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Hyaluronan in chondrogenesis of mesenchymal stem cells

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CONTENTS

1 INTRODUCTION

Cartilage is a highly specialized connective tissue of the mammalian body that ensures friction less movement of joints and protects bones from forces associated with transmission of mechanical load and impact. The avascular character of the tissue together with low cell content is the main cause of its limited regenerative capacity. Current medical approaches to cartilage repair do not provide satisfactory results, especially in large defects and in long-term time periods. (Steinert, Ghivizzani et al. 2007; Khan, Gilbert et al. 2008) High expectations are therefore placed upon tissue engineering. Preparation of a cartilage graft that would fully integrate into the site of the defect and assume the structure and function of a normal healthy cartilage in a time frame of years is in a focus of this strategy. To resolve this task, three main questions have to be answered – the character of the scaffold, the choice of cell type for its seeding and the biological and physical conditions of graft preparation. (Vinatier, Mrugala et al. 2009)

The crucial requirements placed upon the scaffold are its biocompatibility, biodegradability and its ability to incorporate into the site of damage. A large scale of materials has been proposed for cartilage repair up to date. The widely tested synthetic substances, such as polylactic acid (PLA), and polyglycolic acid (PGA), enable easy manipulation of the shape, surface morphology, and inner architecture. On the other side, they often induce inflammatory response *in-vivo* and do not incorporate to the surrounding tissue sufficiently. Biological polymers can be divided into materials that are based upon molecules naturally occurring in a cartilage and others. Out of the natural cartilage polymers, collagens and their derivatives, hyaluronan based scaffold materials, and fibrin glue are probably the closest to clinical use. Alginate beads, silk based materials and chitosan represent the second group. Besides the material, which is the scaffold made of, other features have been shown to be important for its application. These include namely the inner architecture of the graft ensuring homogenous distribution of seeded cells, mechanical properties, the technical requirements for graft preparation and manipulation at surgery, and also the costs of the whole procedure. (Tortelli and Cancedda 2009; Vinatier, Mrugala et al. 2009)

Generally, three cell types are intended for seeding in cartilage scaffolds - autologous chondrocytes, cells isolated from perichondrium and periosteum, and mesenchymal stem cells (MSCs). Autologous chondrocyte transplantation (ACT) has been tested in clinics since early 1990s, however outcomes of this procedure are not quite satisfying. (Grande, Pitman et al. 1989) The main limits of ACT reside in the lack of suitable matrix or scaffold materials and difficulties accompanying autologous chondrocyte isolation and culture. Cells obtained from perichondrium and periosteum may overcome the limits of low cell numbers, however, they did not come to wider use. (Minas and Nehrer 1997) MSCs can be relatively easily isolated from bone marrow (eventually lipid tissue), rapidly expand in culture, and reveal chondrogenic differentiation potential. (Dominici, Le Blanc et al. 2006) The main obstacles limiting their application in cartilage grafts are poor definition of this cell type and lack of information on the process of chondrogenesis and factors directing the cell fate.

Biological and physical factors influencing cell behaviour in a scaffold and the final character and functionality of the graft *in-vivo* are not known in detail, despite of an intensive research. Considering the biological factors directing chondrogenesis of MSCs and perhaps influencing the differentiation status of chondrocytes, the transforming growth factor beta superfamily comprises the most important place. (Tang, Shakib et al. 2009) TGF-β 1 and 3 are known to induce chondrogenesis in a yet not elucidated mechanism in MSCs. Members of the bone morphogenetic protein (BMP) family are involved in chondrogenic as well as osteogenic differentiation and the outcome is probably dependent on their spatio-temporal relative actions. Additionally, fibroblast growth factors and insulin like growth factors also contribute to regulation of the phenotype of chondrogenic cells. (Vinatier, Mrugala et al. 2009) The physical conditions of the graft environment are as important as the biological ones. Cell anchorage in a three dimensional milieu is a crucial condition for chondrocyte phenotype maintenance as well as for chondrogenic differentiation of MSCs. The significance of viscoelastic properties of the scaffold for cell differentiation has been clearly demonstrated e.g. by Engler et al. (Engler, Sen et al. 2006) Additionally, hypoxia, mechanical stress, and cell density are known to influence the overall outcome of graft culture at least *in-vitro*. (Brandl, Sommer et al. 2007; Tortelli and Cancedda 2009)

This thesis summarizes and comments five single studies carried out in a project of development of hyaluronan – based scaffold intended for seeding with MSCs in order to purose a cartilage graft suitable for application in articular joint cartilage repair.

1.2 CARTILAGE

Cartilage is a specific connective tissue type. Its function in adult body is mainly mechanical and structural. Additionally, cartilage plays important role in embryonic development of the skeletal system. This project focuses mainly on terminally differentiated hyaline cartilage localised on the contact surfaces of bones in joints.

1.2.1 HISTOLOGICAL STRUCTURE OF ARTICULAR CARTILAGE

Hyaline joint cartilage is characterised by low cellularity (less than 5% of the volume) and extensive extracellular matrix content, without vascularization and inervation. The load bearing function of the tissue is ensured by the mechanical character of the matrix, whose primary constituents are water, glycosaminoglycans (GAG) and an organising protein network. In cross – section, the articular cartilage displays four layers. The superficial zone, which is in contact with the synovium, contains small discoidal cells organised parallely with the surface; the cells of the transitional zone are round and larger and do not reveal an apparent pattern; the radial zone is characterised with cell arrangement in columns aligned at right angles to the surface. The matrix of the calcified layer is slightly mineralised and interdigitates with the subchondoral bone. (Poole, Kojima et al. 2001)

Protein components of cartilaginous extracellular matrix fulfil mainly organising function networking in the prevailing GAG mass. Collagen fibres provide both the tensile strength to the whole tissue and the capacity to contain the swelling pressure of the embedded proteoglycans. The collagen fibrils consist of cartilage specific collagen type II, collagen type IX, and type XI. The regulation of fibril formation is guided by decorin and partly by collagen type IX. The proteoglycan part of the matrix is represented mainly by aggrecan and link-protein that interact with hyaluronan and form large, highly hydrated aggregates. These macromolecular complexes control the osmotic pressure of the matrix and determine the viscoelastic properties of the tissue. Hyaluronan (hyaluronic acid) – a non-sulphated, non-branched polysaccharide composed of repeating disaccharide units is the most abundant GAG of the cartilage tissue; its structure and function are described in detail below. Versican, brevican and

neurocan complete the list of main cartilage proteoglycans. Sulphated GAGs are represented namely by chondroitin sulphate, heparan sulphate and dermatan sulphate. (Poole, Kojima et al. 2001)

The embryonic origin of articular cartilage tissue is mesodremal. A developing cartilage contains high amounts of hyaluronan enabling chondroblast migration, proliferation and intensive contact at the developmental stage of cell condensation. (Knudson 2003) In contrast, terminally differentiated chondrocytes are isolated from each other, arrested in non- proliferative, non-migratory mode and their function is mainly production and maintenance of the extracellular matrix. The nutrition of joint cartilage is mediated by synovial liquid washing the cartilage surface, which is produced by cells of a surrounding synovial membrane and immigrating cells of other types e.g. immune cells; any active transport of nutrients and metabolites into deeper layers was not described.

1.2.2 CARTILAGE DEFECTS AND REPAIR STRATEGIES

The limited activity of chondrocytes and the avascular character of the tissue explain the very limited ability of cartilage to regenerate. Cartilage defects can be classified into two basic types according to their extent, which conditions the character of their healing. (Steinert, Ghivizzani et al. 2007; Khan, Gilbert et al. 2008) Subchondral/chondral defects affect only the cartilaginous tissue. Due to lack of vascularisation and inability of the resident chondrocytes to migrate to the site of injury, spontaneous healing does not occur. Chondral injuries affect the mechanical function of the tissue and can lead to large scale degeneration in longer time periods. Osteochondral defects reach also the underlying bone plate and thus are associated with bleeding and inflammation. Spontaneous healing starts with hematoma and fibrin clot formation, which is a source of soluble signalling molecules such as platelet derived growth factor and TGF-β1. Mesenchymal stem/progenitor cells originating in bone marrow and circulation penetrate the fibrin clot. The cells proliferate, enter the differentiation pathway and remodel the tissue forming a repair tissue. The resulting fibrocartilage contains cartilage specific collagen type II and proteoglycans, however the ratio of collagen type I is high, the organization of the tissue is poor and the biomechanical

function is inferior. In proceeding time, microfractures could appear, especially at the margins between the host and the repair tissue and degeneration may progress. (Steinert, Ghivizzani et al. 2007; Khan, Gilbert et al. 2008)

The specialised architecture and limited regenerative capacity of articular cartilage coupled with high physical demands placed upon this tissue make it exceedingly difficult to treat cartilage injury medically. The primary goal of articular cartilage repair is restoration of a functioning joint. The newly formed tissue should replicate the biomechanical function of a natural cartilage, allow pain-free articulation, integrate with the surrounding tissue, and prevent further degeneration. Current repair strategies are limited to surgical techniques, as no pharmacological agent stimulating cartilage repair exists. (Steinert, Ghivizzani et al. 2007) Biological attempts to cartilage repair are currently aimed at small size defects resulting from injury rather than large osteoarthritic degradations, which usually require total joint replacement. (Minas and Nehrer 1997)

Focused small size chondral lesions are usually treated by abrasion arthoplasty, when spontaneous healing is stimulated by drilling or microfracturing the subchondral bone plate. These procedures are cost effective and clinically useful, as patients often report reduced pain and improved joint function. However, the resulting fibrocartilage tissue does not comprise for an equal cartilage substitute and in a time frame of years, the problem may appear again. Mosaicplasty depicts transplantation of autologous osteochondral grafts, which has already found its place in clinical practise, however, the donor site morbidity and insufficient incorporation of the graft into the defect site limits its wider use. Similar restrictions occur in transplantations of perichondrium and periost tissues. (Minas and Nehrer 1997) A primarily cell- based approach to cartilage reparation appeared in late 1980s and is known as ACT. (Grande, Pitman et al. 1989) First generation protocols used chondrocyte cell suspensions covered e.g. with perichondrial flaps. A significant benefit was introduced in second generation strategies combining the chondrocytes with suitable matrix designed in order to maintain the cells in the site of repair and provide a simple support for the newly formed tissue. (Marlovits, Zeller et al. 2006) To obtain autologous chondrocytes in a sufficient quality and numbers is the main limit of ACT. The procedure requires surgical intervention and biopsy of a healthy cartilage tissue from a non-load bearing site of a joint, which is

associated with donor site morbidity and a significant discomfort for the patient. Additionally, the proliferative and regenerative potential of cartilage cells in aged or osteoarthritic patients is compromised. Chondrocyte expansion in traditional two dimensional cell culture is further complicated with a gradual loss of the chondrocyte phenotype that can be only partly reversed by transformation into a 3D environment *in-vitro*. (Benz, Breit et al. 2002) Therefore, the current progress in stem cell biology revealing chondrogenic potential in other cell types has driven the attention to MSCs. (Wood, Malek et al. 2006) Bone marrow MSCs are considered to contribute to spontaneous cartilage healing, clearly bear cartilaginous differentiation potential, and are easily expanded *in-vitro*. Their application *in-vivo* is promising, however further research is still necessary.

1.2.3 CHONDROGENIC SCAFFOLDS

All the mentioned therapeutic strategies share similar obstacles in-vivo as reviewed e.g. by Steiner et al., namely differentiation insufficiencies, loss of transplanted cells or tissue, matrix destruction and integration failures. (Steinert, Ghivizzani et al. 2007) At least some of these limits may be overcome by improved design of the scaffold supporting the cells. The requirements for an ideal cartilage scaffold have been set already in early 1990s, nevertheless, they have not changed substantially since that time. (Bell 1995; Safran, Kim et al. 2008) The scaffold must be biocompatible, biodegradable in a controlled way, permeable, and mechanically stable. Temporary scaffolds suitable for continuous natural replacement with a healthy cartilage tissue are preferred to stabile matrices that would find their place only in large defects with limited potential to regenerate. The scaffold should stimulate cell adhesion, enable their migration, proliferation and maturation and promote phenotype stability of differentiated chondrocytes. Considering the clinical application, easy implantation procedure, minimal surgical morbidity accompanying cell harvesting, and efficient and complete integration to the surrounding tissue are desired. (Safran, Kim et al. 2008) Despite of an intensive research in the last two decades, only a few types of scaffolds have approached clinical application up to date. (Tognana, Borrione et al. 2007; Vinatier, Mrugala et al. 2009)

A large variety of scaffold materials based on different chemical and also physical principles is known. Hydrogels can be classified according to several aspects, namely according to the principle of the gelating process and the conditions of the reaction determining the possibilities of the application. The cross-linking reaction is based either on physical interactions or on chemical reactions involving creation of covalent bonds. Both these types of processes can proceed in single or multistep manner and may require special physical conditions. The final step of the procedure is usually closely related to cell seeding. The living cells are either contained directly in the reaction mixture, or they are seeded thereafter utilising passive soaking of the cell suspension into the porous scaffold, directed cell "printing", and active cell migration into the scaffold volume. (Johnstone and Yoo 2001; Sherwood, Riley et al. 2002; Ji, Ghosh et al. 2006) Considering the homogenity of cell localization in the graft and easiness of preparation, the first type of protocol is preferred. In this case, the physical conditions required for the reaction have to be mild and close to physiological environment so as to protect the cells from harmful agents. Materials enabling preparation of simple injectable systems with cross-linking in –situ are highly desired by surgeons. (Khan, Gilbert et al. 2008; Safran, Kim et al. 2008)

1.3 MESENCHYMAL STEM CELLS (MSCs)

Mesenchymal stem cells represent a type of adult progenitor cells that have been isolated from various tissues of the human body, namely bone marrow and adipose tissue. They reveal high expansion potential and as yet not fully explored ability to differentiate along mesenchymal and other cell lineages e.g. endothelial, myogenic, and neural. (Kemp, Hows et al. 2005)

1.3.1 MSC CHARACTERIZATION AND SOURCES

Due to lack of a specific MSC marker, the cells are defined rather functionally. The International Society for Cellular Therapy has stated three conditions characterising this cell type. At first, the cells grow in an adherent pattern in culture. Secondly, the cells must not expose surface molecules typical for contaminating haematopoietic stem cells and lineages. The immunophenotype of MSCs is defined as: CD14-, CD19-, CD34-, CD45-, HLA-DR-, CD44+, CD73+, CD90+, CD105+. The third essential feature of MSCs is their capacity to differentiate along adipogenic, chondrogenic and osteogenic lineages under specific conditions. (Dominici, Le Blanc et al. 2006)

The poor characterization of MSC cell type and their morphological and habitual closeness to fibroblasts and other types of progenitor cells lead to substantial variety among studies of single research groups. (Barry and Murphy 2004; Kemp, Hows et al. 2005) Additional identifying MSC surface markers have been proposed so as to solve this task. Usage of these markers is declared to improve the process of MSC isolation from different tissues and to increase the homogenity of the cultures. (Hachisuka, Mochizuki et al. 2007; Zangrossi, Marabese et al. 2007) However, their significance for application in clinical tissue engineering is mostly not convincing and widely accepted, partly as their benefit does not substantially compensate the increase in the costs of the procedures.

Progenitor cells with mesenchymal differentiation potential have been identified in a variety of tissues including bone marrow, adipose tissue, dermis, hair follicles, etc. (Prockop 1997; Kemp, Hows et al. 2005; He, Wan et al. 2007; Wei, Sun et al. 2007) Two source tissues – bone marrow and adipose tissue dominate the research focused on

cartilage substitutes, mainly as they provide sufficient MSC amounts through a relatively easy isolation procedure.

The canonical source of MSCs is the bone marrow. MSCs are isolated from bone marrow aspirates by their adherence to cell culture plastics, after a ficoll/hypaque cell separation. They are reported to account for less than 0.1% of the mononuclear fraction of bone marrow blood aspirates. (Mageed, Pietryga et al. 2007) They expand rapidly when seeded in extremely low densities (less than 100 cells per cm), reach confluence within circa 10 days and can be held in culture for more than 10 passages. (Sekiya, Larson et al. 2002) In common culture conditions, tens to hundreds millions of cells can be obtained from several millilitres of bone marrow aspirates, which is satisfactory for research and some potential clinical applications. Several cell culture supplements have been proposed to stimulate MSC proliferation, but they do not seem to be necessary, the cells resist well also low serum conditions. (Pochampally, Smith et al. 2004; Mannello and Tonti 2007) The relative undemanding conditions of the culture may be promising for the development of serum and animal-free culture media required for MSC application *in-vivo*. Fully functional cells can be also obtained from frozen bone marrow aspirates and can be cryopreserved themselves. (Bruder, Jaiswal et al. 1997)

Besides that, adipose tissue has recently attracted attention as a promising MSC source. (Wagner, Wein et al. 2005; Wei, Sun et al. 2007) Comparison with bone marrow reveals that adipose-derived MSCs can be isolated from lipoaspirates in higher amounts, they seem to have bigger expansion potential *in-vitro*, and differentiate along the adipogenic pathway more easily. On the other side, their cultures reveal substantial heterogenity and contaminations with enotheliocytes, preadipogenic cells, and fibroblasts, which may lead to inconsistencies among individual cultures. (Dominici, Le Blanc et al. 2006; Wei, Sun et al. 2007) Additionally, their chondrogenic differentiation brings less satisfactory results than in bone marrow derived MSCs. (Mehlhorn, Niemeyer et al. 2006; Bernardo, Emons et al. 2007)

1.3.2 MSC CHONDROGENIC DIFFERENTIATION

Chondrogenic differentiation of MSCs requires cell anchorage in a three-dimensional environment and specific composition of cell culture media containing TGF-β growth factor. Three-dimensional system can be provided by a hydrogel or another type of insoluble scaffold, while the simplest way is comprised by pellet micromass culture, as introduced by Johnstone. (Johnstone, Hering et al. 1998; Yoo, Barthel et al. 1998) The model micromass system has been successfully used in a wide range of studies examining the process of chondrogenesis, despite of its limitations residing mainly in a small volume of the chondrogenic pellets and a compromised metabolite and nutrient exchange resulting in gradual cell death. (Wu, Yang et al. 2007)

The common formula of serum-free chondrogenic medium contains dexamethasone, insulin, transferin, selenium, ascorbate phosphate, prolin, increased glucose concentration, and growth factors belonging to the TGF-β superfamily, at least TGF-β3 isotype. (Tang, Shakib et al. 2009) The TGF-β superfamily encompasses various multifunctional growth factors involved in many developmental and regenerative processes. Chondrogenic differentiation is induced mainly by TGF-β1 and 3, and some members of the BMP group *in-vitro* as well as *in-vivo*. All the TGF-β superfamily growth factors promote their action through a serine-threonine kinase receptor family involving seven type I receptors and five type II receptors, which are shared across the family. Upon ligand binding, type I and type II receptors associate in a heterodimer and further form a tetrameric actively signalling structure. The activated receptor type I subunits trigger pathways involving a series of messenger Smad proteins, resulting in regulation of gene expression. TGF-β1 and TGF-β3 dominantly activate a pathway leading to formation of Smad 2/3 heterodimer complex that is transported into the cell nucleus, where it binds to gene regulatory sequences. The signalling routes triggered by BMP factors are similar, some of which (Smad 1/5/8 pathway) also enhance osteogenesis. (Lawrence 1996; Zhang and Li 2005; Tang, Shakib et al. 2009) Despite of an indisputable TGF-β3 significance for in-vitro chondrogenesis and natural cartilage development and regeneration, its application in cartilage tissue engineering is still scarce. (Tang, Shakib et al. 2009) The main reason is the unresolved task of its continuous delivery during the healing process. For this purpose, TGF- β3 has been already incorporated into hydrogels, PLGA microspheres

and some other constructs so as to slow down its release into the tissue environment and thus prolong its function. (Chou, Cheng et al. 2006; Sohier, Hamann et al. 2007) Another issue is the termination of the TGF-β3 action, so as to avoid adverse side effects, namely chondrocyte hyperthophy demonstrated in massive collagen type X expression, and tissue mineralisation associated with ectopic osteogenic differentiation. Supplementation with BMP factors can substantially enhance the chondrogenic induction at least in MSC micromass culture and some other *in-vitro* models. BMP-2 and BMP-7 have been shown to be the most potent chondrogenic stimulators; due to their high cost and limited availability they are often replaced with BMP-5. Their application *in-vivo* deals with the same constrains as in case of TGF-β, including the risk of adverse osteogenic induction. (Luo, Kang et al. 2004; Tang, Shakib et al. 2009)

Primary transcription factor necessary for mediation of chondrogenic signals in MSCs is the protein Sox 9, which is under a direct control of TGF-β and BMPs. Its expression has been detected even in undifferentiated MSCs, however, for its action on chondrogenic gene regulation other cofactors are necessary. Sox 9 binds to regulatory DNA sequences in a complex with cAMP response element binding protein (CBP), its paralog p300, and Smad 2/3 activated heterodimer in chondrogenic cells. The target genes of this activating structure include namely collagen type II α-1 chain and aggrecan. (Kawakami, Rodriguez-Leon et al. 2006) Stimulation of chondrogenic genes is also regulated by MAPK pathway that is essential for aggrecan expression, and upergulates both collagen type II α-1 chain and Sox 9 gene transcription itself. (Kawakami, Rodriguez-Leon et al. 2006) Sox 9 signalling is also involved in initiation of osteogenesis and other developmental processes, such as in formation of hair stem cell compartment. (Vidal, Chaboissier et al. 2005) The outcome of its action depends mainly on the presence of cofactors and signalling pathway cross-talk, in a yet poorly described mechanism.

The onset of chondrogenic differentiation in MSCs has been roughly described in micromass pellet cultures *in-vitro*. (Barry, Boynton et al. 2001; Karlsson, Brantsing et al. 2007) The process starts with increased expression of the Sox 9 factor followed by cartilage structural proteins collagen type II, aggrecan and protein cores of other proteoglycans, such as versican, and decorin. A massive production of sulphated as well as non-sulphated GAGs appears during the second week of culture and further

increases. Differences from gene expression of articular chondrocytes in micromass system have been described, namely the relatively higher expression of collagen type I and collagen type X in MSCs. (Karlsson, Brantsing et al. 2007)

The biological process of chondrogenic differentiation is strongly regulated by physical conditions prevailing during its progression. They include mainly viscoelastic character of the surrounding matrix, oxygen tension, and mechanical forces. The impact of mechanical features of the cell culture surface on the cell fate decision in MSCs has been clearly demonstrated by Engler et al. (2006). This study shows that the influence of viscoelasticity is fully comparable to the effects of growth factors. The natural hypoxic environment surrounding chondrocytes in avascular cartilage tissue can be mimicked *in-vitro* by cell culture at 5% O_2. This treatment promotes chondrogenic differentiation and chondrocyte phenotype maintenance in micromass cultures as well as in various types of hydrogels and scaffolds. (Ren, Cao et al. 2006; Markway, Tan et al. 2009) Repeated exposal of the chondrogenic tissue to dynamic hydrostatic compression is another way of chondrogenic differentiation enhancement *in-vitro*. (Schumann, Kujat et al. 2006; Mouw, Connelly et al. 2007) Several patterns of mechanical stimulation have been proposed ensuring optimal stimulation of the tissue in relation to the type of supporting graft matrix. This is in harmony with traditional clinical experience reporting improved cartilage repair in joints exposed to gradually increased load.

1.4 HYALURONAN

Hyaluronan (hyaluronic acid) is a non-branched polysaccharide occurring in extracellular matrix in many tissues of the mammalian body. It is composed of repeating disaccharide units of N-N-acetyl-D-glucosamine and D-glucuronic acid connected by β linkages. Its molecular weight ranges from oligosaccharide chains up to megaDalton molecules. (Lee and Spicer 2000)

1.4.1 GENERAL HYALURONAN BIOLOGY

In contrast to other GAGs, hyaluronan is not synthesized in Golgi aparatus, but by plasma membrane anchored hyaluronan synthases facing the cytosol. The polysaccharide is transported out of the cell by way of a mutidrug-resistance transporter system during the process of synthesis. (Stern, Asari et al. 2006) Three hyaluronan synthase genes have been identified in the mammalian genome, that differ in their tissue specifity and in the general size of molecules they produce. (Knudson 2003) The actual molecular weight of the polysaccharide is further regulated by degradative enzymes in the extracellular space, namely hyaluronidases, and some proteases. (Noble 2002; Stern, Asari et al. 2006) Additionally, hyaluronan degradation can also result from the action of reactive oxygen species. (Soltes, Mendichi et al. 2006) The hyaluronan turnover in most tissues is rapid, with a molecule life-span in the grade of hours. Extracellularly cleaved hyaluronan fragments are internalised with an assistance of specific cell surface receptors, such as CD44 and RHAMM and degraded in endosomes. (Stern 2004)

CD44 comprises for a primary hyaluronan cell surface receptor. (Knudson 2003) The CD44 gene sequence is located on the 11^{th} chromosome in humans and chromosome 2 in mice and includes 20 exons, 10 of which show variable usage. At least 23 splice variants of the gene have been identified up to date coding for receptors differing in their ability to bind hyaluronan and other GAGs, involvement in signal transduction networks, and interactions with other cell surface molecules. Another source of extraordinary diversity in the CD44 variants is introduced by posttranslational modifications of the CD44 molecule residing in N- and O- glycosylations on a few sites of the extracellular domain. The so called standard CD44 variant (lacking the

expression of all variant exons v1-v10) belongs to common MSC surface markers, while a more detail study on CD44 varieties in MSCs is missing. The same type of CD44 is known to be exposed on chondroblasts and terminally differentiated chondrocytes, where it contributes to organisation of pericellular GAG matrix. (Knudson 2003) Receptor for hyaluronan mediated migration acronymed as RHAMM (CD168) is another variable hyaluronan receptor. It belongs to GPI-anchored cell surface molecules, at the same time, RHAMM molecules have been detected on intracellular membranes. Besides its function in cell migration, it is known to mediate intracellular hyaluronan translocalization and to engage in hyaluronan independent processes. (Pilarski, Pruski et al. 1999; Maxwell, Keats et al. 2003; Evanko, Parks et al. 2004) The set of hyaluronan binding cell receptors is further complemented by: hyaluronan receptor for endocytosis (HARE), lymph vessel receptor LYVE-1, ICAM-1 (CD54), and tumor necrosis inducible protein 6 (TSG6). (Knudson and Knudson 2004)

Hyaluronan ability to incorporate huge amounts of water in its structure (depending on the size of the molecule) ensures the unique feature of many tissues providing relatively fluidic cell environment without a loss of mechanical stability. This structural function is attributed mainly to high molecular weight hyaluronan, while the smaller hyaluronan fragments reveal stronger direct biological activity. (Stern, Asari et al. 2006) High-mass hyaluronan was shown to inhibit immune reaction (Tamoto, Nochi et al. 1994), and to provide a support for cell migration (Lee and Spicer 2000; Zhu, Mitsuhashi et al. 2006; Docheva, Popov et al. 2007). Low molecular weight hyaluronan stimulates proliferation and natural function of skin cells (Kaya, Tran et al. 2006), promotes angiogenesis (Slevin, Kumar et al. 2002; Slevin, Krupinski et al. 2007), and may stimulate chondrogenic cells (Ohno, Im et al. 2006). Oligosaccharide fragments are traditionally considered to signalise tissue damage and to promote inflammatory response. (Stern, Asari et al. 2006) However, this effect may be a matter of debate, mainly due to inconsistencies in oligosaccharide preparation in different laboratories, potential substance contamination, and details of the model cell systems.

Important functions of GAGs including hyaluronan are the interaction with growth factors and other soluble molecules in the extracellular environment, sequestration of these factors in the tissue, and their protection from the action of activating as well as destructive proteases, and participation in their interactions with

cell surface receptors. (Locci, Marinucci et al. 1995) Hyaluronan has been shown to regulate cell response to the chondrogenic growth factor TGF-β1 in some cell systems. The feature of the interaction is not simple and depends on the type of cells and their receptor expression pattern, hyaluronan molecular weight, and the way of its administration to the cell culture. The outcome may be an enhancement of the TGF-β1 action, such as in myofibroblast differentiation (Meran, Thomas et al. 2007) and breast cancer cells (Bourguignon, Singleton et al. 2002), as well as TGF- β1 signalling attenuation in e.g. renal cells (Ito, Williams et al. 2004).

1.4.2 HYALURONAN IN CARTILAGE REPAIR

Terminally differentiated healthy cartilage contains mainly high –molecular mass hyaluronan exceeding 2 MDa values, which is produced mainly by hyaluronan synthase type 2. Hyaluronan is comprised for the most abundant cartilage GAG accounting for cca 1.2 μg/ml of the cartilage volume in terminally differentiated cartilage, and even more in developing embryonic tissue. (Knudson 2003; Li, Toole et al. 2007) The structure of the polysaccharide network is organised by its interaction with CD44 receptors on chondrocytes and extracellular proteins, so called hyaladherins. Out of these, aggrecan dominates the cartilage tissue complemented by link protein, versican, brevican, and neurocan. A single aggrecan molecule can interact with hundreds hyaluronan chains and thus form huge structures. (Knudson and Knudson 1991) The rate of natural hyaluronan turnover in cartilage is in the time grade of hours. Smaller molecules appearing in the synovial liquid belong to diagnostic markers of cartilage degeneration in osteoarthritis. (Laurent, Laurent et al. 1996) It is a matter of debate, whether they are simple products of general degradative processes, or whether they are capable of active stimulation of inflammation and tissue destruction by interactions with infiltrating macrophages and by promotion of matrix metalloproteinase production.

The abundance and biological importance of hyaluronan in developing and differentiated cartilage have made it attractive for applications in cartilage repair. A simple intrarticular injection of high molecular hyaluronan in rheumatoid arthritis and osteoarthritic patients brings pain relieve and improvement of joint mobility. However,

some studies point out a relatively low efficiency of this treatment. (Waddell 2007; Migliore and Granata 2008) Native hyaluronan was also utilised in ACT protocols as an embedding matrix in cell suspension transplantation.

1.4.3. HYALURONAN CHONDROGENIC SCAFFOLDS

The unique physical features of hyaluronan molecule and its chemical structure allowing a large scale of chemical modifications have encouraged material engineers to study and prepare hyaluronan derivatives with lowered solubility, controlled biodegradability, and various viscoelastic features. Hyaluronan based biomaterials are either prepared by solo polysaccharide derivative or in combination with other synthetic and natural substances. Insoluble hyaluronan derivatives can be obtained by modification of the polysaccharide molecule by addition of side chains, such as alkyl chains of variable length. Another approach is chemical or physical cross-linking of the hyaluronan chains into large complexes. The resulting hydrogels and water insoluble materials can be formed into membranes, sponges, stents, and tubes, and find their use in various medical applications. (Solchaga, Dennis et al. 1999; Tognana, Borrione et al. 2007)

Hydrophobised hyaluronan derivatives prepared by modification of the polysaccharide chain are represented mainly by alkyl derivatives. (Sedova, Knotkova et al. 2007) This class includes also the most successful hyaluronan- based cartilage scaffolding material – a total hyaluronan benzylester depicted as HYAFF®11. This material provides a suitable support to chondrocytes as well as differentiating MSCs as shown *in-vitro* and *in-vivo*. Hyalograft C® depicts a commercially produced graft prepared by combination of HYAFF®11 with autologous chondrocytes, which has been developed by Fidia Advanced biopolymers s.r.l. (Italy). This graft has already found its place in clinical practise in treatments of cartilage lesions in the knee in human patients. (Lisignoli, Cristino et al. 2005; Tognana, Borrione et al. 2007)

Traditional chemically cross-linked derivatives introducing various linking chemicals, hydrogels induced to reaction by changes in pH and temperature, and photocrosslinkable materials have been prepared from hyaluronan. In recent years,

enzymatically cross-linked biomaterials have attracted the attention, as this type of reaction can usually proceed in physiological conditions and thus can be carried *in-situ*. (Darr and Calabro 2009) Hydrogels cross-linked by horse-radish peroxidase conjugation of tyramine have been prepared from hyaluronan as well as from alginate, dextran, and carboxymethylcelulose. The two step enzymatic cross-linking chemistry for production of tyramine based hyaluronan hydrogels was described by Darr et Calabro. (Darr and Calabro 2009) The first step is tyramine substitution on hyaluronan. The obtained derivative is water soluble at physiological pH, salinity and RT, with a viscosity depending on the molecular size of the initial hyaluronan. The final cross-linking reaction engaging horse radish peroxidase (HPR) and hydrogen peroxide is rapid and proceeds well in physiological conditions and can be also achieved *in-situ in-vivo*. The final viscoelastic features of the material can be regulated by modifications of HRP and hydrogen peroxide dosing in the reaction mixture and the degree of hyaluronan substitution. The biocompatibility and stability during 8 weeks of the resulting material has been tested in-vivo in subcutaneous rat model. Any study evaluating cell encapsulation in the hyaluronan-tyramine hydrogel has not been carried out, excluding 7days long experiments showing cell survival in the hydrogel. (Lee, Chung et al. 2009) Recent study on regulated protein embedding and release in this type of material indicates that scaffold enrichment with bioactive molecules promoting cartilage regeneration may be possible. (Lee, Kwon et al. 2007)

Out of the combined materials, several types have been tested for cartilage substitute preparation. The combined scaffolds include synthetic scaffolds enriched with hyaluronan in order to improve their biocompatibility and allow cell migration and embedding into the scaffolds. (Na, Kim et al. 2007; Schagemann, Chung et al. 2009) Hyaluronan has also been incorporated into sponges based on native or modified proteins, such as collagen type II, collagen type I, and silk proteins. (Chajra, Rousseau et al. 2008; Ko, Huang et al. 2009; Ren, Zhou et al. 2009) Another type is comprised by combinations with other polysaccharides and glycosaminoglycans, namely chitosan, chondroitin sulphate, and alginate. (Miralles, Baudoin et al. 2001; Funakoshi, Majima et al. 2005; Fan, Hu et al. 2006; Chou, Cheng et al. 2006)

2 AIMS OF THE PROJECT

Main aim:

To develop a methodological approach of *in-vitro* chondrogenic graft preparation utilising MSCs seeded in a hyaluronan based scaffold.

Specific aims:

To review hyaluronan derivatives with an intention for use in chondrogenic applications.

To compare different methods of cell viability determination so as to choose methods suitable for engagement in MSC research.

To characterize and optimise bone marrow collection sets as a source of MSCs for research applications.

To examine the influence of soluble native hyaluronan at different molecular weights on the onset and early chondrogenesis in micromass MSC culture system.

To induce chondrogenic differentiation of MSCs seeded in hyaluronan based scaffold and to describe its progression in relation to growth factor content in the culture media.

3 RESULTS AND DISCUSSION

3.1 HYALUORNAN DERIVATIVES INTENDED FOR CARTILAGE APPLICATION (review)

Native hyaluronan is water soluble and its life-span in tissues is shorter than demanded for cartilage repair applications. A large range of hyaluronan chemical derivatives have been prepared with the aim to keep the biocompatibility of the molecule, and to obtain a polymer suitable for use in tissue engineering. (Zavan, Cortivo et al. 2003; Safran, Kim et al. 2008; Darr and Calabro 2009) Medical devices made of these derivatives take the form of tubes, stents, membranes, sponges, threads, surgical implants and drug release systems. A review of insoluble hyaluronan derivatives and hydrogels with an intention to cartilage usage has identified two leading groups of preparations.

The largest group of derivatives is represented by alkylated hyaluronan species, as described in detail in the article Water soluble and insoluble alkylderivatives of hyaluronic acid, Review (Sedova, Knotkova et al. 2007), see attachment number 1. Several approaches were used to introduce alkyl chains with a length of 4 to 18 carbons to the native hyaluronan chain. Water soluble and insoluble derivatives have been prepared in varying lengths, degree of substitution, and preservation of polyelectrolytic character. From a chemical point of view, they include esters, ethers, carbamoyl derivatives, amides, and amines. A number of studies concluded that the various derivatives seemed to be promising in different applications, according to their different physico-chemical, mechanical and biological properties. Out of these derivatives, mostly alkyl derivatives prepared by esterification have been employed in cartilage substitute design. The product HYAFF®11 (total hyaluronan benzylester) has already found its use in clinical practice in humans in a form of Hyalograft C®, which is a custom – made combination of autologous chondrocytes seeded in HYAFF®11 scaffold, MSC differentiation has also been successfully tested. (Facchini, Lisignoli et al. 2006; Tognana, Borrione et al. 2007)

The second group of hyaluronan derivatives involves cross-linked hyaluronan hydrogels. Out of these, promising materials are comprised by enyzme cross-linked derivatives, such as HRP-cross-linked hyluronan hydrogel prepared from tyramine

substituted hyaluronan. (Lee, Kwon et al. 2007; Darr and Calabro 2009) This material is biodegradable, biocompatible, the gelation reaction proceeds at physiological conditions, also upon injection *in-situ*. Chondrogenic differentiation of MSCs in this type of derivative, either seeding with chondrocytes has not been examined yet.

3.2 COMPARISON OF CELL VAIBILITY DETERMINATION METHODS

Determination of cell numbers is a crucial step in studies focused on cytokinetics and cell toxicity. Cartilage tissue engineering engages determination of cell number and viability mainly in tests exploring toxicity of the scaffolding materials and potential residuals of chemical agents used during scaffold preparation. Additionally, cell viability determination is important in examination of cell seeding techniques and cell survival in the scaffold environment. Another application is an assessment of cell adherence to different biomaterials. A limiting obstacle in MSC experiments is often their demand for extremely low seeding densities in cultures requiring very sensitive methods. (Colter, Class et al. 2000; Sekiya, Larson et al. 2002) This part of the project focused on comparison of impedance-based method of cell proliferation monitoring with commonly used metabolic-based techniques so as to consider their suitability for different applications (Vistejnova, Dvorakova et al. 2009), see attachment number 2.

The impedance-based analysis xCELLigence System was compared with a colorimetric assay MTT and a chemiluminiscent determination of intracellular ATP production. The xCELLigence System engages an electrical impedance cell sensor technology to measure the level of impedance on the surface of the cell culture/well, which corresponds to the extent of the cell- covered area. This label-free method offers a non-invasive approach to the monitoring of cell adhesion and proliferation. (Zhu, Wang et al. 2006) The other two methods are based on evaluation of cell metabolism. The MTT assay quantifies reduction of tetrazolium salt by mitochondrial enzymes (Goodwin, Holt et al. 1995), the ATP determining method utilises luminiscence (Crouch, Kozlowski et al. 1993). Normal human epidermal keratinocytes and normal human dermal fibroblasts, together with 3T3 mouse fibroblast and HaCaT keratinocyte cell lines were employed in this study as model cell types varying in morphology and cell growth pattern.

The progress of cell growth curves obtained by different methods during 72 hours reflected cell type and cell seeding densities. The impedance-based method was found to be applicable for the determination of the cell proliferation of 3T3 fibroblasts, HaCaT and normal human dermal fibroblasts, since the comparison of this method with ATP and MTT determinations showed comparable results. In contrast, the proliferation of normal human dermal keratinoctyes measured by the impedance-based method did not

correlate with other methodological approaches. This could be accounted to the specific morphological appearance of these cells, as documented by microscopic evaluation.

This study has shown that, specific morphological characteristics of cell lines have to be considered employing the impedance based method for determination of cell proliferation without using other reference methods. This fact comprises for a significant obstacle in MSC studies, as it is known, that these cells substantially change their morphology in cell culture reflecting the level of culture confluence, differentiation status, and cell senescence. (Colter, Sekiya et al. 2001; Sekiya, Larson et al. 2002) The MTT cell viability method is not sensitive enough to detect standard plating MSC amounts, that are lower than 10^2 in a single sample. Therefore, the highly sensitive metabolic method based on luminiscent ATP determination was chosen for the following experiments.

3.3 MSC ISOLATION FROM BONE MARRROW COLLECTION SETS

Bone marrow is an important source of MSCs. Utilization of this MSC source in tissue engineering research is limited by cell yields obtained under standard isolation protocols and ethical concerns of bone marrow aspirate acquirement from human donors. (Kemp, Hows et al. 2005; Sotiropoulou, Perez et al. 2006) Used bone marrow collection sets were evaluated as a valuable source of MSCs in this part of the study, as described in detail in attachment number 3 (Dvorakova, Hruba et al. 2008).

Adherent cells washed out from collection sets remaining after harvesting of bone marrow from healthy donors for transplantation purposes were examined for criteria defining MSCs as stated by The International Society for Cell Therapy. (Dominici, Le Blanc et al. 2006) Significant numbers of cells (median $9x10^6$ per set in passage 1) revealing colony forming activity and high proliferative potential were obtained. Flow cytometry analysis of surface markers has shown, that these cells were positive for essential MSC surface molecules (CD90, CD105, CD166, CD44, CD29) and negative for most haematopoietic and endothelial cell markers (CD45, CD34, CD11a, CD235a, HLA-DR, CD144). The only exception was slightly increased content of $CD14^+$ cells in one of the three donors examined. The cells were capable of differentiation along adipogenic, osteogenic and chondrogenic pathways when cultured in harmony with commonly used differentiation protocols. (Heng, Cao et al. 2004; Otto and Rao 2004; Dominici, Le Blanc et al. 2006) The process of differentiation was examined microscopically, utilizing histological staining and Real Time PCR. Additionally, optimization of cell seeding density was carried out in order to maximise cell yields while maintaining cell morphology attributed to non-senescent cells. (Sekiya, Larson et al. 2002) As a result, the cell seeding density 60 cells per cm^2 was chosen for cell expansion in following experiments.

The results have confirmed previous assumptions, that bone marrow collection sets entrap a specific population of cells, which can have interesting features. (Vicente, Podesta et al. 2006; Mageed, Pietryga et al. 2007) This study has shown that washing out bone marrow collection bags may constitute an undemanding step leading to the enrichment of MSC population. This approach comprises a highly ethical source of MSCs for research purposes and may find its use also in clinical applications.

3.4 HYALURONAN INFLUENCE ON THE ONSET OF CHONDROGENIC DIFFERENTIATION IN MSC PELLET SYSTEM

Hyaluronan is a usual component of three dimensional scaffolds intended for cartilage graft preparation utilising MSCs. Although it has been demonstrated that hyaluronan addition to these structures may improve chondrogenic differentiation of these cells, the fundament of its action is not known. (Allemann, Mizuno et al. 2001; Miralles, Baudoin et al. 2001) At the same time, effect of hyaluronan molecular weight on chondrogenic differentiation is not clear. (Stern, Asari et al. 2006) The aim of this part of the project was to evaluate modulation of MSC early chondrogenesis cultured in a micromass pellet system by hyaluronan of molecular weights 100, 600 and 1500 kDa, as described in detail in attachment number 4 (Dvorakova, Velebny et al. 2008).

The native polysaccharide was applied on MSCs cultured in a pellet system for one, two and three weeks. Chondrogenesis was evaluated by determinations of gene expression of transcription factor Sox 9 and extracellular matrix proteins collagen type II and XI, aggrecan, and COMP by Real-Time PCR and completed with histological analysis. (Barry, Boynton et al. 2001) Upon chondrogenic induction, the pellets revealed active transcription of the chondrogenic genes together with proceeding accumulation of GAG rich extracellular matrix. Sox 9 was also expressed in non-chondrogenic MSC controls. Hyaluronan treated pellets were not significantly influenced on day 7 of culture. However, on day 14, significantly lowered expression of collagen type I, collagen type XI, aggrecan, and Sox 9 appeared in some of the hyaluronan treated chondrogenic pellets in comparison to controls. This effect was associated with a moderately smaller amount of GAG content in histological pellet sections. Nevertheless, the analysis on day 21 has demonstrated that hyaluronan did not affect the outcome of the differentiation by the end of the culture, as any significant differences between the chondrogenic pellets were not observed. At the same time, any difference regarding the molecular weight of hyaluronan was not found.

In conclusion, it could be speculated that hyaluronan induced a time shift in the phase of the dominant matrix protein onset, which has already been localised into the second week of chondrogenic differentiation in pellets. (Barry, Boynton et al. 2001) The initial slow down was in full compensated by the end of the evaluated three week time

period. The obtained data suggest that hyaluronan of any tested molecular weights does not significantly modulate chondrogenesis of MSCs in pellet micromass system. These results are in harmony with previous studies showing that the direct biological influence of soluble hyaluronan on MSC chondrogenic differentiation is mild or hardly distinguishable from other factors. (Allemann, Mizuno et al. 2001; Miralles, Baudoin et al. 2001; Hegewald, Ringe et al. 2004)

3.5 CHONDORGENESIS OF MSC IN A SCAFFOLD BASED ON TYRAMINE DERIVATIVE OF HYALURONAN

The final stage of the project was to evaluate chondrogenesis of MSCs obtained from washed bone marrow collection sets in a three dimensional hydrogel prepared from a biomaterial based on hyaluronan. The whole study is described in detail in attachment number 5. A relatively novel hydrogel system revealing interesting features for biological applications was chosen. This system is comprised by a tyramine derivative of hyaluronan that is cross-linked by an action of horse radish peroxidase in presence of hydrogen peroxide (HA-TA hydrogel). (Darr and Calabro 2009) The gelating reaction can be performed in situ and the resulting material is biocompatible and slowly biodegradable. (Lee, Chung et al. 2009) According to our knowledge, any experiments evaluating chondrogenic potential of this scaffold either with seeded chondrocytes, or MSCs still have not been published.

MSCs seeded in the scaffold (4×10^6 cells per ml) were followed during a time period of three weeks *in-vitro*. Cell viability, gene expression of collagen type II, collagen type X, Sox 9, and aggrecan, and histological character of the scaffolds were evaluated. Three types of cell culture media were compared including standard MSC culture medium, chondrogenic medium, and chondrogenic medium enriched with BMP-5 (0.5 μg/ml).

The results have shown that the cells resist the process of cross-linking when suspended directly in the reaction mixture and remain viable for the whole time period of 21 days. The scaffold itself did not induce chondrogenic differentiation. However it provided an environment enabling MSC anchorage and differentiation in standard chondrogenic conditions. Both types of the chondrogenic media induced gene expression of transcription factor Sox 9, and structural proteins collagen type II and aggrecan, while the collagen type II expression was also detected on a protein level in histological sections. At the same time, substantial changes in cell morphology and localisation inside the scaffold were remarkable in macroscopic and microscopic observations. The expression level of chondrogenic markers in standard chondrogenic media was gradually increasing during the whole time period.

In contrast, BMP-5 enrichment led to a more massive expression with a different time proceeding, mainly in collagen type II. BMP-5 has also stimulated the expression of collagen type X, which is produced by hypertrophic chondrocytes in cartilage ossification. (Tang, Shakib et al. 2009) A similar increase was found in the expression of a marker of osteogenic MSC differentiation - osteopontin. (Meijer, De Bruijn et al. 2007) Histological analysis did not detect accumulation of mineralised extracellular matrix in any of the samples.

The study has shown that the tyramine derivative based hyaluronan scaffold system provides a suitable environment for chondrogenesis of MSCs. The process proceeds well in a standard chondrogenic medium optimised for micromass culture. (Otto and Rao 2004) An addition of BMP-5 factor, which is utilised in some differentiation protocols (Heng, Cao et al. 2004; Tang, Shakib et al. 2009), promotes the initial stages of differentiation. On the other site, it but may have adverse effects on the overall outcome of the potential chondrogenic graft preparation, similarly to some other members of this growth factor family. (Nakashima and Reddi 2003; Tang, Shakib et al. 2009)

4 SUMMARY

The main aim of the project – to develop a methodological approach of *in-vitro* chondrogenic graft preparation utilising MSCs seeded in a hyaluronan based scaffold was fulfilled. MSCs were obtained from a newly evaluated source – bone marrow collection sets. Methodological tools respecting specific aspects of MSC research were optimised and applied in further research. It was shown, that native soluble hyaluronan does not substantially influence their chondrogenic differentiation in micromass culture, regardless of molecular weight of the polysaccharide. On the other side, a relatively novel hyaluronan based biomaterial prepared from tyramine derivative of hyaluronan cross-linked by horse radish peroxidase was demonstrated to provide a sufficient environment for chondrogenic differentiation of MSCs.

ABBREVIATIONS

ACT = autologous chondrocyte transplantation

ATP = adenosine triphosphate

BMP = bone morphogenetic protein

GAG = glycosaminoglycan

HRP = horse radish peroxidase

MSC = mesenchymal stem cell

PGA = polyglycolic acid

PLA = polylactic acid

RHAMM = receptor for hyaluronan mediated migration

TGF = transforming growth factor

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DECLARATIONS

DECLARATION OF THE AUTHOR OF THE THESIS

I, the author, declare that I have not submitted any part of thesis, either the thesis as whole, with an intention to acquire any academic title.

Dolní Dobrouč, 5.3.2010 Jana Dvořáková

DECLARATION OF AUTHOR CONTRIBUTION TO THE PUBLICATIONS

On behalf of the authors of the five scientific publications, which make part of this thesis, I declare, that the contribution of Jana Dvořáková to the single studies was as follows:

1. Water soluble and insoluble alkylderivatives of hyaluronic acid. Review
   - writing out the parts of the text reviewing biological features of the derivatives, editing of the whole text structure; (25%)
2. The comparison of impedance based method of cell proliferation monitoring with commonly used metabolic-based techniques
   - responsibility for MTT experiments on HaCaT and 3T3 cell cultures, contribution to the manuscript preparation; (15%)
3. Isolation and characterization of mesenchymal stem cell population entrapped in bone marrow collection sets
   - design and coordination of the whole study, caring out the experiments evaluating MSC colony forming activity, growth, and differentiation, manuscript core preparation; (65%)

4. Hyaluronan influence on the onset of chondrogenic differentiation of mesenchymal stem cells

   - design and coordination of the whole study, caring out all the experiments, manuscript core preparation; (75%)

5. Hydrogel system based on enzymatically cross-linked tyramine derivative of hyaluronan supports chondrogenic differentiation of mesenchymal stem cells

   - design and coordination of the whole study, caring out most parts of the biological experiments, manuscript core preparation; (50%)

Dolní Dobrouč, 5.3.2010 Vladimír Velebný

ATTACHMENTS

1. Sedova P, Knotkova K, Dvorakova J, Velebny V: Review. **Water soluble and insoluble alkylderivatives of hyaluronic acid.** Progress in Biopolymer research (2007), P. C. Sanchez, Nova Science Publishers, Inc.: 77 -105.

2. Vistejnova L, Dvorakova J, Hasova M, Muthny T, Velebny V, Soucek K, Kubala L: **The comparison of impedance-based method of cell proliferation monitoring with commonly used metabolic-based techniques.** Neuroendocrinology Letters (2009), vol. 30 (suppl 1): 121-127.

3. Dvorakova J, Hruba A, Velebny V, Kubala L: **Isolation and characterization of mesenchymal stem cells population entrapped in bone marrow collection sets.** Cell Biology International (2008), vol. 32 (9): 1116 – 1125.

4. Dvorakova J, Velebny V, Kubala L: **Hyaluronan influence on the onset of chondrogenic differentiation of mesenchymal stem cells.** Neuroendocrinology letters (2008), vol. 29 (5):685-690.

5. Dvorakova J, Kucera L, Foglarova M, Muthny T, Pravda M, Berkova M, Velebny V, Kubala L: **Hydrogel system based on enzymatically cross-linked tyramine derivative of hyaluronan supports chondrogenic differentiation of mesenchymal stem cells.** (manuscript)