are incorporated into existing matrix. So, non-labelled ECM components identified in culture media of cartilage explants represent most probably the degradation products from ECM turnover. In addition, most of the labelled proteins identified in culture media of tissue explants were matrix regulating agents, confirming the high turn-over of ECM when the chondrocytes are maintained in their original matrix.

In paper II we used advanced analytical approaches in proteomics for both qualitative and quantitative comparison of proteins secreted by de-differentiated ACs in monolayers and re-differentiated ACs in spheroids. The spheroids are cartilage micro-tissues assembled by "hanging drop technique", a three dimensional culture of ACs in a hypoxic environment. Previous work from our laboratory showed that the ACs in spheroids undergo partial re-differentiation, enhance matrix deposition and expression of cartilage specific genes such as collagen II and aggrecan [13]. The results are comparable with another gene expression study preformed by Tallheden et al. using three dimensional pellet model [140].

Qualitative proteomic comparison of cells secretome revealed a longer list of proteins in the spent medium from monolayer cultures together with higher metabolic labelling level. This might most probably be caused by lower metabolic activity of ACs while cultured in

spheroids configuration. Besides, in spheroids the cells are caught in the extracellular matrix and are not so exposed to the culture media. Moreover, newly synthesized proteins are most likely not directly released into the culture media like in monolayers, but rather incorporated into developing matrix and thus not identified in the secretome.

The quantitative MS analysis of secretome revealed clearly higher expression of proteins characteristic of de-differentiated chondrocytes such as collagen I and tenascin, together with important growth factors such as CTGF and GAS6 in monolayers. On the other side, re-differentiated ACs in spheroids expressed higher levels of cartilage signature molecules aggrecan and powerful matrix regulators like chitinase-like protein 2 and stromelysin-1 indicating the transition from proliferation to tissue formation during spheroid culture. Our results confirm that in spite of considerably long in vitro culture the de-differentiated chondrocytes are still equipped with tools to regulate matrix homeostasis. In addition, genes and pathways found to be up- and down- regulated in chondrocytes during re-differentiation by a former study using gene expression microarray were in accordance with some findings obtained by our proteomic analysis of cells secretome [140].

Because some of the important matrix regulators and cytokines might be too small to be identified by MS, the study was supplemented be cytokine antibody microarray of secreted proteins. Commercially available membranes were designed to identify 79 different human cytokines and active substances. Although some inter-patient variations were observed, the pattern of secreted cytokines in our study was similar to results of previous studies from other groups [141]. Some of the identified factors like epithelial-derived neutrophil-activating peptide (ENA-78), macrophage inflammatory proteins (MIP-1β), epidermal growth factor (EGF), transforming growth factor beta (TGF-β) and tissue inhibitors of metalloproteinases (TIMP1 and TIMP 2) gave a strong immuno-reaction on array membranes from both de- and re-differentiated ACs. On the other hand, ACs in monolayers showed higher expression of

growth-regulated oncogene (GRO), monocyte chemoattractant protein-1 (MCP-1), IL-8, angiogenin and insulin-like growth factor binding protein-2 (IGFBP-2), while ACs in spheroids revealed higher expression of vascular endothelial growth factor (VEGF), CKb 8-1 and macrophage colony stimulating factor (M-CSF). These results might suggest that ACs in monolayers reveal a more inflammatory, leukocyte activating and cell proliferative profile, while ACs in spheroids acquire a more matrix-producing and matrix-regulatory phenotype.

In paper III, we explored secretory profiles of un-differentiated MSCs and de-differentiated ACs in order to investigate which cell type might be better equipped for cell based biological repair of cartilage defects. Initially, cartilage specific gene expression was examined, and the results confirmed the differentiation status of ACs and MCSs after several weeks of cell growth. Although in much smaller scale than in native cartilage, cartilage specific genes like coll II and sox9 were significantly more elevated in ACs compared to MSCs indicating that de-differentiated ACs have a somehow closer phenotype to cartilage cells then un-differentiated MSCs.

Qualitative proteomic analysis using SILAC methodology revealed similar protein profiles between the two cell types. However, interesting differences with potential impact on cell transplantation strategies were discovered. Most of the proteins expressed by both cell types were matrix components and matrix regulating agents (over 70%) characteristic for fibroblastic phenotype and not proteins like collagen II and aggrecan characteristic for hyaline cartilage. Nine different types of proteases and only five different types of protease inhibitors were identified in the secretion medium of ACs indicating the dominance of catabolic processes over anabolic ones. Moreover, MSCs synthesized a higher number of anabolic agents (12% in MSCs vs. 7% in ACs) revealing a more synthetic profile than ACs. Although previous gene and proteomic analysis of MSCs are scarce, several groups have studied

proteomic profiles of MSCs under adipogenic, osteogenic and chondrogenic differentiation [142-144]. Study of Chiellini et al. focused on secretome of MSCs in early stages of adipogenic and osteogenic differentiation and revealed the same pattern of ECM components and similar pattern of ECM regulators like SPARC, MMP-1 MMP-2, TIMP-1 and serpins as our experiments [145]. However, we could not identify markers specific for adipogenic and osteogenic differentiation thus confirming the un-differentiated phenotype in our cell cultures. To stress the importance of matrix proteases and their natural inhibitors in maintenance of cartilage ECM, the concentrations of specific matrix metalloproteases and tissue inhibitors of metalloproteases in the spent medium were quantified by pre-casted multiplex protein assay. Our results showed significantly higher production of MMPs by ACs compared to MSCs, while the expression of TIMPs was similar in both cell types. This analysis validates the data from proteomic analysis and confirms a more anabolic profile of un-differentiated MSCs.

Overall, in our work we have also been able to identify proteins that have not been identified in the context of chondrocytes and cartilage by proteomic approach, such as sushi (SVEP1), secreted protein acidic and rich in cystein (SPARC), growth arrest specific protein 6 (GAS6), proteoglycan 4 (PRG4) and osteomodulin (OMD).

SVEP1 or Sushi (von Willebrand factor type A, EGF and pentraxin domain-containing protein 1) is a recently identified protein containing several different structural domains and is involved primarily in cell adhesion processes. The expression of SVEP1 has been demonstrated *in vivo* in several tissues like bone, periosteum and bone marrow. *In vitro,* the protein was found to be expressed by mesenchymal stromal cells, but not by cartilage cells. Although the exact function of Sushi in the context of cartilage formation still remains to be clarified, it could constitute an important factor regulating cell-cell and cell-matrix interactions during *ex vivo* growth [146, 147]. In our studies SVEP1 was identified only in the

spent media of ACs in monolayers and not in explants or in spheroids. This observation suggests that SVEP1 might be an aberrantly expressed protein by chondrocytes during *ex vivo* cell growth, and might be considered a new marker of chondrocyte de-differentiation.

Secreted protein acidic and rich in cystein (SPARC) is a matricellular protein that binds to type I, III, and V collagen and is produced predominantly in remodelling organs with a high cellular turnover like gut, bone and tissues responding to injury [148]. It is a multifunctional regulator modulating diverse biologic effects, including proliferation, migration and matrix protein synthesis of soft tissue cells [149]. Both adult and developing chondrocytes express significant amount of SPARC and its function in tissue injury response might be a reason why markedly enhanced synthesis of SPARC has been observed in chondrocytes from OA joints [150, 151]. In our studies SPARC was identified in secretion media of ACs in all culture configurations, indeed as one of the most abundantly expressed proteins confirming its important role in the biology of chondrocytes both in vivo and in vitrol.

Proteoglycan 4 (PRG4) is responsible for low friction properties of hyaline cartilage. The role of PRG4 as a boundary lubricant is supported by its presence at the articular surface of cartilage, its abundance in synovial fluid and reduction of the friction coefficient when applied between natural and artificial materials. Mutated form of PRG4 results in camptodactyly-arthropathy-coxa varapericaritis syndrome. Expression of PRG4 was found to be down-regulated in several animal models of OA suggesting a relationship between loss of PRG4 and development of OA [152, 153]. In our experiments PRG4 was identified in spent media of ACs in all culture settings indicating that chondrocytes do not loose the expression of this critical cartilage protein during ex-vivo cell expansion.

Growth arrest specific protein 6 (GAS 6) is a member of the vitamin K-dependent protein family which has been demonstrated in a number of human tissues including lung, intestine, bone marrow, as well as endothelial cells and fibroblasts. In cartilage, GAS6 function via cell-

matrix adhesive-type interactions in an autocrine signaling pathway promoting cell growth and survival [154, 155]. In our investigations the expression of GAS6 was over two fold up-regulated in monolayer cultures compared to 3D cultures, which emphasizes its function in regulating chondrocyte proliferation and matrix synthesis in cell cultures.

Osteomodulin (OMD) is a keratan sulfate-containing proteoglycan belonging to the small leucine-rich proteoglycan (SLRP) family and might have a role in the regulation of bio-mineralization. It was shown to promote integrin ($\alpha_v\beta_3$)-mediated cell binding. OMD is primarily expressed by osteoblasts has been shown to bind different extracellular matrix components, growth factors, and cells. Immunolabeling for OMD was located to the mineralized bone matrix, with the highest concentration at the border between bone and cartilage [156, 157]. In our experiments OMD was clearly overexpressed in monoleyers configurations when compared with cartilage explants or 3D spheroids suggesting that in vitro expanded ACs undergo a phenotype conversion towards bone forming cells.

Cartilage has been traditionally considered “simple” as judged by its morphology and composition, comprising only one cell type and ECM. In early nineties after the introduction of autologous chondrocyte implantation to achieve biological repair of cartilage defects, the optimistic scenarios considered the cartilage defects issue to be solved in the near future. Nevertheless, after approximately 30,000 patients treated with ACI worldwide, and in spite of new second and third generation repair procedures, the ultimate goal of achieving hyaline cartilage tissue repair with perfect incorporation into surrounding cartilage and bone remains unsolved and a persisting challenge for clinicians and scientists [10-12, 158, 159]. Many of the modern approaches for cartilage repair procedures require expansion of cells in culture prior to implantation [6, 8, 9, 160]. However, other mostly stem cell based approaches apply native cells directly extracted from bone marrow [49, 50, 95]. As a consequence, two main streams have emerged, one preferring MSCs and other ACs for cell based cartilage repair. In disputes over

which cell type is better equipped for cartilage repair, the chondrocyte supporters claim that they are intrinsically programmed to make cartilage, while cartilage differentiation of MSCs is often incomplete [13, 62, 161]. However, this might contrast with postulates that ACs and cartilage are developed from MSCs under embryonal development and postnatal growth of the organism [80, 81]. In addition, it is generally accepted that chondrocytes in adult organisms do not possess such a high proliferative and synthetic capacity as in the growing individuals, and that their main function is conferred to maintain the existing cartilage matrix rather than to synthesize new cartilage tissue [22]. These features, together with the de-differentiation of ACs after cell expansion in culture might not make them an ideal candidate for cell transplantation strategies. On the other side supporters of stem cells based approaches argue that MSCs cells have a higher anabolic potential than ACs, are easier to extract from a donor and keep their differentiation capacity for longer culture periods [92, 162]. However, the process of differentiation is not completely understood, and thus they might differentiate into other tissues than cartilage in cell cultures. In addition, they might be difficult to distinguish from other nucleated cells in bone marrow because of the lack of representative surface receptors [99, 163-165]. With increasing age the anabolic potential of MSCs and their differentiation capacity declines, making it questionable whether MSCs from older individuals still posses the ability to produce high quality hyaline cartilage tissue [166, 167].

Overall, although a more synthetic profile of MSCs may favour their use in autologous cell implantation strategies, results presented in this thesis might not be sufficient to predict the final outcomes regarding the extent of cell differentiation or the quality of the replaced tissue after implantation. Moreover, the biological relevance of the differentially expressed proteins revealed in our investigations remains an area of investigation.

11 Conclusions

11.1 *Paper 1*

We have shown that stable isotope labelling technology is one of the most promising techniques for comparative proteomic studies and was found to be an appropriate method to differentiate between contaminant proteins and proteins secreted by cells. In addition, our group has been the first one using this technique in secretomic studies of cartilage and chondrocytes.

Our results showed differences in the protein secretion pattern of ACs cultured in monolayers and native chondrocytes in cartilage explants. Although most proteins identified in cartilage explants supernatants as newly synthesized were also found in ACs in monolayers, de-differentiation markers encountered in the secretion medium of ACs in monolayer cultures confirmed the de-differentiated phenotype of these cells. Most of the proteins identified in secretion media of cartilage explants were not newly synthesized, but rather degradation products of existing matrix. Chondrocytes in their native matrix were characterized by the production of matrix turn-over molecules; whereas de-differentiated chondrocytes were characterized by the secretion of matrix components and regulatory agents. In addition, in our study we have identified several proteins and factors not previously described by proteomics.

11.2 *Paper 2*

Stable isotope labelling technique followed by quantitative proteomic analysis and supplemented by protein antibody micro arrays allowed us to identify secretion products of de- and re-differentiated ACs. Although certain similarities in the panel of secreted proteins were observed qualitatively, some important differences were identified after quantitative

comparison. The secretory profile of ACs in monolayers revealed a more pro-inflammatory, leukocyte-activating phenotype, and more proliferative-like cell metabolism. On the other side, the secretory profile of ACs in spheroids revealed a more matrix-producing phenotype. Although the cells in spheroids had limited nutritional and oxygen supply they turned on autocrine pathways increasing ECM remodelling and synthesis. Our data point to factors such as chitinase-3-like protein and stromelysin as important mediators during the process of tissue reconstruction.

11.3 *Paper 3*

Our study confirmed the de-differentiated phenotype displayed by MSCs and ACs in monolayer cultures. At the gene level ACs revealed a more chondrogenic gene expression then MSCs. However, a more catabolic phenotype of ACs might negatively influence the synthesis of new tissue after cell transplantation. On the other side MSCs showed a higher anabolic phenotype with significantly lower secretion of the matrix catabolic agents like MMPs compared to ACs. Both cell types show advantages and disadvantages for their use in cell transplantation strategies, although a more anabolic tendency of MSCs in culture might favour the use of this cell type in cartilage resurfacing approaches.

12 References

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Paper I

Paper II

PAPER III

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