Influencing CHONDROGENESIS in bone marrow STROMAL CELLS

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A good word is like a good tree, whose roots are firmly fixed and whose top is in the sky - Quran

There is no higher or lower knowledge, but one only, flowing out of experimentation. - Leonardo da Vinci (1452-1519)

Contents

Prologue

In the beginning there were stem cells. This is how every story of organogenesis start. Chondrogenesis is one of the most unique amongst them involving extremely fascinating characters and stages of development. Condensation of mesenchymal stem cells with epithelial cells, commitment to the lineage, formation of chondrocytes which in turn generate cartilage specific extracellular matrix (ECM); then the cells attain prehypertrophic stage which leads to hypertrophy and in the end a phoenix like death of chondrocytes giving way to the birth of bone, and the life goes on.

This thesis deals with one small part of the chondrogenesis story, i.e. the beginning. It probes the biological factors which may have an influence on induction and maintenance of chondrogenesis *in vitro*. The rationale for this and other similar studies lies in the irreparableness of the cartilage tissue. Cartilage does not repair itself neither offers any easy way for aided repair. Thus, comes tissue engineering in to the picture. Like every other engineering, tissue engineering also needs at first a blue print of the structure to be constructed. Construction materials and tools are required and everything has to be done efficiently in the most cost effective manner as quickly as possible. For cartilage tissue engineering, time limitation is further intense because a human being is suffering while we are tinkering in the lab. Joint forces of biomechanics, biomaterials and cell biology provide us with the tools. Immense concentrated effort is directed to develop an ideal material to be used as anlage, and to attain enough cells to start the *in vitro* synthesis of cartilage. We are trying our best to engineer cartilage tissue as efficiently as possible but our knowledge of the blue print is limited and incomplete; we know a lot, still there are mysteries unknown to us. We know growth factors like IGF´s, TGF´s and BMP´s help chondrogenesis but we are struggling to employ them fruitfully. We have identified some biomaterials like fibrin and have constructed many synthetic biosorbable scaffolds but we have still not optimized the use of these materials to obtain the desired type of cartilage tissue *in vivo*. We can isolate chondrocytes and embed them in the scaffolds to tip off cartilage construction but the chondrocytes tend to become fibroblasts in our labs. We know that mesenchymal stem cells (MSCs) are chondroprogenitor cells and that they reside in the bone marrow but we do not know sufficiently how their proliferation and differentiation is regulated by the factors from their native environment. In short, there are many open issues and a collective effort is called

for to provide pain free and agile life for osteoarthritis patients and injured sportsmen. This thesis is yet another effort to fill some of the gaps in our collective knowledge of chondrogenesis induction and maintenance of the desired phenotype.

The focus of this study is the regulatory effect of the surrounding environment on chondrogenic differentiation of MSCs. We have been able to show that both the bone marrow microenvironment and cartilage tissue influence chondrogenesis at different stages. The effect itself could be shown at molecular as well as on biochemical level. The involved cell types and various paracrine factors were also identified. At the last stage, one of the effected molecules, a major transcription factor was knocked down and an experimental study model was setup for future studies on chondrogenesis related genes.

This thesis is structured in a series of four major titles (chapter 2-5), each title is a short complete account based on different aspects of chondrogenesis in MSCs. In the **sixth and last chapter** the data presented in the preceding chapters are collectively concluded and analyzed in the light of 'influencing chondrogenesis'. The **first chapter** is a prelude to the main topic, an in depth introduction of the molecules and processes appearing in the following chapters. Here, we have discussed articular cartilage and related molecules and the physiological process of chondrogenesis. The importance of MSCs pertaining chondrogenesis is also in detail explored along with the bone marrow microenvironment. MSCs are the main tools of this study therefore; in the **second chapter** MSCs are investigated in depth. Here we establish osteo-chondro progenitor status of MSCs by doing osteogenic and chondrogenic differentiation studies. The genes and proteins which may predestine MSCs to become committed chondroprogenitor cells are screened by quantitative PCR (qPCR) and antibody microarray. Thus, this chapter constitutes a foundation on which the next chapters are constructed. The **third chapter** deals specifically with chondrogenesis of MSCs in 3-D high density alginate cultures *in vitro*. MSCs source of origin, native environment and *in vitro* behaviour is explored with immunofluorescence and FACS. With the help of MACS and qPCR we demonstrate how the other cells of bone marrow microenvironment influence chondrogenesis. In the **fourth chapter** effect of cartilage tissue on the differentiating MSCs is studied. The behaviour of chondrogenically differentiating MSCs under the influence of articular cartilage explants is investigated in a novel coculture model. The differences and the putative responsible factors have been identified by qPCR, antibody arrays, zymography, immunobloting and collagen preparations. The fourth chapter revealed transcriptional factor *Sox9* as an effected molecule therefore, in the **fifth chapter** a method was developed to knock down *Sox9* by RNA interference. *Sox9* is an integral regulator of chondrogenic lineage

differentiation therefore, this retroviral based *Sox9* gene silencing experimental model system can be used to identify direct and indirect role of *Sox9* in chondrogenic regulation.
The methods and identified molecules described in this thesis collectively make up one more step in the direction of successful regenerative therapy for damaged cartilage.

Specific questions

- Do undifferentiated multipotent MSCs express osteo-chondro lingeage specific genes?
- Does the native bone marrow environment influence chondrogenesis in MSCs?
- Does cartilage affect chondrogenic differentiation of MSCs?
- Can we achieve efficient chondrogenesis via biological factors?
- How integral is Sox9 for chondrogenesis?

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Regensburg, Germany
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Abbreviations

3-D	Three dimensional
α-MEM	Minimum Essential Medium Eagle, alpha modification
Amp	Ampicillin
bFGF	Basic fibroblast growth factor
ßNGF	ß Nerve growth factor
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
Cbfa-1	Core binding factor-1, a transcription factor
CD	Cluster of differentiation
CINC-2	Cytokine induced neutrophil chemoattractant
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra acetic acid
FACS	Fluorescence activate cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothyocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
HC	Haematopoietic cells
Hox	Homeobox containing transcription factors
HSCs	Haematopoietic stem cells
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
Ihh	Indian hedgehog
IL	Interleukin
ITS™	Insulin, transferrin and selenious acid containing supplement
Km	Kanamycin
MACS	Magnetic activated cell sorting, Miltenyi Biotech™

MAPC	Multipotent adult progenitor cells
MCP	Monocyte chemoattractant protein, aka CCL2
MMPs	Matrix metalloproteinases
mRNA	messenger RNA
MSCs	Marrow stromal cells/ Mesenchymal stem cells
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTHrP	Parathyroid hormone-related peptide
Puro	Puromycin
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Riboneuclease (RNA degrading enzymes)
Rpm	Rotations per minute
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sox	Sex related homeobox containing transcription factors
shRNA	Short hairpin loop containing RNA
TGF-ß	Transforming growth factor ß
TEMED	Tetramethylethylenediamine
TIMPs	Tissue inhibitors of matrix metalloproteinases
TNFα	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor

Chapter 1

State of the art

Abstract

Articular cartilage disorders and injuries often end up as life long chronic pain and compromised quality of life. When it comes to local articular cartilage defects modern medicine is limited to short term pain relief and inflammation control. In extreme cases the affected tissue is surgically removed and replaced by synthetic prostheses carrying an expiry date. Cell based therapies to regenerate articular cartilage are in use since 1994. Such therapies provide a healthy population of cells to the injured site and require differentiated chondrocytes from uninjured site as base material. Use of healthy chondrocytes often lead to donor side morbidity and generate rigid fibrous cartilage when more flexible hyaline cartilage is required. The major restrictive factor for such methods is inadequate number and limited proliferation capacity of chondrocytes *in vitro*. The discovery of adult marrow stromal cells /mesenchymal stem cells (MSCs), their unlimited proliferation potential and proven capability to differentiate into chondrocytes is therefore significant. However, for optimal harnessing of MSCs as chondroprogenitor cells basic background information regarding commitment to the lineage, cartilage differentiation and the regulatory factors and molecules is essential. The current knowledge of cartilage differentiation has lots of open ends. This review covers the latest information regarding cartilage developmental pattern. Though MSCs are no longer considered as panacea, still the vision of autologous *ex-vivo* created hyaline cartilage tissue may come true with tissue engineering of MSCs.

Key words: Chondrogenesis, mesenchymal stem cells, articular cartilage, Sox9, bone marrow

Introduction

Tissue engineering uses living cells, biomatrices and signalling molecules to provide new functional tissue hence it combines cell biology, engineering, material sciences and surgery. The potential of tissue engineering is endless and ranges from cardiac valve generation to *ex-vivo* cartilage construction. Damaged articular cartilage specifically requires tissue engineering based therapeutic methods because of its minimal self-repair capacity (Solchaga et al., 2004).

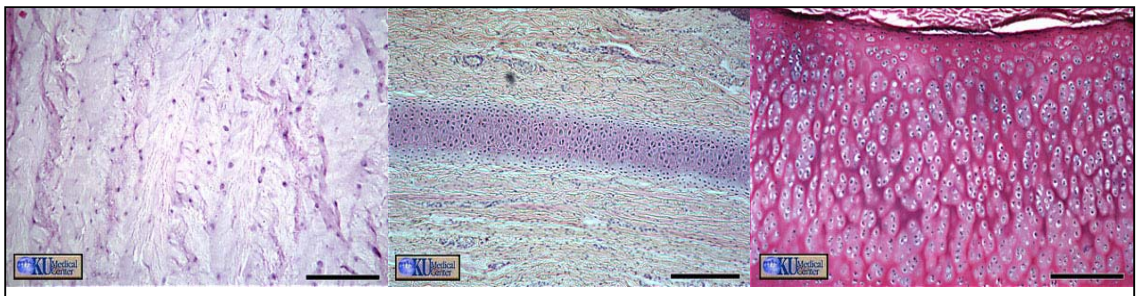
Defects in cartilage structure, biosynthesis, and assembly lead to severe diseases or abnormalities such as osteoarthritis, campomelic dysplasias or multiple epiphyseal dysplasia etc (Ge et al., 2006; Mansour et al., 1995; Thur et al., 2001). Any defect or external injury leading only to chondral lesions (more then 5 mm) is detrimental to normal living because it does not spontaneously heal due to the avascular nature of the tissue and, more importantly, because of chondroprogenitor cells deficiency. Even if the injury is osteochondral in nature and penetrates through the vascularized subchondral bone it usually results into fibrous cartilage formation which is quite rigid and lacks the characteristic features of chondrocytes derived hyaline matrix. Therapies are usually restricted to surgical intervention as autologous osteochondral transfer (OCT), fresh osteochondral allograft and microfracturing. All of the surgical methods have limitations and high risk potential to the donor and recipient tissue. OCT or mosaicplasty is limited to the damaged area of less then 2 cm^2, lack of compatible donor tissue is the major limitation of allogenic osteochondral graft and microfracturing cues off fibrocartilage formation (Cancedda et al., 2003). A classical cell based therapeutic option in use since 12 years is autologous chondrocyte transfer (ACT). The most important factor for this and any other tissue engineering method is the source and origin of the appropriate cells. For ACT autologous chondrocytes from healthy tissue of minor weight bearing areas are isolated by enzymatic digestion, expanded in culture and then injected at the injured cartilage site under a periosteal flap or a synthetic matrix (Brittberg et al., 1994). However, the generated tissue is mostly fibrous and rigid and the risk of permanent damage to the donor site is too large. Furthermore, differentiated chondrocytes do not proliferate *in vitro* and attempts of induction of proliferation leads to dedifferentiate towards fibroblast like cells. Thus, the need of specific chondroprogenitor cells, with high proliferation capacity in combination with good differentiation potential, is evident for regenerative therapy. The discovery of skeletal stem cells or MSCs has opened new horizons for bone/cartilage reconstructive procedures (Cancedda et al., 2003).

1. Cartilage

Cartilage is an essentially avascular highly specialized connective tissue of mesenchymal lineage. It is widely distributed throughout the body and has multiple pre- and post-natal functions. In adults the most highly manifested function is in assisting bones to withstand compressive forces; in addition it has a vital role in skeletal development and growth. During embryogenesis most of the bones, with exception of craniofacial bones are developed by a process called "endochondral ossification" in which first a cartilaginous mould is formed which is later converted into bone (Goldring et al., 2006).

Cartilage comprises chondrocytes embedded in self contrived extracellular matrix (ECM). Primary molecules of ECM are collagens which constitute 60% of cartilage protein bulk. The most dominant (90-95%) form of cartilaginous collagen is collagen type II which forms a heterofibrillar structure together with (~5-10%) type XI and type IX collagens (Mendler et al., 1989). In addition, cartilage ECM also contains large proteoglycans as aggrecan and hyaluronic acid and several small leucine rich proteoglycans (SLRPs) e.g. decorin and biglycan (Iozzo, 1999). Non-collagenous adhesive glycoproteins as fibronectin, tenascin, matrilins and cartilage oligometric protein (COMP) often function as adapter proteins connecting fibrillar structures with the extra fibrillar matrix that may contribute to the stability and structural integrity of the ECM (Budde et al., 2005).

On the basis of biochemical composition, morphology and composition of ECM cartilage is divided into three divisions of hyaline, elastic and fibrous cartilage (Eikenberry and Bruckner, 1999).



Fibro cartilage Elastic cartilage Hyaline cartilage

Figure 1: Histological analysis of different cartilage types. The three kinds of cartilage can be differentiated by haematoxylin eosin (H&E) staining. Fibrous cartilage shows bundles of fibres, elastic cartilage shows elastic fibres. Hyaline cartilage exhibits characteristic ECM structures with intermittent chondrocytes in the lacunae. The bar represents 250µm.
Courtesy of http://www.kumc.edu/instruction/medicine/anatomy/histoweb/cart/cart.htm

Hyaline cartilage is the most abundant type of cartilage present in the skeleton of all vertebrates. It is mainly found in diarthrodial (synovial) joints forming a smooth surface to reduce friction; this hyaline cartilage is termed as articular cartilage. Hyaline cartilage also exists inside the bones and forms the growth plate which serves as the template for endochondral ossification. Articular cartilage is optically uniform mainly composed of chondrocytes and extracellular matrix. It is avascular thus diffusion of nutrients from the surrounding diarthrodial fluid is the only means of sustenance. Oxygen tension in articular cartilage gets as low as 1-3% compared with 24% in the normal atmosphere (Eikenberry and Bruckner, 1999). The more flexible elastic cartilage is mainly found in the pinna of ear and lining of the tubes like larynx; it keeps the tubes permanently open. It is similar to hyaline cartilage but contains more elastin in the matrix. Fibrous cartilage is tougher and is found in the areas which require more tensile strength as intervertebral discs. It contains denser collagenous fibrillar network as compared with the hyaline cartilage and lacks perichondrium (Eikenberry and Bruckner, 1999) **(Fig.1)**.

ECM turnover by MMPs and Inhibitors

For stability of functionally competent ECM different proteases and their inhibitors play a regulatory role. Among them different matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs) are crucial. MMPs are Ca^{2+}- and Zn^{2+}-dependent endopeptidases which cleave most of the ECM components. About 25 different MMPs have been so far identified and according to the substrate preference have been divided into four major classes: collagenases, gelatinases, stromelysins, membrane-type (MT-MMP). In contrast, only four TIMPs are known, but they control the activities of all the MMPs. TIMPs are significant for tissue development and remodelling. Not well explored hints of regulatory activity of TIMPs in cell growth and mesenchymal growth also exist (Mannello et al., 2005). Both the MMPs and TIMPs have a large activity portfolio, from ECM regulation to embryonic development, morphognesis and cells and tissue development and modulation of gene expression. They have also been known to affect cell differentiation (Vu and Werb, 2000).

Development of axial and appendicular skeleton during embryogenesis occurs via endochondral ossification and depends on chondrocytes fashioned cartilaginous template. Ossification begins with chondrocyte hypertrophy and cell death; parallel to severe calcification and partial degradation of the template which facilitates vascular invasion, essential for skeletogenesis (Haeusler et al., 2005). The remodelling of the cartilaginous

template is mainly achieved by MMPs employing their proteolytic activity. MMP-13 or collagenase-3, a highly expressed collagenolytic MMP detected in primary centre of ossification during embryonic development has a critical role in cartilage turnover (Mitchell et al., 1996). Absence of MMP-13 hinders hypertrophic differentiation of chondrocytes and causes increased length of growth plates and complete distortion in alignment of rows of chondrocytes leading to delayed ossification (Inada et al., 2004). Therefore, for normal skeletal development and maturation coordinated regulation of the anabolic and catabolic ECM associated genes is critical (Von der, 1999).

2. Marrow stromal cells

All cells arise from a single population of progenitor cells traceable to the fertilized egg or zygote, the totipotent embryonic stem cells (ES). In 1970s *Friedenstein et al* isolated pluripotent MSCs with ES properties from adult tissues. The colonogenic adherent cells could replicate many folds in culture while retaining their differentiation competence (Friedenstein et al., 1976).

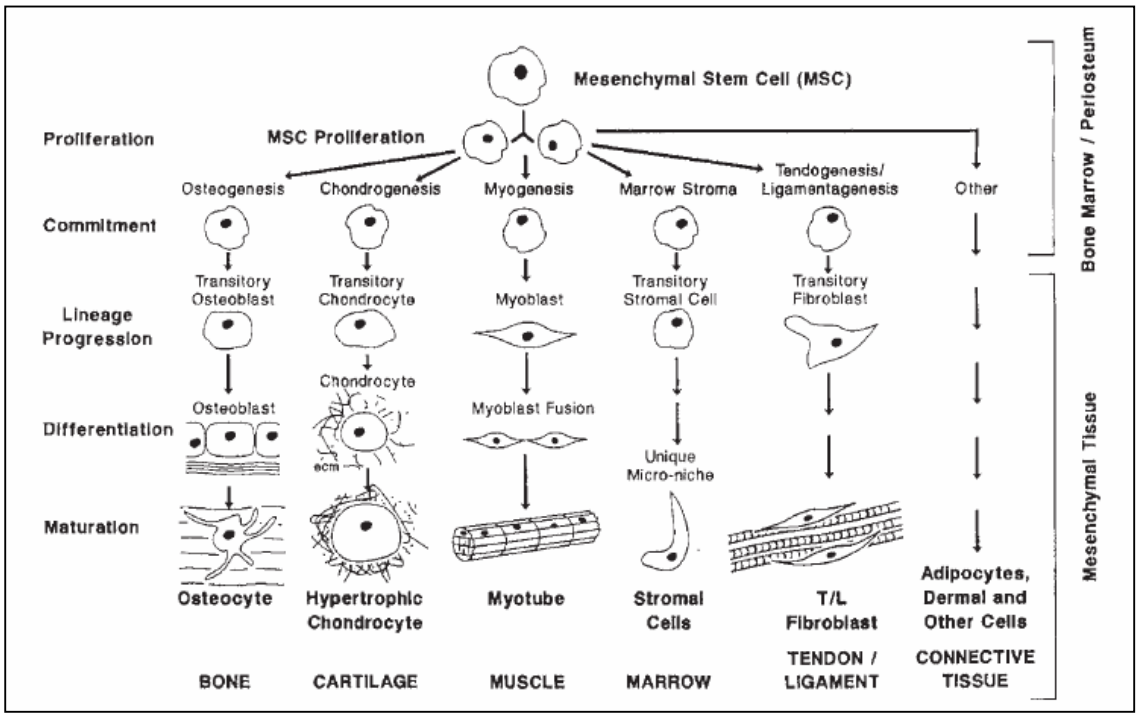


Figure 2: Multilineage potential of MSCs: MSCs have multiple lineage potential to differentiate into bone, cartilage, muscle, marrow stroma, tendon/ligament, fat and other connective tissues. Each differentiation involves multiple steps controlled by growth factors and cytokines. Reproduced from *Caplan 2005* (Caplan, 2005).

The original discovery was brought in lime light by *Pittenger et al* in 1999 who showed the true promise of MSCs; in their multi-differentiation potential and in the fact that they do not harbour ethical questions related with embryonic stem cells research (Pittenger et al., 1999).
MSCs are a fundamental unit of bone marrow not only as mesenchymal progenitors but also as support for haematopoiesis (Bianco et al., 2001). They possess three distinctive characteristics of a stem cell; they can be expanded *in vitro*, they have an unlimited proliferation capacity and they can differentiate into multiple lineages namely, osteocytes, chondrocytes, adipocytes, astrocytes and myocytes **(Fig.2)** (Caplan, 2005). The greatest importance of MSCs lie in the fact that under specific culture and physical conditions a particular differentiation pathway can be induced at will *in vitro* as well as *in vivo* (Pittenger et al., 1999; Cancedda et al., 2003).

Isolation:

Initially MSCs were discovered in bone marrow as part of the marrow stroma. It is now known that MSCs also exist in umbilical cord blood, teeth, skin, adipose tissue, periosteum, trabecular bone and peripheral blood. However, bone marrow still remains the major source for MSCs which retain their pluripotency even after 6-10 passages *in vitro* (Magne et al., 2005). Iliac crest of pelvis is usually the site of bone marrow extraction in humans and other larger animals (Pittenger et al., 1999). In rodents they are easily harvested from mid-diaphysis of the tibiae and femora and the marrow extract is directly cultured in tissue culture flasks (Maniatopoulos et al., 1988). The bone marrow aspirate from iliac crest is first subjected to density gradient centrifugation for separation of mono-nucleated cells and only the MSCs fraction is cultured. So method of choice for MSCs isolation mainly depends upon the species and source of extraction (Pittenger et al., 1999). Using low seeding density and proper culture conditions MSCs can be separated from the other cells of bone marrow due to their adherent nature. Distinct colonies of spindle shaped fibroblast like cells termed as colony forming unit-fibroblast (CFU-F) are major characterization criteria for MSCs (Bianco et al., 2001). Further characterization is based on their antigenic profile and differentiation potential. Under proper culture conditions and mechanical stimulus MSCs can be induced to differentiate towards terminally differentiated cell lineages of mesenchyme *in vitro*. However most of the MSCs populations are heterogenic, comprising of naïve MSCs with multidifferentiation capacity and progenitor MSCs which have reached different stages of commitment to a particular lineage. Depending on the commitment status these cells exhibit restricted lineage potential (Bianco et al., 2001).

Surface markers:

Not all stromal cells are stem cells however, until to date no unique antigenic cell surface marker has been discovered which can positively identify MSCs. Therefore, to characterize marrow derived MSCs a consortium of positive and negative markers is required. Stro-1, CD29, CD44, CD49a, CD71, CD73, CD90, CD106 are some of the generally accepted positive markers (Baksh et al., 2004; Pittenger et al., 1999; Barry, 2003). The consensus is that MSCs stain negative for markers of haematopoietic lineage like CD4, CD14, CD34 and CD45 (Magne et al., 2005; Baksh et al., 2004). Different techniques like fluorescence associated cell sorting (FACS) and magnetic associated cell sorting (MACS) are used to sort the cells on the basis of their surface marker profile. Both the techniques are crucial to establish the so called "stemness" of the cultured MSCs. An advantage of MACS over FACS is that after MACS separation cells can be further cultured and their differentiation and proliferation behaviour can be monitored (Majumdar et al., 2000). MACS is routinely used for negative selection of bone marrow extracted cells, such cell populations are cultured and studied to decipher involvement of other cells on MSCs proliferation and differentiation *in vitro*.

3. Bone marrow microenvironment in vivo

The bone cavity of mammalian bone is filled with soft bone marrow (BM) and blood vessels. BM is the only organ so far identified which is host of two types of functionally cooperating stem cells. The main population of haematopoietic stem cells (HSCs) is supported by bone marrow stroma containing a small population of non-blood forming MSCs. In the stroma or bone marrow microenvironment, MSCs coexist with endothelial cells, macrophages, adipocytes, fibroblasts, osteoprogenitor cells and HSCs and their progeny etc. (Dorshkind, 1990; Yin and Li, 2006).

Stem cell niche is where the stem cells reside and undergo self-renewal and/ or differentiation, the MSCs niche in the marrow is not well explored. However, existence of two distinct stem cell niches has been well argued; an osteoblastic niche for osteoprogenitor cells and a vascular niche for HSCs where the mature haematopoietic cells are released into the vascular system **(Fig 3)**. Since 1978 HSCs niche has been known and since then the role of their physiological microenvironment as structural support and in mediation of cell signalling has been studied in depth. MSCs exist in different commitment and differentiation states most likely the so called naïve MSCs with true stem cell attribute reside as part of the stroma but the MSCs with

committed osteoblastic progenitor status reside in the osteoblastic niche (Moore and Lemischka, 2006).

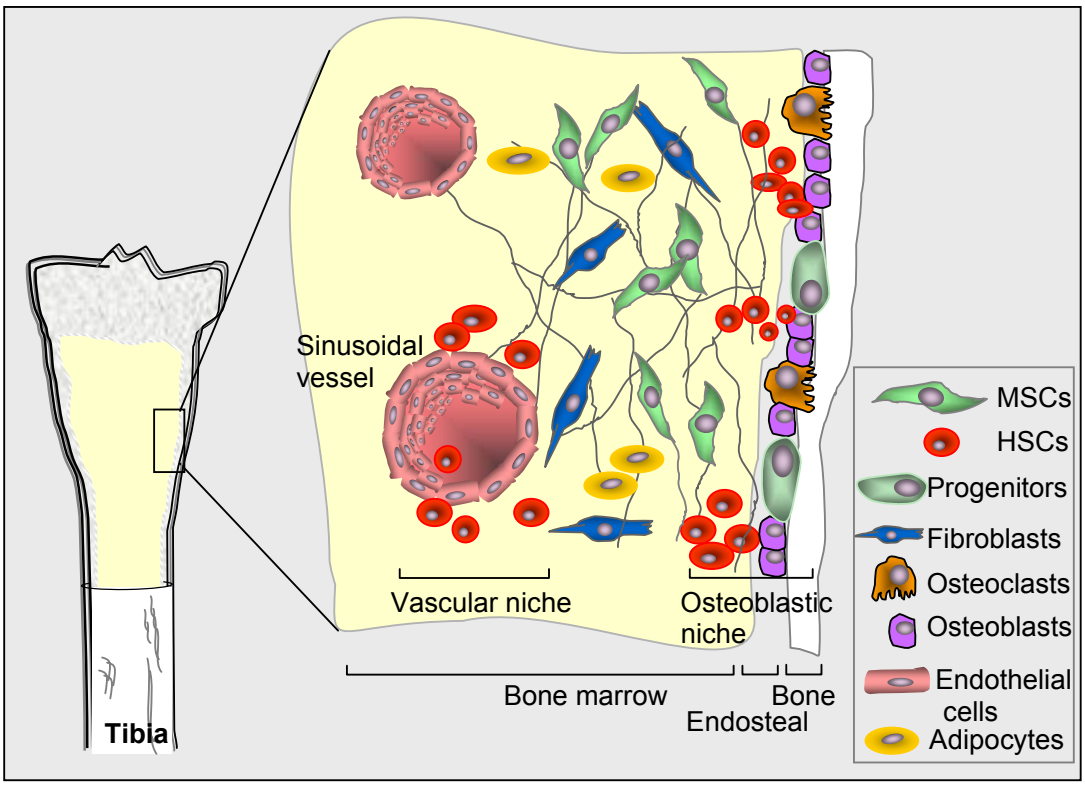


Figure 3. Bone marrow microenvironment. Bone marrow contains 99% HSCs and <1% MSCs. HSCs reside mostly in the osteoblastic niche and move towards the vascular niche at the time of differentiation to enter the circulation. Multipotent naïve MSCs are part of the stroma however, as the MSCs become committed progenitors they move towards the osteoblastic niche. Exact spatial relationships of the bone marrow cells are not well defined (Moore and Lemischka, 2006; Yin and Li, 2006).

Both *in vivo* and *in vitro* plasticity of MSCs greatly depends on the microenvironment. It has been convincingly shown that heterogeneity of the extracted cell population determines their differentiation potential. Historically, MSCs as part of BM stroma have been known to support haematopoiesis even before their mesodermal progenitor cells status was established (Bianco and Robey, 2001). Now it is also known that removal of the native soluble and cell-contact signalling network of the bone marrow reduces plasticity and proliferation capacity of

MSCs *in vitro*. Such discoveries indicate that the signalling cues, cytokines and growth factors from the environment are vital for differentiation, proliferation and maintenance of differentiation status of HSCs and MSCs (Bianchi et al., 2001). Thus, to provide an optimal chondrogenic favourable culture microenvironment *in vitro* it is necessary to characterize the bone marrow microenvironment *in vivo.*

4. Chondrogenic favorable microenvironment in vitro:

For *in vitro* chondrogenic differentiation MSCs are needed to be kept in high density 3-D environment. This can be attained by aggregating the cells in mircomass pellets or as suspension in alginate. Different synthetic or biological scaffolds like agarose, collagen suspensions, fibrin gels and biopolymers can also be used, depending upon the aim of study (Bruckner et al., 1989; Kavalkovich et al., 2002; Hunziker, 2002). Alginate bead culture is an excellent tool for chondrogenic differentiation studies *in vitro*. Alginate is a linear polysaccharide which is soluble in aqueous solutions and cells can be homogenously suspended in it. It is cross-linked in presence of calcium or other divalent ions to form a polymerized hydrogel. Importantly, it can be easily resolubilized by a chelating agent (EDTA) to separate cells from the ECM (Hauselmann et al., 1994).

Chondrogenic differentiation is a complicated process requiring well defined conditions, therefore, external fetal calf serum (FCS) normally used for proliferation of MSCs has to be substituted by defined medium supplement, like widely used ITS™. It contains insulin, transferrin, selenious acid and linoleic acid suspended in solution of bovine serum albumin. Insulin as a hormone supplement is necessary for survival of cells as it is involved in fatty acid and glycogen synthesis. Transferrin is an iron-binding protein for hormones and nutrients, therefore, it is vital for *in vitro* cell growth and selenious acid is a cofactor for glutathione peroxidase necessary for cell membrane integrity. Linoleic acid is an integral component of chondrogenic medium, like many unsaturated fatty acids it is an integral membrane component and important for cell growth. It is not a specific chondrogenic differentiation factor but in combination with Tgfß has profound stimulatory effect on chondrogenesis. Tgfß alone and in combination with dexamethasone exerts profound stimulatory effect on chondrogenesis (Johnstone et al., 1998; Lennon et al., 1995). The chondrogenic medium has to be enriched with proline because this amino acid is found in very high concentrations in extracellular matrices. Pro-x-gly-pro sequence motif appears very frequently in collagens where x is usually a neutral amino acid. Ascorbic acid is required as an electron donor, thus it contributes to collagen synthesis by acting as a co factor for lysine and

proline hydroxylation essential for the formation of typical-triple helical collagen structures (Chepda et al., 2001).

As all the required conditions are met MSCs start to loose their fibroblast like characteristics and start expressing chondrocyte specific ECM which can be monitored by expression of chondrogenic markers.

5. Chondrogenesis

Chondrogenesis, one of the major differentiation pathways of MSCs, is the process which leads to formation of cartilage anlagen during endochondral ossification in skeletal development. It occurs autonomously in three separate mesenchymal lineages, cranial neural crest, sclerotome cells and lateral plate mesoderm cells. From cranial neural crest cartilage and bone of the head region arise, sclerotome gives rise to vertebrae and ribs while the lateral mesoderm generates limb cartilage (Goldring et al., 2006). It is important to note that in every case the same chondrogenic differentiation program is used independent of cell source or type of cartilage. Subsequently the same program is adapted for skeletal regeneration following bone fractures in adults (Ferguson et al., 1999).

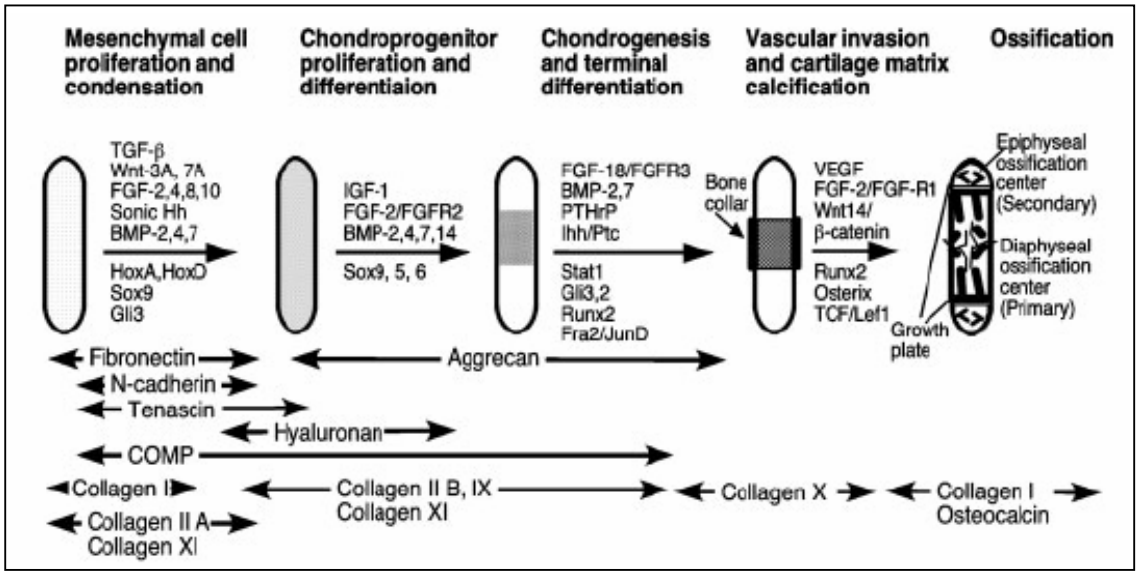


Figure 4. Chondrogenic differentiation during endochondral bone formation. Schematic representation of different stages; growth and differentiation factors are listed on top of the arrows and transcription factors below the arrows. Stage specific ECM marker proteins are listed at the bottom of the figure. Reproduced from *Goldring 2006* (Goldring et al., 2006).

This osteochondral differentiation is a multistep process where every succeeding step is guided by the predecessor and its integrity is controlled by interplay between genetic and biomechanical forces. Every stage of the process is identifiable because of differentiation stage specific and tissue specific biochemical markers. Defined cell surface markers may be employed to identify a specific differentiation stage on the cellular level while mRNA can be utilized for gene expression analysis (Hall and Miyake, 2000). The first stage of chondrogenic differentiation is conversion of undifferentiated MSCs to committed osteochondroprogenitor cells and migration to the site of differentiation where they interact with epithelial cells. This leads to cell condensation (formation of high cell density regions) and growth arrest. Condensation on its own is a multistep process and involves intiation, establishment of boundary conditions, cells adhesion, proliferation, growth and eventual cessation of growth. Cells present at the centre of this condensation nodule first form pre-chondrocytes and then chondrocytes which start to produce cartilage matrix. In the growth plate chondrocytes undergo successive changes and after unidirectional proliferation become hypertrophic and attain the ability to calcify the matrix which is followed by cell death and replacement by osteoblasts **(Fig.4)** (Goldring et al., 2006; de Crombrugghe et al., 2000). Progression from condensation to overt chondrogenic differentiation requires down regulation of proliferation associated genes (e.g. *N-CAM*) and up regulation of differentiation associated genes as *Col2a1, Col9a2*, *Col11a2, COMP and aggrecan*. It is governed by many extracellular ligands and their receptors, nuclear receptors, transcription factors, DNA-binding proteins, matrix proteins, matrix modifiers as metalloproteinases and adhesion molecules (de Crombrugghe et al., 2000; Mundlos and Olsen, 1997; Lefebvre et al., 2001). Collagen X is the distinguishing marker of terminal differentiated chondrocytes, able to synthesize mineralized matrix followed by vascular invasion and cell death (Von der Mark et al., 1992). Ossification can be overtly detected by collagen I and osteocalcin gene expression **(Fig.4)**.

The family of homeobox containing (Hox) transcription factors play integral roles in epithelial-mesenchymal interaction. Major growth factors involved in condensation initiation are members of the transforming growth factor ß family (TGFß-1, 2 and 3) and fibronectin. Up regulation of differentiation after condensation is directly under influence of bone morphogenic proteins (BMP2-7) and fibroblast growth factors (FGF1, 2 and 9). Parathyroid hormone and parathyroid hormone-related peptide receptor (PTHrP) are induced by Indian hedgehog (Ihh) for transition of chondrocytes to hypertrophic chondrocytes.Transcription factor families with paired type DNA-binding homeodomain (Pax) and *SRY*-type high mobility group box DNA binding domain (Sox) have been shown to play important roles in

condensation and transition from condensation to overt differentiation (Hall and Miyake, 2000; Shum and Nuckolls, 2002). For chondrogenic differentiation the most important transcription factor by far is Sox9 (Akiyama et al., 2002; Bi et al., 1999).

6. Transcription factor Sox9

Expression of all chondrogenesis associated genes including *Col2a1, Col9a2, Col11a2, aggrecan, COMP* and most of the *Sox* family genes, is suggested to be regulated by transcription factor *Sox9* (Akiyama et al., 2002; de Crombrugghe et al., 2001). Hence, Sox9 is designated as master regulator of chondrocyte lineage formation. It has been reported to be expressed in all chondroprogenitor cells and also in differentiated chondrocytes. Its expression starts in chondroprogenitor cells, reaches a peak in resting and proliferating chondrocytes and is completely absent in hypertrophic chondrocytes. The pattern of *Sox9* expression precedes *Col2a1* gene expression in developing chondrocytes and it has been observed that MSCs can not condense without Sox9 (Chimal-Monroy et al., 2003). Heterozygous mutations in and around *Sox9* cause skeletal dysmorphology syndrome and haploinsufficiency resulting in campomelic dysplasia, a lethal skeletal malformation syndrome and XY sex reversal (Mansour et al., 1995). Another role for *Sox9* is suggested in gene regulation of cell-adhesion molecules. In skeletogenesis down regulation of *Sox9* is required for transition of proliferating chondrocytes to hypertrophic chondrocytes which ultimately leads to endochondral ossification and chondrocytes death. Cbfa1, an integral transcriptional factor indespensible for osteoblastic differentiation also seems to be under direct or indirect control of Sox9 (Akiyama et al., 2002; de Crombrugghe et al., 2001). Several studies carried out in chondrocyte cell lines, mesenchymal stem cells, primary chondrocytes and on genes of chicken, mice, rats and human, have identified *Sox9* as the key player of chondrocyte phenotype induction and maintenance. In short, Sox9 is not only the primary transcription factor involved in chondrocyte differentiation but it also has an earlier active role in chondrocyte progenitor cell determination, conserved across vertebrates (Chimal-Monroy et al., 2003; Bi et al., 1999; de Crombrugghe et al., 2001). Sox9 integrates several signalling pathways involved in regulation of cartilage differentiation associated gene expression and activity. For example, positive regulatory signals from FGFs (via MAPK) and BMP-2 (via cAMP), matrix degradatory signals from cytokines (JAK/STAT) or retinoic acids and PTHrP signalling in prehypertrophic chondrocytes are all involved in Sox9 mediated gene regulation. An important factor to consider is that the activity of Sox9 in these pathways may depend not only on the signalling molecules but also on the level of *Sox9* gene expression itself, as it is

seen in human single allele mutation studies (Bi et al., 2001). Due to post natal fatality, attempts for generation of *Sox9* null homozygous transgenic mice have been abandoned thus, *Sox9* expression studies are limited to mouse embryo chimeras derived from *Sox9*-/- ES (Akiyama et al., 2002). Hence, use of new technologies and novel ideas for understanding Sox9 function and mode of action is vital.

7. Animal models

Many aspects of biology are similar in most or all organisms and often it is easier to study a particular aspect in one organism than in others. These much-studied organisms are commonly referred to as model organisms. Mammals share many basic biological functions, such as the regulation of cell division, the development of organ systems, and immune response. The rat is a principal model organism to link function to genes. The large number of inbred rat models and the vast amount of data available are helpful for studies of human physiology and pathology. These animals are a unique resource for studying and identifying genetic pathways relevant to some human diseases. Tissue engineering based approaches require pre clinical *in vitro* and *in vivo* studies. Though the mouse model is better characterized and is less cost and space intensive, a big advantage of the rat over the mouse model is bigger animal size which results in more cellular material. The rat system is also ideal for tissue engineering studies due to less inter-individual variation and easy availability of the cells. (http://www.ncbi.nlm.nih.gov/About/model/mammal.html).

8. Future

MSCs based therapeutic approaches for cartilage repair exhibit ample promise. The more effort goes in deciphering regulatory mechanisms of stem cells proliferation and differentiation the quicker we will reach the goal. Optimal application of MSCs for cartilage repair requires biodegradable scaffolds and proper signalling to maintain the articular phenotype of the newly formed cartilage. Factors and the involved pathways which influence geno- and phenotypical changes in MSCs during differentiation could be vital for *ex vivo* cartilage regeneration. Hence, studies like this are important for the future of cartilage tissue engineering.

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Chapter 2

Gene expression and protein secretion profile of naïve and differentiated rat marrow stromal cells

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Abstract

Adult mesenchymal stem cells (MSCs) are adherent stromal cells of non-haematopoietic origin. Upon *in vitro* expansion they retain their self renewal capacity as well as their potential to differentiate into tissues of mesenchymal lineage including bone, cartilage, muscle, tendon and connective tissue. Amongst these cartilage is the only tissue which lacks self renewal capacity thus MSCs are an excellent tool for therapeutic regeneration of focal cartilage lesions. For optimal manipulation of MSCs identification and better understanding of

molecular mechanisms regulating differentiation pathways is needed. While many studies on genetic profiles of human MSCs exist; basal gene and protein profiles of adult rat MSCs has rarely being investigated. Rat is a widely used mammalian experimental model for preclinical studies. Therefore, this study undertook a comprehensive profiling of mRNA expression of osteo- / chondrogenesis related genes in undifferentiated and differentiated rat adult MSCs by using quantitative RT-PCR technology. A differential gene expression pattern was observed depending on osteo-chondral differentiation status of the cells. At protein level TIMP-1, MCP-1 and VEGF164α were detected in culture supernatant and CINC-2 and ß-NGF in the cell lysate of MSCs after an antibody array analysis. Our results provide a foundation for a more reproducible and reliable quality control of rat bone marrow derived MSCs used for osteo-chondro differentiation studies.

***Key words*:** rat marrow stromal cells, profiling, cytokines, gene expression, antibody array

Introduction

Cartilage cannot regenerate upon injury or degeneration, the exact reason of this incapacity is unclear, but lack of access to progenitor cells is often pointed out as a main reason. Adult osteo-chondroprogenitor bone marrow stromal or mesenchymal stem cells (MSCs) are known progenitors of tissue of mesenchymal lineage. These chondroprogenitor MSCs residing as part of bone marrow stroma in the medullary cavity of the bone are inaccessible to the avascular cartilage (Cancedda et al., 2003). They make up less then 1% of the bone marrow cellular population which is otherwise dominated by haematopoietic cells. *In vitro* expansion of bone marrow cells leads to separation of the stromal MSCs relying on their capacity of adherence to the plastic surface of the tissue culture vessels. Upon expansion these cells retain not only their self renewal capacity but also their differentiation competence (Friedenstein et al., 1976). This intrinsic competence can be invoked under controlled nutritional and mechanical conditions to differentiate MSCs into bone, cartilage, muscle, tendon and connective tissue (Bruder et al., 1994). MSCs have shown promise for *ex-vivo* cartilage regenerative medicine and for treatment of large bone defects (Quarto et al., 2001; Horwitz et al., 1999).

Lack of common standards and of a precise definition of initial cell preparations is a major obstacle for MSCs based research and application. Genetic profiling of human MSCs for

trans-differentiation capacities has provided data on differential gene expression depending on origin and commitment status of MSCs from different tissue sources (Wagner et al., 2005). However, very little is known about the proteomic and genomic profile of MSCs which can qualify as osteo-chondroprogenitor cells. Knowledge of changes in expression pattern of matrix associated structural genes, of proteases and their inhibitors, transcription factors and adhesion molecules during osteogenesis and chondrogenesis are essential for providing proper conditions for *in vitro* differentiation of MSCs.

It has been known that together with the other cells of bone marrow microenvironment MSCs contribute to an interactive network of cytokines, growth factors and matrix proteins to sustain existence and differentiation of haematopoietic stem cells (Dorshkind, 1990). However, what role the network plays in proliferation and differentiation of MSCs is still unknown. Some reports indicate differential gene expression of cytokines as IL-1, IL-6 and GM-CSF in MSCs. However, the studies are mainly restricted to gene expression analysis and do not address the protein. (Majumdar et al., 1998; Kim et al., 2005). Knowledge of a basal cytokine protein profile in MSCs is essential to understand the effect of these cytokines on MSCs and their differentiation.

Mammalian animal models like rat share many aspects of the human genomic, cellular and immunological structure. Large numbers of inbred models and subsequent vast amount of available data parallel to the small size and rapid development are advantages of employing rat MSCs for *in-vitro* studies. However, genetic and proteomic expression profiling of rat MSCs is incomplete. Keeping in mind that the basic background profile of human MSCs cannot be taken at face value for rat MSCs, this study undertook a comprehensive profiling of baseline mRNA level of osteo-chondro related genes in undifferentiated adult rat MSCs which is subsequently compared with the changes in gene expression upon osteo-chondrogenic differentiation. Secondly, a basic protein profile of factors generated from undifferentiated MSCs was compared with that of the differentiated cells.

Material and Methods

Isolation and cell culture of MSCs

MSCs were isolated from rat bone marrow as described earlier (Ahmed et al., 2006). Briefly, bone marrow was removed from tibiae and femora of 6 week old male Sprague-Dawley rats by centrifugation (2000rpm for 3min). Homogenized bone marrow was cultured in 175cm² tissue culture flasks in proliferation medium containing 5% glutamate, 1% antibiotics/antimycotics and 10% FBS (Gibco, Invitrogen, UK) in α-MEM (Sigma Aldrich, Germany). Non-adherent cells were removed on the 3rd day and the adherent CFU-cells were proliferated until reaching 70% confluence. Magnetic associated cell sorting procedure (MACS- Miltenyi Biotech, Germany) was carried out according to the manufactures instructions; in short, expanded cells were incubated in suspension with 4µl of selected antibody / 10^6cells for 5min at 37°C followed by washing and incubation with goat anti-mouse secondary antibody coupled with magnetic beads for 15min at 4°C. The suspension was passed through a magnetic column (Miltenyi Biotec´s LS-MACS columns); while labelled cells were retained by the magnetic field the flow through containing the unlabelled negative fraction was collected. After removing the column from the magnetic field the antibody labelled cells (positive fraction) was also flushed out. For both fractions cells were counted and stained. Antibodies used for MACS were directed against CD45 and CD49a (Chemicon, Germany), CD71 and CD106 (BD Bioscience, USA).

Chondrogenic and osteogenic differentiation of MSCs

For chondrogenesis, cells were cultured for 21 days in high density 3-D alginate cultures. To prepare the culture 10^7 cells/ml were suspended in 1.2% alginate. The cell- alginate amalgam was dropped into 102 mM $CaCl_2$ solution via a syringe which resulted in formation of beads with a diameter of 2-3mm containing approx. 10^5 cells/bead. Beads were cultured in 2.3ml chondrogenic medium in 12 well tissue culture plates. 10 alginate beads were used for RNA isolation and gene expression analysis. Cells were released from alginate by incubation at 37°C for 30min in 55mM sodium citrate and 0.15M sodium chloride buffer followed by cell recovery by 3min spin at 750xg. Chondrogenic medium contained: ITS+ premix (6.25µg/ml insulin, 6.25ng/ml selenium acid, 6.25µg/ml transferrin, 1.25mg/ml BSA and 5.35µg/ml linoleic acid (BD Biosciences, USA.), 110µg/ml pyruvate, 40µg/ml proline, 0.1µM dexamethasone, 50µg/ml ascorbic acid and 10ng/ml TGFß-3 (Johnstone et al., 1998) (R&D Systems) in α MEM high glucose (Gibco, Invitrogen, UK). Alginate sodium salts were

acquired from Sigma Aldrich, Germany (Cat# A0682-100G). Osteogenesis was induced for 15 days in 6 well culture plates, 250,000 cells/ well were cultured as monolayer in osteogenic medium containing, 10% FCS, 1% pen/strep, 10nM dexamethasone, 50µg/ml Ascorbate 2-PO_4 and 10mM ß-Na glycerophosphate.

Immunofluorescence

For immunofluorescence analysis alginate beads were irreversibly polymerized by replacing $CaCl_2$ with 100mM $BaCl_2$ (Hauselmann et al., 1994). The beads were fixed with 4% paraformaldehyde and after sequential dehydration embedded in paraffin. 4µm sections were acquired and the deparaffinized and rehydrated sections were used for staining after hyaluronidase digestion. Undifferentiated MSCs were fixed with 4% paraformaldehyde. Slides were blocked for 1h at 37°C in 5% normal goat serum and 1% BSA in PBS containing Complete Mini 1:5 protease inhibitor solution (Roche, Germany). After washing with PBS cells were stained overnight at 4°C with monoclonal antibodies directed against collagen II, 1:1000 diluted (Acris, Germany), CD49a, diluted 1:50 (Chemicon, Germany) and D7fib, diluted 1:50 (Acris, Germany). The appropriate Alexa568 or Alexa488 conjugated secondary antibodies (goat anti mouse, 5 µg/ml; Molecular Probes, USA) were added for 1h at RT. After washing slides were permanently mounted with DAKO fluorescent mounting medium (DAKO, USA) and covered with cover slips. Slides were evaluated with scanning laser microscopy (C1 confocal microscope from Nikon, Germany) and photos were taken with a Nikon C4 camera and software.

Histological analysis

Osteogenically differentiated cells were washed with PBS and fixed with methanol for 10 min. After rinsing with water staining was carried out for 2min with 1% alizarin red prepared in 25% ammonia. Stained cells were thoroughly washed and photographed with a Nikon C4 camera after complete drying.

RNA Isolation and reverse transcription

RNA was isolated by an affinity column chromatography method with Ambion's RNAqueous4-PCR kit according to the manufacturer's protocol. For removal of possible DNA contamination *DNase*I enzyme (DNA-*free*, Ambion, USA) was used. RNA concentration was determined at 585nm wavelength with RiboGreen RNA quantification kit (Molecular Probes, USA). Conversion of 0.5-1µg of RNA to cDNA was done with

SuperScript II reverse transcriptase kit (Invitrogen, UK) in 20µl of total reaction volume in the presence of 40 units/µl recombinant ribonuclease inhibitor (RNase OUT®), 500µg/ml of Oligo-dT primers, 10mM dNTPs and 200 units of SuperScriptII enzyme in First-Strand Buffer and 0.1M DTT for 50min at 42°C followed by an extension period of 15min at 70°C.

Relative quantitative PCR

Relative quantitative PCR was performed using the SYBR Green Dye I on ABI 7000 Prism Sequence detection system (AB Systems, USA) according to manufacturer's instructions. Briefly, 1µl of cDNA was amplified in 50µl final volume of 0.2µM of each primer suspended in SYBR green master mix (AB Systems, USA). Amplification parameters were identical for all primer pairs and were repeated for 40 cycles, denaturation occurred at 95°C for 0.15min and annealing at 60°C for 1min. ΔCt values, i.e. difference in mRNA expression level of genes of interest to that of an endogenous control, ß-actin, was evaluated from three independent experiments and plotted on an inverse scale so that 0 indicates expression of the endogenous control and thus taken as the reference point for the genes of interest. Mean relative quantification (RQ) values were calculated by the software "RQ study application v1.1" (ABI Prism 7000 SDS software v1.1) according to the ΔΔCt method using ß-Actin as endogenous control and undifferentiated MSCs (day 0) as calibrator. Primers were designed either with freeware Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or "Primer Express" software supplied by AB. All primers were manufactured at MWG – Biotech, Germany and are listed in table 1.

Antibody array analysis

RayBio™ (Tebu-bio, France) rat cytokine antibody array I for conditioned medium was used according to the manufacturer's instructions. Briefly, first the membranes were blocked for 30min in 5% BSA in 0.01M Tris buffer with 0.15M NaCl (pH 7.6) followed by overnight incubation at 4°C in 1ml of cell culture supernatant or cell lysates. After washing and incubation with biotin coupled anti-cytokine antibodies for 2h a second series of washing was performed and then the membranes were incubated with horse reddish peroxidase-conjugated streptavidin for 2h. Signals were detected with the provided detection solution using a CURIX 60 film developer (Agfa, Germany).

Statistical Analysis

One way ANOVA and student t test were employed for quantitative PCR experiments performed in triplicate. The level of significance was determined by Turkey´s test with 2 group comparison.

Table 1: Primers used for quantitative PCR

Gene	RefSeq#	Amplicon (bp)	Forward primer (5´ - 3´)	Reverse primer (5´ - 3´)
Alpha-10	XM_001063132	188	-tttcttcgggaaatcagagc	-tggatggagaagccaatctc
Alpha-11	XM_001075650	170	-tggaggtccaacacttcctc	-gggtttcagtccctcctctc
Aggrecan	NM_022190	224	-ggccttccctctggatttag	-ccgcactactgtccaac
BMP-7	AF100787	167	-gaaaacagcagcagtgacca	-gtggcgttcatgtaggagt
ß-Actin	NM_031144	104	-gtagccatccaggctgtgtt	-ccctcatagatgggcagagt
Cbfa1	XM_34016	86	-gccgggaatgatgagaacta	-agatcgttcaacctggccact
Col1a1	RGD61817*	59	-tccagggctccaacgaga	-ctgtaggtgaatccactgttgc
Col2a1	NM_012929	60	-cccctgcagtacatgcgg	-ctcgacgtcatgctgtctcaag
Col10a1	AJ131848*	247	-ccctattggaccaccaggta	-tctctgtccgctctttgtga
Col16 a1	M92642	97	-gcctggtaccaaaggtgaaa	-catagcctggaggaccttga
COMP	NM_012834	167	-tgacttcgatgctgacaagg	-gaacgatctccattccctga
Ihh	XM_343590	103	-atgaagacggccatcactcag	-cgcgccagtagtccgtacttat
MMP-2	NM_031054	111	-gaccggtttatttggcgga	-ggcctcatacacagcgtcaat
MMP-13	XM_343345	93	-acctgggatttccaaaagagg	-acacgtccttccctgagaaga
Sox4	XM-344594	58	-ggcccatgaacgcctttat	-ctggatgaacgggatcttgtc
Sox6	XM_215016	51	-gaaatccatgtccaaccaggac	-cgggcctgctcttcatagtaag
Sox9	XM_343981	140	-ctgaagggctacgactggac	-tactggtctgccagcttcct
Tbox2	XM_220810	71	-gcccactctccgtttgtatgag	-aggacgaggcatcggattc
TIMP-1	NM_053819	136	-gattcgacgctgtgggaaat	-tttccgttccttaaacggcc
TIMP-2	NM_021989	140	-ggcaagatgcacattaccctct	-atgtagcatgggatcatagggc
Tgfß-3	NM_013174	86	-ttccttcttggccgtatttcc	-tgtgtgggatccagaatcca
VEGFα	NM_031836	71	-tggctttactgctgtacctcca	-tttctgctccccttctgtcgt
VEGFR-2	NM_013062	95	-ttgcctagtcaagcagctcgt	-cgatggtctcaccaatggttg

*Locus ID

Results

Cellular morphology and surface antigen markers of MSCs

Formation of CFU-F is one of the basic classification of bone marrow derived stem cells along with rapid adhesion and extended proliferation (Bianco et al., 2001). After *in-vitro* expansion of bone marrow isolated cells, colony forming units of fibroblast like cells (CFU-F) designated as MSCs readily adhered to the culture flasks and showed typical fibroblast like morphology (Fig 1a). Immunofluorescence analysis of the adherent cells exhibited positive staining for fibroblast marker D7fib and alpha 1 integrin marker CD49a (Fig. 1b).

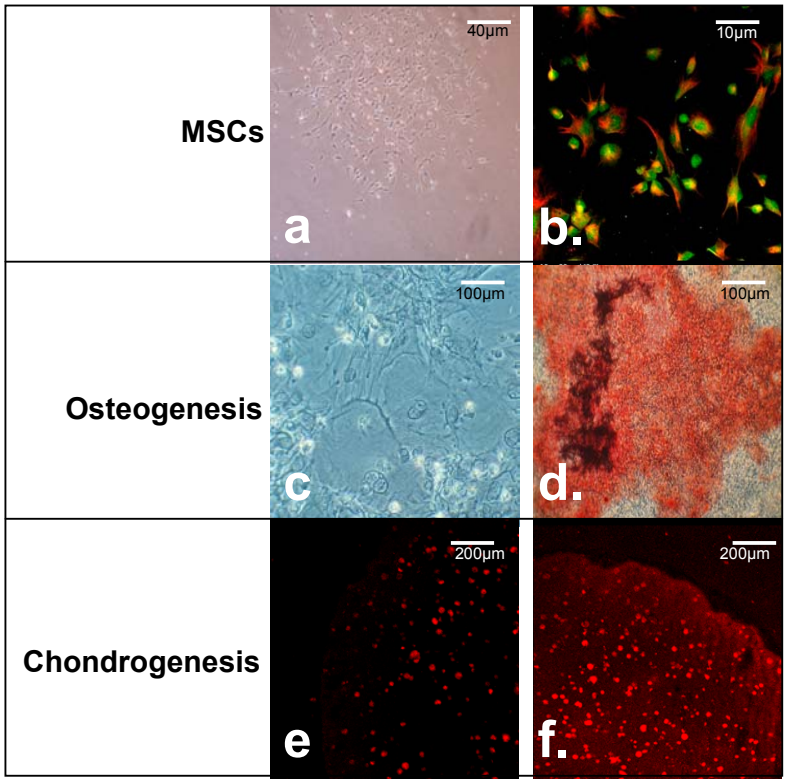


Figure 1: Morphological characterization of MSCs CFU-F like marrow derived adherent cells (a) double stained with D7fib (red) and CD49a (green) by immunofluorescence (b) exhibit typical stem cells morphology. Dark red staining with alizarin red indicates calcified matrix formation after 15 days of osteogenesis in monolayer while the control culture remained unstained (c, d). Chondrogenically differentiated cells kept in alginate were stained with collagen II antibody. After 21 days (f) the positive signal was stronger when compared with the beads stained on day 0 (e).

One of the major characterization criteria for MSCs is the retained differentiation potential upon expansion. MSCs were induced for 15 days in monolayer culture for osteogenesis and 21 days for chondrogenesis in 3-D high density alginate cultures. Osteogenesis cultures stained positive for alizarin red indicating formation of calcified matrix while the un-induced

control cultures did not stain (Fig 1c-d). Chondrogenically induced cells exhibited clear collagen II staining of the surrounding matrix on day 21 of the culture as compared with day 0 (Fig 1e-f). As a unique MSCs marker is not yet identified culture purity is usually determined by employing a consortium of positive and negative markers. In this study adherent cells were screened by magnetic cells sorting (MACS™) using three positive markers CD71, CD106 and CD49a. In addition, CD45, a haematopoietic marker used for negative selection revealed partial purity of MSCs with 75-85% of CD45-negative cells. 60-65% of the cells were CD71 positive and 75% were positive for CD106 and CD49a (Fig. 2).

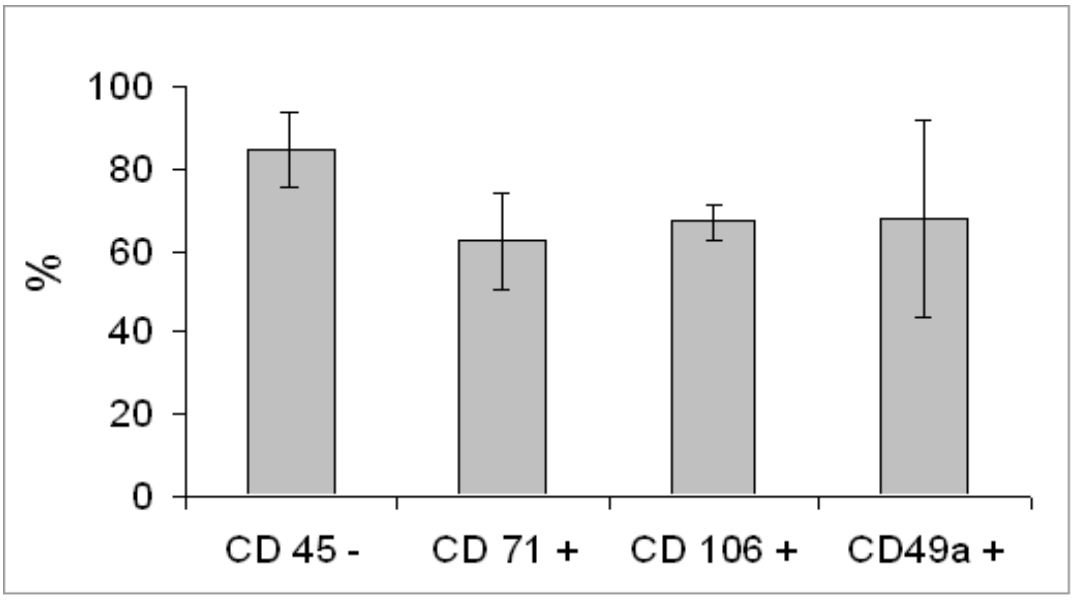


Figure 2: MACS sorting of bone marrow derived CD45+ cells Separation by magnetic labelling of cells exhibiting CD71, CD49a and CD106 antigen and lack of CD45 antigen revealed 75-85% CD45- negative stromal cells with 60-75% of CD71, CD49a and CD106 positive cells. N=3-5

Gene expression profiling

With the help of quantitative RT-PCR and specific primers (Table 1) baseline mRNA expression of selected genes was determined. Subsequently, the MSCs were induced to differentiate into osteogenic and chondrogenic lineages and mRNA level was compared with the expression in un-induced undifferentiated cells.

Among the most highly expressed genes in undifferentiated MSCs were integrin *Alpha-11, VEGFa, Sox9, Sox4, Cbfa1,TGFß-3, TIMP-1, TIMP-2 and MMP-13; Col1a1* exhibited the highest expression of all analysed genes. Lowest expressed genes were *VEGFr-2 (kdr), Ihh, Col2a1,* and *Col10a1*. Expression of integrin *Alpha-10*, *Sox6, Col16a1, Aggrecan, COMP* and *MMP-2* was also observed (Fig. 3a).

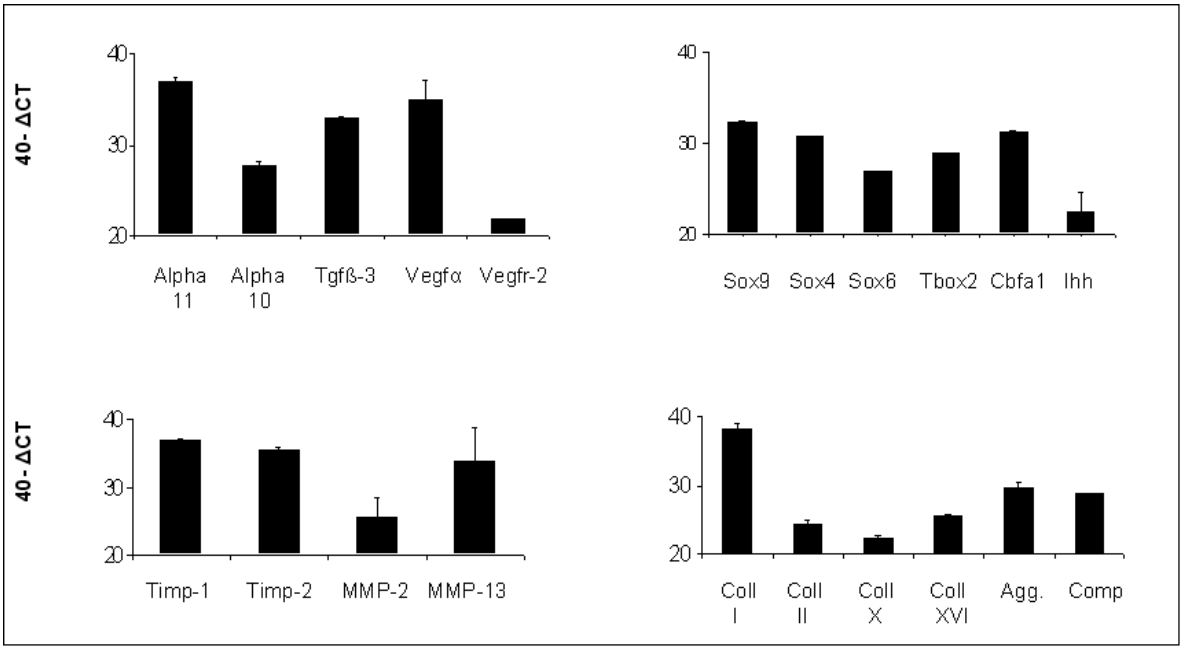


Figure 3a: Selected gene expression profile of adherent undifferentiated MSCs Quantitative mRNA expression level of selected groups of genes in undifferentiated MSCs (black bars) was normalized to ß-actin employing the ΔCT method using SYBR I Green dye. The calculated ΔCT value was subtracted from 40, the total number of PCR cycles. This value was plotted into a graph to show a positive correlation between bar length and gene expression. N = 3.

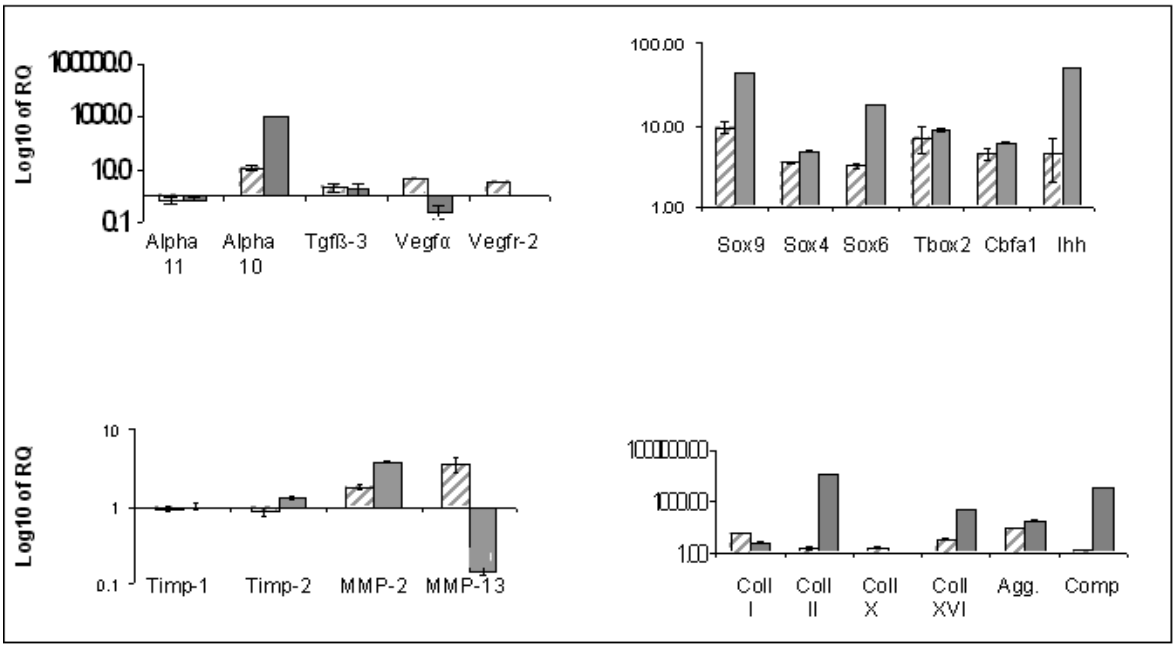


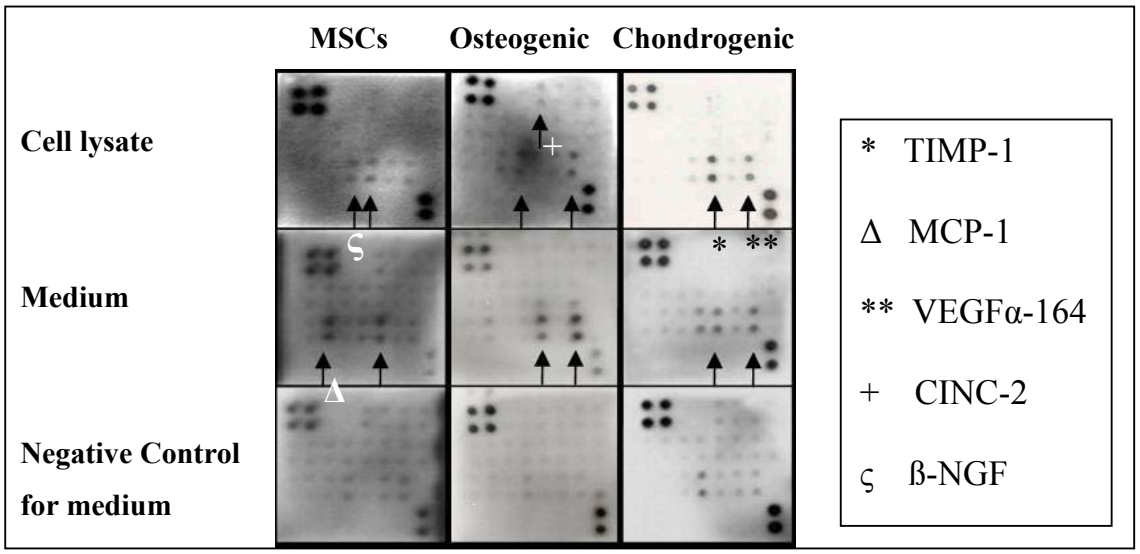
Figure 3b: Selected gene expression profile of adherent undifferentiated MSCs compared with differentiated cells Relative quantitative mRNA expression level of selected genes was determined by the ΔΔCT method using ß-actin gene expression as endogenous control. Expression level of each gene was compared after osteogenesis (stripped grey bars) and chondrogenesis (grey bars) with that of undifferentiated MSCs used as calibrator (set to 1). N=3

Upon induction of osteogenic and chondrogenic differentiation gene expression of *Alpha 11, TIMP-1 and TIMP-2* and *Col10a1* remained mostly unaltered. After 21 days of chondrogenic favourable conditions mRNA level of integrin *Alpha-10, Sox9, Sox6, Ihh, Col2a1, Col16a1, COMP,* and *MMP-2* were up regulated compared with uninduced MSCs. Culturing MSCs for 15 days in osteogenic medium induced mRNA level of *VEGF, VEGFr-2,Col1a1,* and *MMP-13.* Differentiation in general caused about 10 folds increase in mRNA level of *Tbox2 and Cbfa1* independent of the differentiation pathway while *Sox4* and *Aggrecan* expression was slightly higher at the end of chondrogenic differentiation. Chondrogenesis reduced the mRNA level of *VEGFa* and *MMP-13* while osteogenesis had no significant negative effects on gene expression level (Fig. 3b).

Cytokine and growth factor profile of undifferentiated and differentiated MSCs

Cell culture supernatants and cell lysates from undifferentiated and differentiated MSCs were analyzed for secretory molecules using an antibody array covering 15 cytokines plus ß-NGF, TIMP-1, CNTF, leptin and VEGFα-164. Only tissue inhibitor of matrix metalloproteases (TIMP-1) was secreted from undifferentiated as well as from differentiated MSCs, while monocyte chemoattractant protein (MCP-1) was exclusively secreted from undifferentiated MSCs. Secretion of vascular endothelial growth factor (VEGFα-164) was observed after induction of osteogenic and chondrogenic differentiation. When the same antibody array was examined with the cell lysate two additional molecules were identified. Nerve growth factor (ß-NGF) was detected in undifferentiated as well as in cells after osteogenic differentiation and cytokine induced neutrophil chemoattractant (CINC-2) was detected in low amounts after osteogenesis, both factors were undetectable after chondrogenic induction (Fig. 4).

A.



B.

	MSC	Osteo	Chondro
TIMP-1	+	+	+
MCP-1	+	-	-
VEGFα-164	-	+	+
CINC-2	-	+	-
ß-NGF	+	+	-

Figure 4: Comparison of cytokine profile of undifferentiated and differentiated MSCs Antibody array containing 19 different cytokines was used to compare protein profile of proliferated MSCs with MSCs after 15 days of osteogenic induction and after 21 days of chondrogenic induction **(A.)**. Dot intensities higher than the negative control were accepted as positive expression. Among the 5 molecules with differential induction pattern TIMP-1 was present in all three conditions, MCP-1 was specific to undifferentiated MSCs. The induction profile of the remaining three factors was influenced by the differentiation state **(B.)**. N=2

The remaining 14 proteins were undetectable in all culture conditions analysed, these include, CINC-3, Ciliary neurotrophic factor (CNTF), fractalkine, GM-CSF, IFN-γ, IL-1α, IL-1ß, IL-4, IL-6, IL-10, lipoplysacchride induced c-x-c chemokine (LIX), leptin, macrophage inflammatory protein (MIP-3α), and TNF-α.

Discussion

Cellular morphology and surface antigen markers of MSCs

In the medullary cavity of bone marrow MSCs coexist with haematopoietic stem cells. *In-vitro* the two cell populations can be easily separated because MSCs tend to adhere to the tissue culture plastic and form small colony like units arising from single cells (Pittenger et al., 1999). Formation of these colony forming units of fibroblast like cells (CFU-F) is one of the basic classification of bone marrow derived stem cells parallel to rapid adhesion and extended proliferation (Bianco et al., 2001). The CFU-F forming rat bone marrow derived MSCs used in this study showed CD $45^{-/low}$, $CD106^{+}$. $CD49a^{+}$ and CD 71^{+} antigen profiles, as determined by MACS after 7 days of expansion. CD106 (V-CAM) and CD71 (transferring) are well established positive markers for bone marrow derived osteo-chondro progenitor cells. CD49a alpha 1 integrin is expressed only on MSCS and not on other cells of bone marrow. CD45 is mainly expressed on haematopoietic cells and MSCs are known not to express this antigen.

Immunofluorescence analysis further confirmed the identity of MSCs which stained positive for fibroblast marker D7fib and CD49a. The D7fib antigen is described as a specific marker for MSCs as suitable as STRO-1 and CD105 (Jones et al., 2002).

BM-derived cells obtained by sole adherance to plastic surfaces are not a pure population with regard to their CD marker profile. MACS™ cell sorting shows that the cells are a heterogeneous population comprising of a small proportion of CD45-positive cells of haematopoietic origin and a large fraction of CD45-negative cells. It is described that both cell populations exert a mutual influence important for differentiation pathways. The role of MSCs in providing a supportive microenvironment for HSCs is well established and in some cases even direct interaction has been observed (Prockop, 1997). However, vice versa influence of haematopoietic cells in the differentiation of MSCs is still not answered satisfactorily, co transplantation of HSCs with MSCs in hematologic malignancy patients have indicated faster engraftment and low graft versus host disease (Lazarus et al., 2005). In

addition, we could demonstrate a positive effect of haematopoietic cells on the chondrogenic differentiation of MSCs *in vitro* (Ahmed et al., 2006). Therefore, investigation of gene and protein expression profile of MSC populations frequently isolated solely according to plastic adherance will be helpful for basic characterization.

Gene expression profile

Little information is available about baseline mRNA expression profile of rat multipotent MSCs. Here, we present profiling of osteo-chondrogenic related gene expression in undifferentiated and differentiated MSCs including growth factors, transcription factors, proteases and their inhibitors, structural components and adhesion molecules.

Integrin subunit *Alpha-11* was amongst the highly expressed genes, whereas only little *Alpha-10* mRNA was detected in undifferentiated MSCs. Integrin *Alpha-11* is highly expressed in various mesenchymal tissues around the cartilage anlage in the developing skeleton. Its additional expression in MSCs of the intervertebral discs and in keratinocytes in the cornea is compatible with its role as receptor for interstitial collagens (Tiger et al., 2001). Integrin Alpha-10 is a chondrocyte specific major collagen II and IX -binding integrin integral to cartilage development and mature hyaline cartilage (Camper et al., 1998; Camper et al., 2001). In rat MSCs neither osteogenic nor chondrogenic differentiation alter *Alpha-11* gene expression, contrary to *Alpha-10* mRNA level which increased about 1000 folds during chondrogenesis. The ratio between *Alpha-11* / *Alpha-10* integrin gene expression makes these genes useful markers for monitoring the progression of MSCs differentiation towards particular lineages.

Transcription factors which regulate osteo- and chondrogenic lineage as, *Tbox2 (Brachyury), Sox9, Sox4* and *Cbfa1* were also expressed in un-induced naïve MSCs. In early vertebrate embryonic development *Tbox2* is involved in determination of mesodermal cell fates and patterning during limb bud formation, where it is restricted to subridge mesoderm (Liu et al., 2003). Both, *Sox9* and *Tbox2* are essential components for the BMP-dependent onset of chondrogenesis (Hoffmann et al., 2002). Increase in *Tbox2* gene expression after differentiation substantiates its role in osteo-chondrogenic cell fate determination. So far involvement of *Tbox2* in the osteogenic pathway of MSCs has not been described and needs further investigations. *Sox9* is expressed in all essential stages of chondrogenic development starting from pre-condensation nodules and including proliferating chondrocytes, however, the expression is abrogated in hypertrophic chondrocytes (Zhao et al., 1997). At that terminal differentiation stage inhibition is possibly regulated due to a negative feed back mechanism

accompanied by the activation of the PTHrP and Ihh signal cascade in pre-hypertrophic chondrocytes (Akiyama et al., 2002); (de Crombrugghe et al., 2000). After chondrogenic differentiation gene expression of both, *Sox9* and *Ihh* was upregulated, indicating cells might be arrested in a pre-hypertrophic stage, corroborated by lack of gene expression of collagen X which is restricted to terminally differentiated hypertrophic chondrocytes (Schmid et al., 1990). Interestingly, master regulators of both the osteogenic pathway, Cbfa1, and chondrogenic lineage, Sox9, are both already expressed in un-induced MSCs. This implicates that the progenitor cells probably have entered a specific commitment stage which may have been triggered by cell culture conditions. Differential expression of both the master transcription factors might serve as marker for harvesting osteo-chondroprogenitor cells at different states of commitment thus allowing a separation of these populations.

Factors of the Tgfß super family have been identified as important inducers and promoters of differentiation in human MSCs (Barry et al., 2001a). Therefore, high expression of Tgfß-3 in un-induced MSCs might be considered as a prerequisite for ECM structural components regulation during differentiation. Structural components of the cartilaginous extracellular matrix like collagen II (Mendler et al., 1989), aggrecan and COMP (Hedbom et al., 1992; DiCesare et al., 1994) are known to be upregulated in MSCs during the chondrogenic differentiation pathway (Barry et al., 2001b). This makes them suitable as markers for discriminating between undifferentiated and chondrogenically differentiated MSCs. In undifferentiated rat MSCs collagen II and X displayed low basal gene expression indicating lack of chondrogenic differentiation, however, gene expression of aggrecan and *COMP* was observed. On the other hand collagen I showed undoubtedly the highest gene expression amongst all screened molecules which further increased during differentiation. Reduction of relative gene expression of collagen I during chondrogenic differentiation is desirable and underscores the development of a cartilaginous matrix. Little is known about expression profile of fibril-associated collagens with interrupted triple helices (FACIT) in MSCs, gene expression of collagen XVI in undifferentiated MSCs indicates a yet unknown role in proliferation or differentiation processes. Notably, collagen XVI mRNA level is upregulated during chondrogenesis compared with osteogenesis implicating a specific role in chondrogenesis.

The results demonstrate high gene expression of VEGFα in un-induced MSCs with an increase during osteogenic differentiation. During osteoblasts differentiation angiogenic factor VEGF modulates matrix mineralization in an autocrine manner and up regulates the expression of its receptor (Mayer et al., 2005). VEGFα gene expression level appears as a

suitable marker for osteogenic committed MSCs while chondrogenic differentiation requires downregulation of VEGFα mRNA.

The ability of MMPs to alter the activity or function of numerous proteins by proteolytic procession suggests that they are also involved in various cellular functions and matrix formation during development. Gene expression of *MMPs* and *TIMPs* in MSCs cultures has been reported earlier in studies on human MSCs of different origin (Silva, Jr. et al., 2003; Mannello et al., 2005). *MMP-13* together with its inhibitors, *TIMP-1* and *-2* were highly expressed in un-induced MSCs indicating a role in stem cell migration as assigned to MMP-2 (Cronwright et al., 2005). While *TIMP-1* and *-2* were not regulated during MCSs differentiation *MMP-13* was controversely regulated during osteo-chondrogenensis. Downregulation of *MMP-13* gene expression after chondrogenesis correlates with arrest of cells in a pre-hyperthrophic stage. Relatively higher *MMP-13* mRNA level after osteogenesis indicates progressing mineralization as *MMP-13* along with alkaline phosphatase and osteocalcin is known to be involved in endochondral and intramembranous ossification (Tuckermann et al., 2000). Notably, *MMP-2* gene expression was elevated during chondrogenesis which may indicate beginning of late stage chondrogenesis as proteolytic activity of *MMP-2* is vital for ECM turnover prior to hypertrophy (Aigner et al., 2001).

Protein profiling of undifferentiated and differentiated MSCs

Secretory cytokines, proteases and growth factors are key players in cell proliferation and differentiation. Mixed cultures of BM isolated MSCs are known to secrete haematopoiesis supportive cytokines. We speculated that the protein profile should differ according to the differentiation state of the MSCs. TIMP-1 was the only identified molecule to be secreted from undifferentiated as well as differentiated MSCs while monocyte chemoattractant protein (MCP-1) was exclusively secreted from undifferentiated MSCs. TIMP-1 is a multifunctional molecule apart from its biological activity as inhibitor of metalloproteinases,it is thought to be involved in regulation of cell growth and differentiation. Cell surface binding of TIMP-1 and -4 has been reported, but the identification of specific cell surface receptors is missing (Stetler-Stevenson and Seo, 2005). Therefore, TIMP-1 secreted by MSCs may act as inducer or repressor of specific factors necessary for commitment to specific differentiation pathways of MSCs. MCP-1, which recruits monocytes into ischemic tissue and stimulates chemotaxis, is secreted exclusively from un-induced MSCs. MSCs are described to secrete this chemokine which is speculated to be involved in homing of circulating stem and progenitor cells to the region of injuries and thus contribute to healing (Kinnaird et al., 2004; Liu and Hwang, 2005)

Notably, osteoarthritic chondrocytes secrete MCP-1 and it is suggested that it contributes in triggering inflammatory mechanisms involved in persistence of joint diseases (Pulsatelli et al., 1999). MCP-1 secretion could be used as a marker for un-induced MCSs which becomes attenuated during chondrogenic and osteogenic differentiation processes.

Secretion of VEGFα-164 was induced only after the cells were subjected to osteogenic and chondrogenic differentiation. VEGF influences vascular permeability and is a strong angiogenic protein indispensable for neovascularisation during embryonic skeletal development (Goldring et al., 2006). VEGF as a multifaceted protein may have a role in induction of metalloproteinases during late stage of chondrogenesis marking the onset of hypertrophy (Pufe et al., 2004). Lack of VEGF secretion in undifferentiated MSCs might be taken as an indicator for a relatively pure MSC population moistly devoid of hematopoietic and endothelial progenitor cell contamination. Latter stem cells are known to express and secrete VEGF (Rehman et al., 2003; Iba et al., 2002) The discrepancy in low gene and high protein secretion profile of VEGFα after chondrogenic differentiation is very likely due to post translational regulation. VEGF production is known to be regulated at translational level as overexpression of transcription factor c-Myc related with rapid induction of tumors, also leads to 10 fold higher VEGF production while the gene expression level remains unaltered. The mechanism of action is most probably higher ribosome biogenesis which increases translation efficiency of selected mRNA (Mezquita et al., 2005).

When the same antibody array was examined with the cellular lysates, two additional molecules were identified, ß-NGF was detected in undifferentiated as well as in cells after osteogenic differentiation and cytokine induced neutrophil chemoattractant (CINC-2) was detected only after osteogenesis. CINC-2 attracts neutrophils and has been suggested to inhibit acute inflammation indicating its role is osteogenesis could be anti-inflammatory (Nakagawa et al., 1994). So far this chemkine has not been determined in MSCs but in bone marrow derived macrophages exposed to pro-inflammamtory cytokins (Crippen et al., 1998)

It is known that early non-committed MSCs with high cologenic potential bind to antibodies against NGF receptor (Quirici et al., 2002) and that undifferentiated bone marrow derived cells express NGF together with other neurotrophic factors (Yaghoobi and Mowla, 2006). This is in line with the production of ß-NGF in undifferentiated MSCs. The naïve MSCs loose ß-NGF upon chondrogenic differentiation but keep it under osteogenic conditions which suggests a role of ß-NGF in osteogenesis as a trophic factor stimulating collagen synthesis and expression of specific osteogenic marker as alkaline phosphatase (Yada et al., 1994). In

mandibular distraction osteogenesis elevated expression of NGF has been demonstrated (Park et al., 2006).

In conclusion, we were able to demonstrate gene expression and secretion of molecules from a mostly CD45-negative bone marrow derived cell population which may be crucial for MSCs fate determination and sustenance. Identification and better understanding of the molecular events characterizing differentiation of MSCs which allows discrimination among specific differentiation stages is a prerequisite for manipulation of MSCs to regenerate complex tissues as cartilage and bone.

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Chapter 3

CD 45-positive cells of haematopoietic origin enhance chondrogenic marker gene expression in rat marrow stromal cells

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Abstract

Adult mesenchymal stem cells (MSCs) can be readily isolated from bone marrow, expanded in culture and subsequently subjected to differentiation into various connective tissue lineages. In general, for animal studies separation of MSCs from other bone marrow derived

cells is achieved by sole adherence to plastic surface of tissue culture flasks, however, this procedure produces a heterogeneous cell population containing CD45-positive haematopoietic cells (HCs) and haematopoietic stem cells (HSCs) as well. It is well known, that such mixed cell cultures consisting of cocultures of differentiated somatic cells with adult stem cells promote differentiation towards specific cell lineages. For determining the effect of the CD45-positive cell population on the differentiation potential of MSCs we have sorted out the bone marrow derived adherent cells by immunomagnetic technique (MACS^{TM}) to attain a subpopulation of CD45-depleted cells. Here, we show that the presence of adherent CD45-positive HCs not only promote expression of the chondrogenic marker genes *Col2a1*, *COMP* and *Sox9,* but also of *Col1a1, Col10a1* and to a certain degree *Cbfa1* in MSCs when cultured in an appropriate three-dimensional environment. These observations constitute a step towards unravelling the influence of haematopoietic cells on chondrogenic differentiation of MSCs.

Key words**:** rat marrow stromal cells, MACS, chondrogenic differentiation, haematopoietic cells, CD45

Introduction

Adult bone marrow stromal cells regarded as mesenchymal stem cells are progenitors of connective tissue cells, thus are excellent candidates for chondrogenic differentiation studies. The progeny of adult stem cells includes both daughter stem cells and committed progenitors with a more restricted differentiation potential. These progenitors in turn give rise to differentiated cell types. Mesenchymal stem cells (MSCs) can differentiate under specific cultural and physical conditions into multiple mesenchymal lineages namely, osteocytes, chondrocytes, adipocytes, astrocytes and myocytes (Pittenger et al., 1999b; Cancedda et al., 2003). Bone marrow consists of primarily non-adherent haematopoietic cells and haematopoietic stem cells (99%) along with a minor population of MSCs (≤1 %). Both of the stem cell types are known to co-exist and have been suggested to cooperate in one another's differentiation (Bianco et al., 2001). The role of MSCs in haematopoietic microenvironment formation is beginning to unravel and it is assumed that the presence of adherent MSCs and their progeny facilitates HSCs differentiation into granulocytes and erythrocytes both *in vivo* and *in vitro* (Galmiche et al., 1993; Prockop, 1997a; Majumdar et al., 1998b). Conversely, in

depth studies concerning the role of HSCs in MSCs commitment and differentiation towards a distinct lineage are scarce.

Adult rat MSCs are routinely isolated from tibio-femoral bone marrow by relying solely on their adherence to the plastic surface of tissue culture flasks (Friedenstein et al., 1976; Caplan and Bruder, 2001). The isolated cells constitute a heterogeneous population which always contains HCs therefore; obtaining a pure population of non-haematopoietic cells remains difficult. This haematopoietic cell fraction varies depending on the species; being relatively high in initial cultures of mouse marrow cells (Phinney et al., 1999) and less than 30% in human cell cultures. In rat marrow stromal cells this trend has not been studied thoroughly, although the presence of haematopoietic cells in primary (7 days) and secondary (18 days) bone marrow cultures has been indicated (Herbertson and Aubin, 1997). Human and murine MSC studies show subsequent loss of haematopoietic cell surface markers when cultures are maintained for 2 or 3 weeks. Prior to differentiation experiments the haematopoietic cell population is often separated from MSCs by employing a magnetic associated cell sorting (MACS) system. The system immunologically separates different cell types by labelling cell surface antigens with magnetic beads followed by sorting through a magnetic column. As a unique identifying marker for MSCs is still lacking a negative selection protocol is carried out using CD34 (My10) or CD45 (leukocyte common antigen) for exclusion of undesired positively labelled haematopoietic cells.

One of the major differentiation pathways of MSCs occurs along the chondrogenic lineage which can happen autonomously in three separate mesenchymal cell lines, cranial neural crest, sclerotome cells and lateral plate mesoderm cells (von der, 1999). The first stage of chondrogenic differentiation is conversion of undifferentiated MSCs to committed osteochondroprogenitor cells leading to cell condensation and growth arrest. The major proteins involved in condensation initiation are either fibronectin or belong to the transforming growth factor ß family of proteins (TGFß-1, -2 and -3). Cells present at the centre of the condensation nodules first form pre-chondrocytes and then chondrocytes which start to produce cartilage matrix marked by up regulation of structural protein genes as *Col2a1, Col9a2*, *Col11a2, aggrecan and COMP*. Expression of chondrocyte marker genes is controlled by members of the Sox-family, in particular Sox9 has been characterized as a master transcription factor with essential direct or indirect regulatory effects exerted along the entire chondrogenic differentiation pathway. It is expressed in all chondroprogenitor cells and also in differentiated chondrocytes (Bi et al., 1999; de Crombrugghe et al., 2000b; Mundlos and Olsen, 1997; Lefebvre et al., 2001) .

In this study we have evaluated the degree of haematopoietic cell coexistence in primary cultures of rat MSCs and their influence on the chondrogenic differentiation potential of MSCs. We have determined the difference in chondrogenic marker gene expression of heterogeneous MSCs isolated by sole adherence to plastic surfaces compared to that of a more homogeneous subpopulation of MACS sorted CD45-depleted cells.

Material and Methods

MSC isolation and primary cell culture

A modified version of the MSCs isolation procedure from rat bone marrow was optimized (Dobson et al., 1999; Maniatopoulos et al., 1988). Briefly, 6 week old male Sprague - Dawley rats were killed with CO_2 and tibiae and femora were aseptically removed. The bones were cut from the middle and centrifuged in a 1.5ml tube at 2000 rpm for 3min (mini centrifuge-Eppendorf). The centrifuged bone marrow cells were collected and homogenized with 18g, 21g and 23g needles and seeded at the rate of 2.5 x 10^5 cells/cm^2 in 175cm² tissue culture flasks in proliferation medium containing 5% glutamate, 1% antibiotics/antimycotics (Gibco.) and 10%FBS (Gibco, Invitrogen, UK; Lot No.: 40F7430K) in α-MEM (Sigma Aldrich, Germany). All non-adherent cells were removed on the 3rd day and the adherent cells were proliferated.

Magnetic separation of cells (MACS)

When the flasks were ~75% confluent (9-11 days) cells were harvested with trypsin digestion. Separation procedure was carried out according to the manufactures instructions; in short, cells were incubated with 4µl of CD45 antibody/10^6cells for 5min at 37°C followed by washing and a second incubation with goat anti-mouse secondary antibody coupled with magnetic beads (Miltenyi Biotech) for 15min at 4°C. The suspension was passed through a magnetic column (Miltenyi Biotec´s LS-MACS columns); while labelled cells got coupled to the magnetic field the flow through containing unlabelled CD45-negative fraction was collected.

Immunofluorescence

20,000 cells/chamber were seeded on chamber slides (Biocoat slides, BD, Bioscience) and incubated overnight at 37°C and 5% CO_2. Cells were blocked for 1h at 37°C in Complete Mini 1:5 (Roche, Germany) plus 5% normal goat serum and 1% BSA in PBS. After washing

with PBS cells were stained with monoclonal antibodies directed against CD45 (Cb1 1502, Chemicon), CD68 and D7Fib (Acris, Germany) in 1:50 dilution ratio overnight at 4°C. The appropriate Alexa568 or Alexa488 conjugated secondary antibodies (goat anti mouse, 5 µg/ml; Molecular Probes, US) were added for 1h at RT. After washing slides were permanently mounted with DAKO fluorescent mounting medium (DAKO, USA) and covered with coverslips. Slides were evaluated with scanning laser microscopy (C1 confocal microscope from Nikon) and photos were taken with a Nikon C4 camera and software.

Flow cytometric analysis

Cells obtained from 1 animal (approx. 8x 10^7 cells, as determined after NH_4Cl lysis of the erythrocytes) were seeded in 4x 150cm^2 flasks (5x 10^5 cells/cm^2). The cells were left to adhere 1, 2, 3 or 4 days before non adherent cells were removed. Cells were trypsinized on day 7 after the isolation and resuspended in PBS. The cell suspension was first blocked for 15 min at 4°C with sheep serum followed by incubation with antibodies against rat CD45-PE (mouse IgG1 against rat CD45, Acris Antibodies, Germany), CD29-FITC (Hamster IgM against rat CD29, BD Pharmingen, USA) and with corresponding isotype controls for 30min at 4°C. Cells were washed with PBS containing sodium azide and bovine serum albumin and resuspended. Shortly before the FACS – analysis propidium iodide was added to each sample in order to discriminate the dead cells which were excluded from the evaluation. All measurements were performed on a FACSCalibur® instrument (Becton Dickenson, USA) with CellQuest data acquisition software (Becton Dickenson, USA). Data analysis was preformed with WinMDI 2.8 software (free access on http://facs.scripps.edu/software.html). A minimum of 1x10^4 viable cells was acquired per data set.

Chondrogenic differentiation

A batch of adherent cells (undepleted) was subjected directly to chondrogenesis favourable condition, the other batch was depleted of CD45-positive cells by MACS prior to chondrogenic differentiation experiments. For chondrogenic differentiation, high density 3-D cultures were attained by preparing cell-alginate amalgam in 1.2% alginate at a concentration of 10^7 cells/ml. 2-3mm cell-alginate beads containing approx.10^5 cells/bead, were formed by dropping the suspension into 102 mM $CaCl_2$ solution. The culture was carried out in 12 well tissue culture plates for 4 weeks; medium was changed every 2-3 days. 10 alginate beads were removed every 7^{th} day for RNA isolation and gene expression analysis. To release the cells from alginate the beads were incubated for 30min at 37°C on a shaker in 55mM sodium

citrate and 0.15M sodium chloride buffer; cells were recovered after 3min spin at 750xg. The chondrogenic medium contained; ITS+ premix (6.25µg/ml insulin, 6.25ng/ml selenium acid, 6.25µg/ml transferrin, 1.25mg/ml BSA and 5.35µg/ml linoleic acid. BD Biosciences, USA.), 110µg/ml pyruvate, 40µg/ml proline, 0.1µM dexamethasone, 50µg/ml ascorbic acid and 10ng/ml TGFß-3 (Johnstone et al., 1998) (R&D Systems) in α MEM high glucose (Gibco). Alginate sodium salts were acquired from Sigma Aldrich, Germany (Cat# A0682-100G).

RNA Isolation and reverse transcription

RNA was isolated by an affinity column chromatography method with Ambion's RNAqueous4-PCR kit according to the manufacturer's protocol. For removal of possible DNA contamination, isolated RNA was incubated for 1 h at 37°C in 2 Units of *DNase*I enzyme (DNA-*free*, Ambion). RNA integrity was determined on 0.8% Agarose-formaldehyde gels. The RiboGreen RNA quantification kit (Molecular Probes) was used for determination of RNA concentration at 585nm wavelength. For RNA conversion to cDNA, Invitrogen´s SuperScript II reverse transcriptase kit was used with 0.5-1µg of RNA in 20µl of total reaction volume in presence of 40units/µl recombinant ribonuclease inhibitor (RNase OUT®). Reverse transcription was carried out with 500µg/ml of Oligo-dT primers, 10mM dNTPs and 200 units of SuperScriptII enzyme in First-Strand Buffer and 0.1M DTT for 50min at 42°C followed by an extension period of 15min at 70°C.

Relative quantitative PCR

Relative quantitative PCR was carried out with SYBR Green Dye I on ABI 7000 Prism Sequence detection system (Applied Biosystems) according to manufacturer's instructions. Briefly, 1µl of cDNA was amplified in 50µl final volume of 0.2µM of each primer suspended in SYBR green master mix (AB Systems).Amplification parameters were same for all primer pairs and were repeated for 40 cycles, denaturation at 95°C for 0.15min and annealing at 60°C for 1min. Mean relative quantification (RQ) value evaluated from three independent experiments was plotted on a semi logarithmic graph. RQ values were calculated by the software "RQ study application v1.1" (ABI Prism 7000 SDS software v1.1) according to the ΔΔCt method using ß-Actin as endogenous control and undifferentiated MSCs (day 0) as calibrator. Primers were designed with either Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or "Primer Express" software supplied by AB. All primers were manufactured at MWG – Biotech and are listed in table 1. Prior to RQ studies, a CT value (cycle threshold) standard curve, with 10 folds diluted cDNA, was plotted to determine

PCR efficiency of each primer pair only 90-100% efficient primers were used. The efficiency was determined with $10^{(1/-S)} - 1$, where S is the slope of the curve (Ginzinger, 2002).

Statistical Analysis

One way ANOVA and student t test were employed for quantitative PCR experiments performed in triplicate. Results obtained by flow cytometry are the average of 4 independent experiments ± standard error of mean. The level of significance was determined by Turkey´s test with 2 group comparison.

Table 1: Primers used for quantitative PCR

Gene / RefSeq#	Amplicon (bp)	Primer (5´ - 3´)
ß-Actin	104	forward -GTAGCCATCCAGGCTGTGTT-3´
NM_031144		reverse -CTGTAGGTGAATCGACTGTTGC-3´
Col1a1	59	forward -TCCAGGGCTCCAACGAGA-3´
RGD61817*		reverse -CTGTAGGTGAATCGACTGTTGC-3´
Col2a1	60	forward -CCCCTGCAGTACATGCGG-3´
NM_012929		reverse -CTCGACGTCATGCTGTCTCAAG-3´
Col10a1	247	forward -CCC TAT TGG ACC ACC AGG TA -3´
AJ131848*		reverse -TCT CTG TCC GCT CTT TGT GA -3´
COMP	167	forward -TGACTTCGATGCTGACAAGG-3´
NM_012834		reverse -GAACGATCTCCATTCCCTGA-3´
Sox9	140	forward -CTGAAGGGCTACGACTGGAC-3´
XM_343981		reverse -TACTGGTCTGCCAGCTTCCT-3´
Cbfa1	86	forward -GGCCGGGAATGATGAGAACTA-3´
XM_34016		reverse -AGATCGTTGAACCTGGCCACT-3´

*Locus ID

Results

MSC isolation procedure and CD expression profile

To determine the influence of adherence time of rat bone marrow cells on the presence of CD45-positive haematopoietic cells we left the cells to adhere for 3, 4 and 5 days before non-adherent and weakly attached cells were removed. Upon immunofluorescence analysis the adherent cells showed a D7fib^{+}, CD68^{+} and CD45$^{+/low}$ expression profile regardless of the time of removal of non-adherent cells. Figure 1 shows the representative immunomicrographs of the cells left to adhere for 3 days and compares the staining of non-sorted and MACS-sorted cells after the first passage. Non-sorted cells, left for 3 days to adhere before medium removal, are contaminated with CD45-positive cells (Fig.1A-C). After magnetic sorting, most of the CD45-positive cells were excluded, leaving a CD45-depleted cell fraction (Fig.1D-F). However, we still observe few rounded cells among the CD68 positively stained fraction (Fig.1D) of unknown surface antigen characterization. The CD45-positive cell fraction includes cells which stain for CD68 and D7Fib (Fig.1G-I).

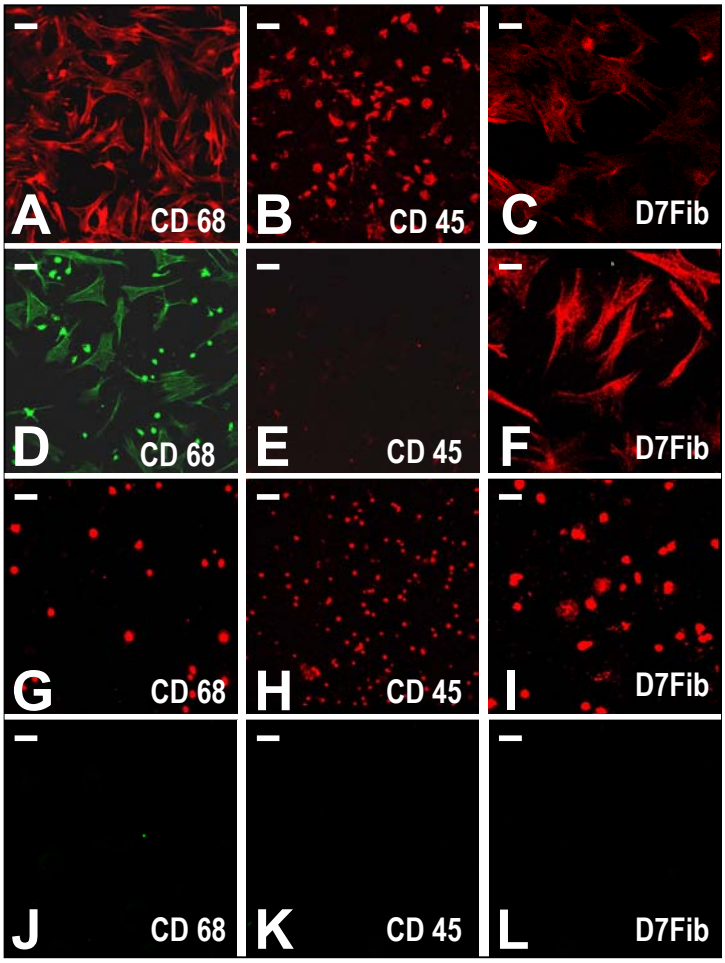

Figure 1: Morphological characterization of MSCs Immunofluorescence imaging of heterogeneous adherent MSCs (A, B, C) and CD45-depleted MACS sorted cells (D, E, F), showing typical fibroblast like spindle shaped cells, while CD45-positive cells (G, H, I) are small and rounded in appearance. The negative controls for all three antibodies used are unstained (J, K, L). All 3 cell types are CD68- and D7fib- positive while the CD45-depleted cell population does not exhibit CD45 harbouring cells (E). Magnification is 20x for all panels, bars are 10µm.

Quantification of CD45-positive cell contamination of primary MSC cultures

In order to quantitate the ratio between haematopoietic CD45-positive cells and MSCs we employed three colour flow cytometry (FACS) which allowed for a distinction between the two different cell types according to their surface antigens (CD45, CD29) and dead cells (Fig.2A).

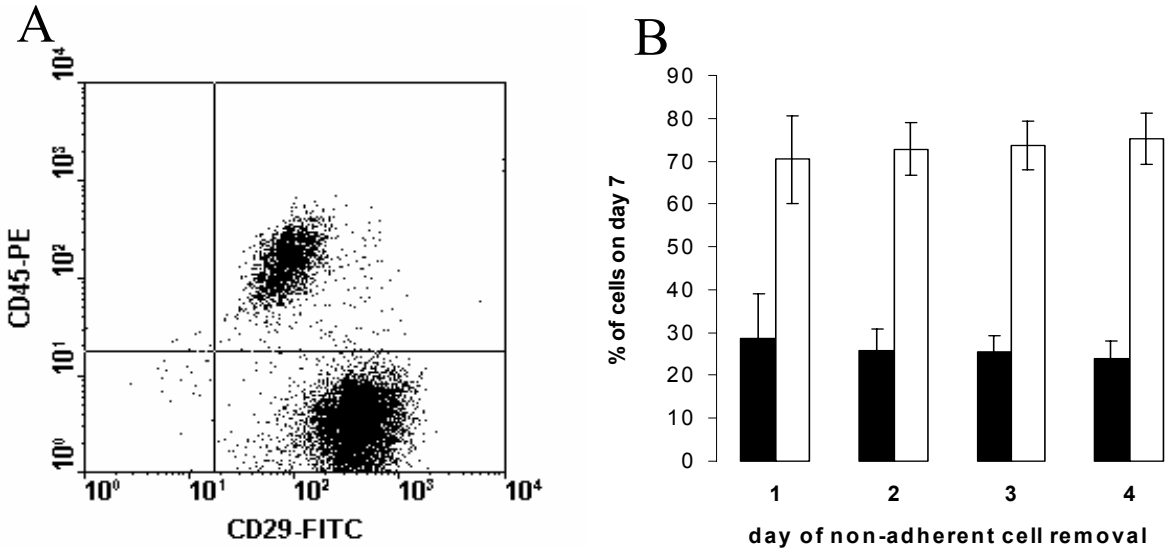

Figure 2: FACS analysis of bone marrow derived stem cells prepared by adherence to plastic Representative dot-plot (out of 3 experiments) of rat MSCs isolated by adherence to plastic after 7 days of cell culture. The lower right quadrant contains CD29 –highly positive and CD45-depleted MSCs while the upper right quadrant contains HCs with a CD45 – positive and CD29-low expression profile (A).
Proportion of CD45-positive (black bars) and CD45-depleted (open bars) cells from rat MSCs isolated by adherence to plastic in relation to the time of the first medium change and therefore, removal of non-adherent cells (B).
Total yield of CD45-depleted (open bars) and CD45-positive (black bars) bone marrow derived cells after 7 days of cell culture (*p< 0,05; **p< 0,01). Yield of cells is normalized to cell number achieved when the non-adherent cells were removed on day 4 (C).

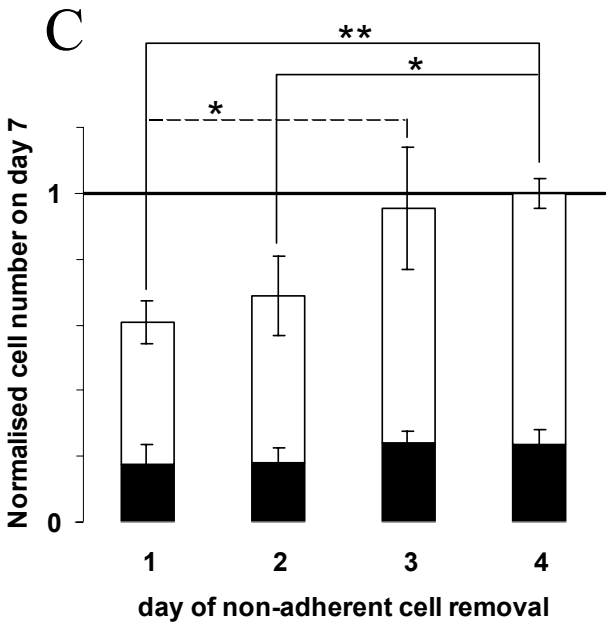

Figure 2B shows that the fraction of CD45-positive haematopoietic cells was not influenced by the adherence time after cell isolation. We mostly found about 20-30 % of the attached cells in bone marrow cell cultures to be CD45-positive when selected according to sole adherence to plastic surfaces. Flow cytometric results were concurrent with the observations made after immunofluorescent staining. In one out of four experiments we detected variations between 10 - 40% in individual flasks. We observed that CD45-depleted cells expressed CD29 stronger than CD45-positive cells (Fig.2A) which offers an additional selection parameter. In cultures where non-adherent cells were removed on days 3 and 4, we obtained a significantly increased number of desired CD45-depleted MSCs compared to removal on day 1 and 2 (Fig.2C). Apparently, 3 to 4 days were a more appropriate duration for the adherent fraction to get acclimated to the cell culture conditions, hence exhibit a firmer attachment to the cell culture plastics.

Effect of CD45-positive cells on gene expression levels of *Col1a1, Col2a1, Col10a1, COMP, Sox9* and *Cbfa1*

During the time course of cell culture we observed a strong increase in relative mRNA levels of COMP and collagen II, while Sox9 mRNA levels exhibited a moderate up regulation (Fig.3A, B, D). Collagen X gene expression remained undetectable the first three weeks of cell culture, however, at day 28 expression of this collagen was found to be induced (Fig.3C). The internal control gene, ß-Actin, remained unaltered during the complete time course of cell culturing (Fig. 3G). Comparing gene expression of CD45-depleted cells and the non-sorted population, we found collagen I gene expression to be increased in the non-sorted cell population during the culture period, while it became decreased in CD45-depleted MSCs. However, in CD45-depleted cells collagen I was still expressed at a relative higher level as in freshly isolated hip articular chondrocytes (Fig.3E). Notably, the non-sorted cells displayed a strikingly higher up regulation of collagen II, COMP and Sox9 gene expression compared to the CD45-depleted MSCs throughout the culture period. In the non-sorted population collagen II gene expression is up regulated at average about 25 fold, COMP gene expression about 10 fold, collagen X expression about 23 fold and Sox9 mRNA levels about 3 fold if compared to CD45-depleted MSCs. For collagen I gene expression we have observed a significant difference only between the two groups at day 14 and 28, where the adherent MSCs demonstrated an up regulation resulting in a 3-5 fold higher gene expression if compared to CD45-depleted MSCs.

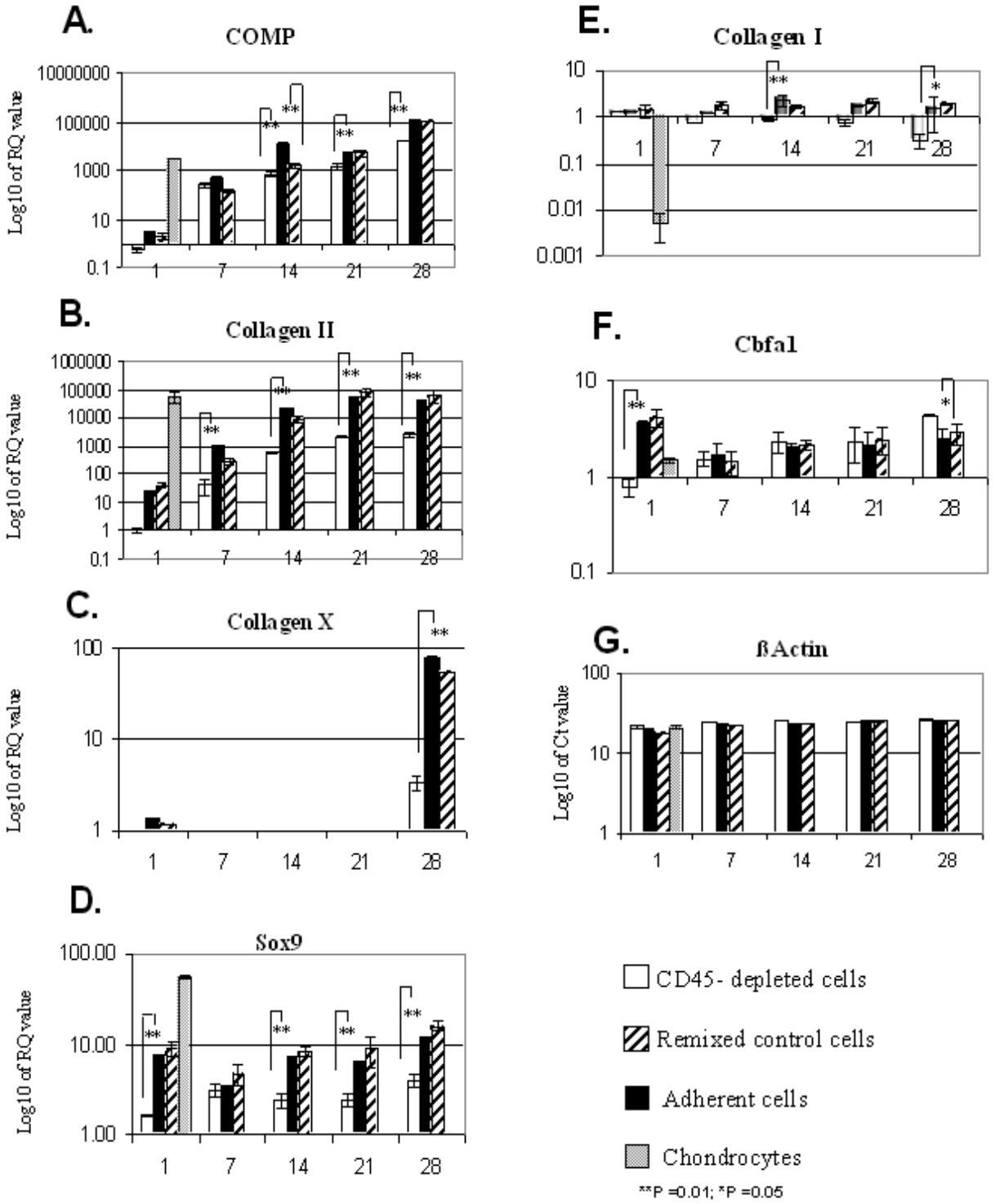

Figure 3: Chondrogenic gene expression profile of adherent MSCs, CD45-depleted MSCs and remixed control cells. Relative quantitative mRNA expression level of chondrogenesis specific genes as *COMP* (A) *Col2a1*(B) and *Col10a1* (C), *Sox9* (D), *Col1a1*(E), and *Cbfa1* (F) was determined by employing the ΔΔCT method using SYBR I Green dye. Expression level of housekeeping gene, *ß-actin* was determined as a controlling indicator of cell viability (G) and is expressed as positive logarithm of CT-values. Expression level of each gene was compared between adherent (black bars), CD45-depleted (open bars) and remixed control cells (crossed bars). Grey shaded bars indicate relative gene expression level of freshly isolated hip articular chondrocytes. Number of experiments = 3

Collagen I remained up regulated from day 14 on in adherent cells while CD45-depleted MSCs exhibit an increasingly down regulation during the time course of cell culture. We also observed a striking difference in the collagen X expression pattern between non-sorted and CD45-depleted cells. The collagen X mRNA expression profile of CD45-depleted cells experienced only a moderate increase of about 3.3 fold at the end of the culture period while adherent cells up regulated their mRNA level about 76 fold compared to day 0 (Fig. 3C). Notably, Cbfa1 gene expression is differentially regulated with respect to the two cell populations (Fig.3F). On day 1 Cbfa1 mRNA level is significantly higher in adherent cells (4.7 fold) and also in chondrocytes (ca. 2.0 fold) compared to CD45-depleted cells. This pattern became inverted at day 28 with CD45-depleted cells exhibiting an elevated expression (1.7 fold) compared to the adherent MSCs. In between these time points, relative gene expression of Cbfa1 is similarly regulated in both cell populations.

In remixed control cell populations (30% CD45-positive cells with 70% CD45-depleted cells) collagen II, X, I, Sox9 and Cbfa1 gene expression profile resembles closely that of adherent cells (Fig.3B, C, D, E, F) and exhibits no significant differences. Only COMP gene expression at day 14 of the culture period significantly differs from adherent cells but the level at day 21 and 28 shows no difference to non-sorted cells (Fig. 3A). ß-actin gene expression profile is similar for all three culture conditions (Fig.3G).

Discussion

Adult bone marrow is the major source of haematopoietic stem cells and mesenchymal stem cells (Pittenger et al., 1999a). In animal studies, adherence to tissue culture plastic is accepted as a satisfactory parameter for attaining marrow stromal cell population depleted of haematopoietic cells. However, when employing FACS analysis for surface antigen characterization we observed a contamination of these non-sorted MSCs with 20-30% of CD45-positive cells. For additional characterization of sorted and non-sorted cell population we applied immunofluorescence staining for CD68 and D7Fib. The D7Fib antigen constitutes a fibroblast specific molecule of yet unknown function, supposedly a specific marker for MSCs as suitable as STRO-1 and CD105 (Jones et al., 2002). We observed expression of the D7Fib antigen on adherent CD45-positive cells; however, these cells displayed a rounded morphology compared to the fibroblast-like CD45-depleted cells. CD68 and its murine analogue macrosialin, a member of the lamp family, appear to have a rather limited tissue distribution, being found on macrophages, Langerhans cells and dendritic cells (Ramprasad et

al., 1996). Notably, we have found all adherent cells CD68-positive suggesting that in non-committed, undifferentiated cells, this protein might exhibit a different distribution pattern and possibly exert functions related to cell–cell or cell–ligand interactions (Fukuda, 1991).

CD45-positive haematopoietic cells were always detected in MSC cultures, regardless of the time of the first change of culture medium. This indicated a persistent presence and attachment of CD45-positive cells in the MSC environment when no further selection based on specific surface antigens is carried out. Adherence to the tissue culture plastic alone is a weak discriminating criterion for a homogeneous MSC population, because neural cells, monocytes and macrophages are also known to be isolated according to their adherence to plastic surfaces (Delles et al., 2002; Kiefer et al., 1991; Majumdar et al., 1998a). The presence of CD45-positive haematopoietic cells and the variation of their proportion in primary cultures of MSCs could explain the occurrence of extensive variations usually seen in differentiation experiments with bone marrow derived cells.

The fact, that CD45-depleted cells expressed CD29 stronger than CD45-positive cells offered us an easier separation of two populations and limited the possible existence of more than two major subpopulations in our cultures. The integrin ß1 subunit CD29 exhibits a broad tissue distribution, including lymphocytes, endothelia, smooth muscle, and epithelia (Springer, 1990) and plays an important role in cellular processes, e.g. embryogenesis and HSC development. Although we did not find the haematopoietic cell fraction to vary according to the time of removal of non-adherent cells we observed a correlation between adhesion time and yield of CD45-depleted marrow stromal cells. In parallel, cell adhesion plus proliferation could also be promoted by the prolonged presence of the non-adherent fraction via cytokine release and / or cell-cell contacts. A comparable effect of haematopoietic cells on MSC proliferation has been observed for megakaryocytes and platelets (Castro-Malaspina et al., 1981). According to our results we decided to leave the cells to adhere for 3 days before performing differentiation experiments.

Differentiation studies of haematopoietic stem cells have demonstrated, that the small number of adherent MSCs present in total bone marrow aspirates provide an important microenvironment for growth of HSCs and their differentiation into several blood borne cell types. In long-term cultures of HSCs, these adherent cells even interact directly with the haematopoietic precursors (Prockop, 1997b). Here, we could show that vice versa adherent CD45-positive haematopoietic precursor cells create a microenvironment which may enhance expression of particular marker genes in MSCs. For studying the effects of contaminating CD45-positive cells we prepared a relatively homogeneous population of CD45-depleted

MSCs by immunogenic depletion of CD45-positive cells. Both, the heterogenic adherent cells and the CD45-depleted subpopulation were subjected to chondrogenic favourable 3-D environment by formation of a cell alginate amalgam. For control purposes in order to assure that difference in gene expression profile between sorted and unsorted MSCs is due to depletion of CD45-positive cells and not because of general depletion of differentiation competent cells or due to cell damage caused by the sorting process, we prepared a third population of remixed cells (30% CD45-positive mixed with 70% CD45-depleted cells) following MACS preparation which was treated according to the differentiation protocol used for adherent and sorted cells.

We monitored relative gene expression of collagen II (Mendler et al., 1989) and COMP (Hedbom et al., 1992; DiCesare et al., 1994) as examples for structural extracellular matrix proteins highly specific for hyaline cartilage. Additionally, we have investigated Sox9 as a master transcription factor imperative for chondrogenic differentiation (de Crombrugghe et al., 2000a) and collagen I as differentiation control for a gene normally not active in healthy hyaline cartilage (Rossert et al., 2000) but occurring together with collagen II in fibrocartilage (Nerlich et al., 1998). Gene expression of collagen X, specific for terminal differentiated hypertrophic chondrocytes in the calcifying zone of cartilage and secondary ossification centres within the growth plate (Schmid et al., 1990) indicates maturation of MSCs towards an undesired cartilage condition.

Gene expression of Cbfa1, a transcriptional activator for osteoblastic differentiation during the process of endochondral ossification is analyzed for monitoring differentiation towards osteogenic condition. Cbfa1 is also expressed in pre-condensation stage during early development in a cell type with the potential to become either a chondrocyte or an osteoblast. In later developmental stages expression of this transcription factor is restricted to prehypertrophic and hypertrophic chondrocytes where it acts as a hypertrophic differentiation inducer (Karsenty, 2001; Takeda et al., 2001).

The observed gene expression profile indicates not only a beneficial influence of CD45-positive cells on the differentiation process of MSCs towards the chondrogenic lineage. It also shows that when culture time is extended beyond 28 days chondrogenically differentiating MSCs might enter the prehypertrophic / hypertrophic differentiation cascade and proceed towards terminal differentiation. Interestingly, usage of chondrogenic culture medium does not prevent up regulation of Cbfa1 and collagen X. This indicates either a not yet defined influence of medium components (i.e. TGF-ß or dexamethasone) on intrinsic cell derived factors (i. e. signalling molecules, proteases, transcription factors) which are involved in

regulation of the chondrogenic differentiation cascade or a medium independent process occurring by default through paracrine interaction of MSCs. A variety of growth factors i.e. members of the TGF-ß superfamily, IGF-1 and bFGF could account for gene modulating effects in our system. (Bohme et al., 1995) (Recklies et al., 1998) (Murakami et al., 2000) (Horton, Jr. et al., 1989).

In conclusion, we demonstrated that bone marrow derived fibroblast like CD45-depleted rat MSCs share a heterogeneous cellular environment with ~20-30% CD45-positive HCs, if no other selection procedure than adherence to plastic is employed. Our differentiation results indicate that adherent CD45-positive haematopoietic cells may create a microenvironment promoting differentiation of MSCs towards the chondrogenic lineage by up regulating specific genes. However, expression of undesired genes as collagen I and collagen X is likewise promoted by HCs. On the contrary, at early condensation stage HCs strongly increase gene expression of Cbfa1 thus supporting chondrogenic differentiation of MSCs, while at a later differentiation stage when Cbfa1 plays an integral role in hypertrophy the regulation appears to be independent of the presence of HCs.

We speculate that the stromal CD45-positive population *in toto* is responsible for regulation of gene expression. The heterotrophic nature of bone marrow derived cells suggest that *in vivo* interactions of different cell types is very likely and should be addressed accordingly. Characterization of additional subpopulations of CD45-positive cells according to their surface antigens and determination of their potential influence on gene expression may elaborate this point further more. Studies using conditioned media are needed to clarify whether physical contact between cells or paracrine factors, alone or in concurrence, are necessary for promoting gene expression leading to chondrogenic differentiation and maturation of MSCs. Identification and isolation of factors which are capable of modulating genes involved in differentiation and dedifferentiation processes of chondrocytes is vital for *in vitro* engineering of cartilage tissue repair.

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Chapter 4

Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of adult marrow stromal cells

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Abstract

Limited regeneration capacity of cartilage is mainly due to lack of access to progenitor cells. The only known chondroprogenitor cells for adult cartilage tissues are multipotent marrow

stromal or mesenchymal stem cells (MSCs). *In vitro* lineage differentiation studies on these precursor cells usually require a cue from externally added growth factors of the TGFß superfamily. We hypothesise that signalling factors secreted by cartilage tissue may substitute for external growth factors. To study such paracrine communication between allogenic rat articular cartilage and MSCs embedded in alginate beads a novel coculture system without external growth factors has been established. Successful chondrogenic differentiation in MSCs was induced and the impact of cartilage on differentiating MSCs was observed at two culture stages. Firstly, elevated expression of Sox9 at the initial stage indicated early induction of chondrogenic differentiation. Secondly, late stage repression of collagen X indicated arrest of differentiation prior to hypertrophy. Four soluble signalling molecules were identified in the culture supernatant: vascular endothelial growth factor alpha (VEGF-164α), matrix metalloprotease (MMP) -13 and tissue inhibitors of MMPs (TIMP-1 and TIMP-2). These factors could be traced back either directly to the cartilage tissue or to the MSCs influenced by the presence of cartilage. The identified factors may have a mutual impact on the control and regulation of differentiation of both the chondrocytes and MSCs.

***Key words*:** rat mesenchymal stem cells, chondrogenic differentiation, coculture, signalling factors, cartilage, MMP-13, collagen X

Introduction

A defect in articular cartilage caused by aging, chondral injury or a degenerative disease is seldom repaired, causes persistent pain and restricts mobility. Generally, the avascular nature of cartilage is held responsible for the lack of spontaneous rejuvenation; more specifically it is probably due to the deficiency of chondroprogenitor cells (Magne et al., 2005). As chondrocytes are the only cell type of cartilage tissues and bone marrow derived MSCs are the only known chondroprogenitor cells, it is in interest of cartilage regenerative medicine to understand and identify molecular events characterising their growth and differentiation (Lefebvre and Smits, 2005).

Under specific culture conditions and proper stimulation MSCs can differentiate into multiple mesenchymal lineages including osteocytes and chondrocytes. The first stage of chondrogenic differentiation is conversion of undifferentiated MSCs to committed osteo-chondroprogenitor cells leading to cell condensation and growth arrest. The major proteins involved in condensation initiation are fibronectin and proteins of the transforming growth factor ß

superfamily (TGFß1-3 and BMP1-7) (Barry et al., 2001). Cells present at the centre of the condensation nodules first form pre-chondrocytes and then chondroblasts / chondrocytes which start to produce cartilaginous extracellular matrix (ECM) marked by upregulation of structural protein genes as *col2a1, col9a2*, *col11a2* and *aggrecan*. Collagens make up to 60% of the hyaline cartilage protein bulk; among them the most dominant (90-95%) form of cartilaginous collagen is collagen type II which forms a fibrillar alloy structure together with type XI and IX collagens and non-collagenous proteins (Mendler et al., 1989). Gene expression of collagen type X which according to the concept of endochondral ossification indicates the final step of chondrocyte maturation serves as a marker for hypertrophic and terminally differentiated chondrocytes (Reddi, 1994).

The molecular basis of regulatory mechanisms involved in chondrogenic lineage signalling cascade are not sufficiently understood, however, they are known to involve numerous growth factors, proteases and cytokines in a strictly controlled time dependent manner. Major players imperative for proper skeletogenesis belong to proteases of MMP-family and growth factors of VEGF family. For example, activity of MMPs and their endogenous inhibitors (TIMPs) is not only fundamental to skeletal growth and repair, but also to their pathology. One member of this large family of matrix degrading MMPs, MMP-13 is predominantly expressed both, in osteoarthritic cartilage and in hypertrophic and calcifying cartilage of the mammalian growth plate (Wu et al., 2002; Johansson et al., 1997). Together with other collagenases, and their endogenous inhibitors like TIMP-1, MMP-13 plays a significant role in collagen turnover by cleaving collagen II, additionally, it also degrades collagen X (Knauper et al., 1996; Mitchell et al., 1996). VEGF, expressed by hypertrophic chondrocytes, is considered as an essential coordinator of chondrocyte death, chondroclast function, ECM remodelling, angiogenesis and bone formation in the growth plate thereby hold responsible for proper endochondral ossification (Gerber et al., 1999). Little is known how these soluble factors affect differentiation pathways decisions and integration processes of chondroprogenitor MSCs.

Chondrogenesis is controlled by several transcription factors, important players are members of the Sox- and Cbfa- family. In particular, Sox9 has been characterized as a master transcription factor with essential direct or indirect regulatory effects along the entire chondrogenic differentiation pathway. It is expressed in all chondroprogenitor cells and in differentiated chondrocytes but not in hypertrophic cells (Bi et al., 1999; de Crombrugghe et al., 2000; Mundlos and Olsen, 1997; Lefebvre et al., 2001). Direct control of hypertrophy involves Cbfa1, also known as RUNX2, the major osteogenic transcription factor. Expression of Cbfa1 increases during maturation of chondrocytes and is necessary for inducing gene

expression of collagen X (Inada et al., 1999; Goldring et al., 2006) and MMP-13 in terminally differentiated chondrocytes (Porte et al., 1999). For a normal skeletal development and maintenance a co-ordinated regulation of these chondrogenic differentiation associated genes is critical.

This *in vitro* study aimed to identify soluble signalling factors mediating a paracrine cross talk of differentiated cartilage tissue with undifferentiated MSCs. We have designed and set up a co-culture system of rat MSCs and allogenic articular cartilage chips under serum-free chondrogenesis favourable 3D- conditions. Our results indicate that externally added growth factors are dispensable for the induction of the chondrogenic differentiation pathway in rat MSCs embedded in alginate beads. Coculture of MSCs with differentiated cartilage induces up regulation of *Sox9* gene expression in MSCs and causes suppression of collagen X biosynthesis and secretion presumably mediated by cartilage secreted factors. Using zymographic techniques and an antibody array we have detected VEGF-164α, TIMP-1, -2 and MMP-13 in the coculture supernatant. These molecules originate in part from cartilage explants and could be important players in regulation and control of chondrogenic differentiation.

Materials and methods

Cell isolation, coculture and differentiation

MSCs from rat bone marrow were isolated as described earlier (Ahmed et al., 2006c). Briefly, bone marrow removed by centrifugation from tibiae and femora of 6 weeks old male rats was seeded at a concentration of 2.5x 10^5 cells/cm^2 and proliferated in α MEM medium containing 5% glutamate, 1% pen/strep and 10% FBS (Invitrogen, UK). Cells from ~75% confluent flasks were harvested and high density 3-D cultures were attained by preparing a cell-alginate amalgam in 1.2% alginate (Sigma, USA) at a concentration of 10^7 cells/ml. Alginate beads of 10^5 cells/bead were formed by dropping the suspension into 102mM $CaCl_2$ solution and 10 beads per insert (1µm pore size, BD Bioscience, USA) were placed in 12-well tissue culture plates. One hip cartilage explant cut in 3 pieces was placed in the bottom well. The culture was carried out for 3 weeks in serum-free chondrogenic medium with medium change every 2-3 days. The chondrogenic medium contained ITS+ premix (6.25µg/ml insulin, 6.25ng/ml selenium acid, 6.25µg/ml transferrin, 1.25mg/ml BSA and 5.35µg/ml linoleic acid; BD Biosciences, USA), 110µg/ml pyruvate, 40µg/ml proline, 0.1µM dexamethasone, 50µg/ml

ascorbic acid (Johnstone et al., 1998) in α MEM high glucose (Invitrogen, UK). Additionally, 10ng/ml TGFß-3 (R&D Systems, USA) was added to the culture medium of control monocultures. Samples of cells, tissue and culture supernatant were taken once a week for gene and protein expression analysis. Alginate beads were depolymerised by 30min incubation in 55mM sodium citrate and 0.15M sodium chloride buffer at 37°C on a shaker; cells were recovered after 3min centrifugation at 750xg. Osteogenesis was induced for 19 days in 6 well culture plates, 250,000 cells/well were cultured in osteogenic medium containing, 10% FCS, 1% pen/strep, 10nM dexamethasone, 50µg/ml ascorbate 2-PO_4 and 10mM ß-Na glycerophosphate. At the end of the culture time cells were fixed with methanol for 10min and stained for 2min with 1% Alizarin Red.

RNA isolation, reverse transcription and quantitative PCR analyses

All molecular biology techniques were carried out as described earlier (Ahmed et al., 2006b). Briefly, RNA was isolated by an affinity column chromatography method with Ambion's RNAqueous4-PCR kit according to the manufacturer's protocol. Contaminating DNA was removed with *DNase*I enzyme (DNA-*free*, Ambion, USA). Invitrogen´s SuperScript II reverse transcriptase kit was used for conversion of 0.5-1µg of RNA into cDNA with 500µg/ml of oligo-dT primers and 200 units of SuperScriptII enzyme. Relative quantitative PCR was carried out in triplicate with SYBR Green Dye I master mix using an ABI 7000 Prism Sequence detection system (Applied Biosystems, USA). Amplification parameters were as follows, denaturation at 95°C for 15sec, annealing at 60°C for 1min in 40 cycles repeats. Relative quantification (RQ) values were calculated by using ABI Prism 7000 SDS software v1.1 employing the ΔΔCt method with ß-actin as endogenous control and undifferentiated MSCs freshly suspended in alginate (day 0) as calibrator. Mean RQ values were plotted on a semi-logarithmic graph. Primers (table 1) were designed with either Primer3 or "Primer Express" software (Applied Biosystems, USA). All primers were manufactured at MWG – Biotech, Germany.

Immunofluorescence

Alginate beads were incubated for 30min in 100mM $BaCl_2$ for irreversible polymerization (Mok et al., 1994). Then the beads were fixed with 3% paraformaldehyde (PFA), sequentially dehydrated with alcohol and then embedded in paraffin. 4-8µm sections were cut with a microtome (Leica, Germany) and 2 sections per slide were mounted. Slides were fixed again in 3% PFA and submerged in xylol for 5min to remove paraffin. Sections were sequentially

rehydrated and incubated for 1h in blocking buffer containing PBS with 1% BSA. 1:50 dilution of collagen II polyclonal antibody (R1039, Chemicon, USA) was used for overnight incubation followed by incubation with Alexa488 conjugated secondary antibodies (goat anti rabbit, 5µg/ml; Molecular Probes, US). Slides were permanently mounted with DAKO fluorescent mounting medium (DAKO, USA) and evaluated with scanning laser microscopy using a C1 confocal microscope (Nikon, Germany). Photos were taken with a Nikon C4 camera and analyzed with the associated software.

Table 1 Primers used for quantitative PCR

Gene / RefSeq#	Amplicon (bp)	Primer (5´ - 3´)
ß-Actin	104	forward -GTAGCCATCCAGGCTGTGTT-3´
NM_031144		reverse -CCCTCATAGATGGGCACAGT-3´
Cbfa1	86	forward -GGCCGGGAATGATGAGAACTA-3´
XM_34016		reverse -AGATCGTTGAACCTGGCCACT-3´
Col1a1	59	forward -TCCAGGGCTCCAACGAGA-3´
RGD61817*		reverse -CTGTAGGTGAATCGACTGTTGC-3´
Col2a1	60	forward -CCCCTGCAGTACATGCGG-3´
NM_012929		reverse -CTCGACGTCATGCTGTCTCAAG-3´
Col10a1	247	forward -CCC TAT TGG ACC ACC AGG TA -3´
AJ131848*		reverse -TCT CTG TCC GCT CTT TGT GA -3´
Sox9	140	forward -CTGAAGGGCTACGACTGGAC-3´
XM_343981		reverse -TACTGGTCTGCCAGCTTCCT-3´

*Locus ID

Collagen isolation

Secreted collagens were isolated from the culture supernatant after pepsin digestion (Bruckner et al., 1989b). 1ml of medium was incubated for 2h on a rotary mixer with 10mg/ml pepsin followed by overnight salt precipitation in 4.5M NaCl. After centrifugation at 14,000xg for 30min at 4°C the protein pellet was precipitated overnight with absolute ethanol at -20°C. After a second centrifugation step the protein pellet was dissolved in 60µl of loading dye containing 0.8M urea. Samples were separated under denaturing conditions on a 4.5%-15% gradient SDS-PAGE gel followed by Coomassie Brilliant Blue R250 staining (Serva, Germany).

Pulse-Chase

After 21 days in chondrogenic favourable environment alginate suspended cells cultured in the presence of TGFß-3 were metabolically labelled for 1h in labelling DMEM-HG medium without methionine and cystein (#21013-024, Invitrogen, UK), as described previously (Grassel et al., 1996). Briefly, cell culture inserts containing the alginate beads were removed from cocultures and pre-incubated in the labelling medium without radioactive isotopes for 1h. Afterwards beads were labelled in absence of cartilage with freshly added 2ml labelling medium plus 3.7MBq of [^{35}S]- methionine and cystein (Tran ^{35}S- label from Qbiogene/ICN-USA) for 1h at 37°C (pulse). Labelling medium was then removed and after 2 washing steps with PBS the labelled cells were placed back in the culture vessel; in case of coculture together with cartilage. Newly secreted collagens were chased by continuing the culture in chondrogenic medium for 4h at 37°C. 1ml cell culture supernatant was retrieved after 0.5h, 1h, 2h and 4h of chase time. Secreted collagens were extracted, as explained above and samples (each ~155 cpm) were separated on a 4.5-15% gradient SDS-PAGE under reducing conditions followed by Coomassie Brilliant Blue R250 staining. The destained and fixed gels were incubated for 30min in Amplify™ solution (Amersham Biosciences, UK), dried and developed for max. 21 days at -80°C. The radiographic images (Hyperfilm™ MP- Amersham Biosciences, UK) were acquired with CURIX 60 film developer (Agfa, Germany).

Immunoblotting

Western blotting was carried out with MSCs, cartilage explants and alginate cell fraction lysates. 0.5 mg cartilage explants were pulverized in a freezer mill and the finely powder extract was treated similar as the cells. Briefly, ~10^6 cells were lysed by suspending the cell pellet in 80µl of 250mM Tris buffer (pH 6.8) containing 6% EDTA, 4% SDS, 40mM DTT, 20% glycerine, 0.002% bromophenol blue and 1:10 protease inhibitors (Complete Mini, Roche, Germany). Cells were heated for 5min at 60°C immediately followed by incubation for 10min at 100°C. Cell debris was precipitated by centrifugation at 14000xg for 30min at 4°C and protein containing supernatant was TCA-precipitated and subjected to 4.5-15% SDS-polyacrylamide gel electrophoresis. Prestained protein standards within a range of 7.5 to 207kDa (BioRad, Germany) were used as molecular weight markers. Proteins were electrotransfered to a nitrocellulose membrane for 3h at 4°C. To reduce unspecific binding the membrane was blocked for 1h in blocking buffer (5% dry milk in PBS containing 0.1% Tween 20). Blots were incubated overnight with polyclonal antibodies against collagen I, (R1038 Acris, Germany), MMP-2 (AB 809, Chemicon, Germany, 1:100) and MMP-13

(RP1MMP13, Triple Point Biologics, USA, 1:1000). Detection was performed using Horseradish-peroxidase coupled secondary anti-rabbit antibodies (A2074, Sigma, USA; 1:50000). Signals were visualized with ECL reagent (Pierce, USA) and the membrane was exposed to blue light sensitive autoradiography film (Pierce, USA). For dot blot analysis, 1ml of cell culture supernatant was extracted with 20% TCA solution for 30min at 4°C. Extracted protein was pelleted by centrifugation at 14000xg for 5min and washed twice with chilled acetone. After the final washing step the pellet was resuspended in 40µl PBS. 1µl of the resuspended protein was spotted and air dried on a nitrocellulose membrane. After blocking for 1h the membrane was incubated overnight with an antibody against TGFß-1/2/3 (sc-7892, Santa Cruz, USA, 1:200) and developed as above.

Antibody array analysis

RayBio™ rat cytokine antibody array I for conditioned medium (Tebu-bio, France) was used according to the manufacturer's instructions. Briefly, the membranes were blocked for 30min in 5% BSA in 0.01M Tris buffer with 0.15M NaCl (pH 7.6) followed by overnight incubation at 4°C in 1ml of cell culture supernatant. After washing and incubation with biotin coupled anti-cytokine antibodies for 2h membranes were again washed and afterwards incubated with HRP-conjugated streptavidin for 2h. Signals were detected with the solution provided with the kit, exposed ECL films (Amersham Bioscience, UK) were developed with CURIX 60 film developer.

Zymography

For gelatine and reverse gelatine zymography, aliquots of culture medium were mixed with equal volumes of two fold concentrated sample loading buffer (2mM EDTA, 2% SDS, 20% glycerol, 0.02% bromophenol blue, 20mM Tris/HCl, pH 8.0) and subjected to electrophoresis on 1% gelatine containing SDS-polyacrylamide gels. For gelatine zymography 4.5 - 15% gradient gels were used. Reverse zymography was carried out on 15% SDS-polyacrylamide gels in which 25% conditioned macrophage medium was included as MMP-source. Both types of gels were washed twice for 30min in 2.5% Triton-X 100, rinsed in distilled water, and developed for 16h at 37˚C in 50mM Tris/HCl pH 8.5 containing 5mM $CaCl_2$. Finally, the gels were stained with Coomassie Brilliant Blue R250 (Serva, Germany) to visualize protease activity and were photographed.

Incubation of 14C-labelled collagen X with culture supernatant from MSCs

(^{14}C)-proline labelled collagen X was produced and isolated from IGF-I stimulated cranial chicken sternum chondrocytes as described elsewhere (Bohme et al., 1995b). Equal amounts (1000 cpm) were incubated with aliquots of differently conditioned cell culture media or PBS for 7h at 37°C and analysed by 4.5-15 % gradient SDS-PAGE gel electrophoresis under reducing conditions followed by fluorgraphy as described (Bruckner et al., 1989a).

Statistical Analysis

One way ANOVA and student t test were employed for quantitative PCR experiments which were performed in triplicate.

Results

The coculture model

Differentiation potential of the isolated MSCs was determined by cell culture under osteogenic and chondrogenic favourable conditions. Positive Alizarin Red staining indicating osteogenesis and collagen II staining indicating chondrogenesis confirmed differentiation potential of the multipotent MSCs (Fig. 1). Cellular composition of the MSCs was screened previously by immunofluorescence and magnetic sorting for selected surface markers. These MSCs were shown to be CD45^{low}, D7Fib^{+} and CD49a^{+} (Ahmed et al., 2006a).

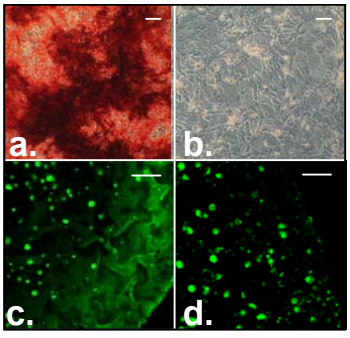


Figure 1. Multidifferentiation potential of bone marrow extracted MSCs. After 19 days culture in osteogenic differentiation medium accumulation of calcified ECM is evident by positive staining with Alizarin Red (a) compared with the MSCs cultured in proliferation medium (b). Chondrogenic differentiation potential of MSCs was established by positive collagen II staining after 21 days in chondrogenic favourable 3-dimensional environment (c) compared with day 0 culture (d). Bars represent 50µm.

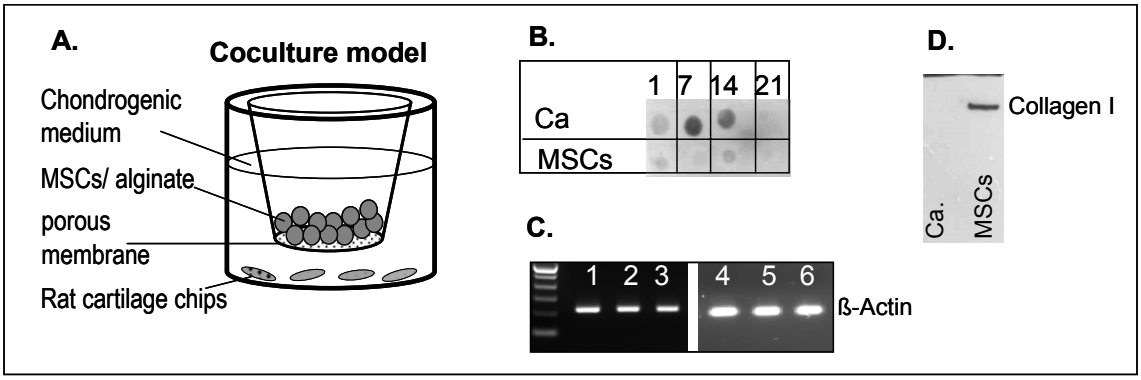


Figure 2. The coculture model Schematic representation of the coculture model carried out in serum-free chondrogenic medium (A). Dot blot analysis of TGFß secretion into the culture supernatant by monocultured cartilage and monocultured MSCs during the period of culture (B). mRNA expression levels of *ß-actin* in the cartilage explants and in alginate suspended MSCs were determined by RT-PCR as an indicator of cell viability. Lane 1-3: coculture MSCs from day 7, 14 and 21. Lane 4-6: cartilage explants from day 7, 14 and 21 (C). Western blot analysis of cartilage explant shows lack of collagen I secretion; in comparison MSCs secrete collagen I (D). Ca = explant culture of rat hip cartilage.

A coculture system of cartilage tissue explants and multipotent MSCs suspended in 1.2% alginate was set up in tissue culture inserts placed into 12 well culture plates under serum-free conditions. The inserts separated MSCs from cartilage chips by a 1µm porous membrane, thus restricting the contact between the explant and MSCs to soluble diffusible molecules (Fig. 2A). Cartilage explants secrete growth factor TGFß until day 14, from day 21 on TGFß secretion is strongly reduced and becomes undetectable in culture supernatant. MSCs totally lack TGFß secretion (Fig. 2B). The viability of both, the cartilage explants and MSCs was determined at mRNA level with RT-PCR. The unchanged band intensities for the housekeeping gene *ß-actin* indicate sustained vitality of MSCs and cartilage tissue throughout the culture period (Fig. 2C). Cartilage tissue samples were screened for contaminating bone tissue by western blot analysis of collagen I in culture supernatant. Lack of collagen I secretion in cartilage explants indicate absence of bone tissue (Fig. 2D).

Effect of cartilage on gene expression profile of *Sox9* and *Cbfa1*

To account for the regulatory effect of Sox9 and Cbfa1 in chondrogenic differentiation of MSCs, expression of these transcription factors was investigated by qRT-PCR. Application of chondrogenic culture conditions resulted in an early increase in gene expression level of transcription factor *Sox9* on the first day of culture (day 1). Cartilage exerted an impact on

Sox9 gene expression within 7 days as the cocultured MSCs showed significant increase in *Sox9* mRNA expression when compared with the monocultures. The increment was equivalent to the mRNA level of control monocultures of MSCs carried out with TGFß-3.

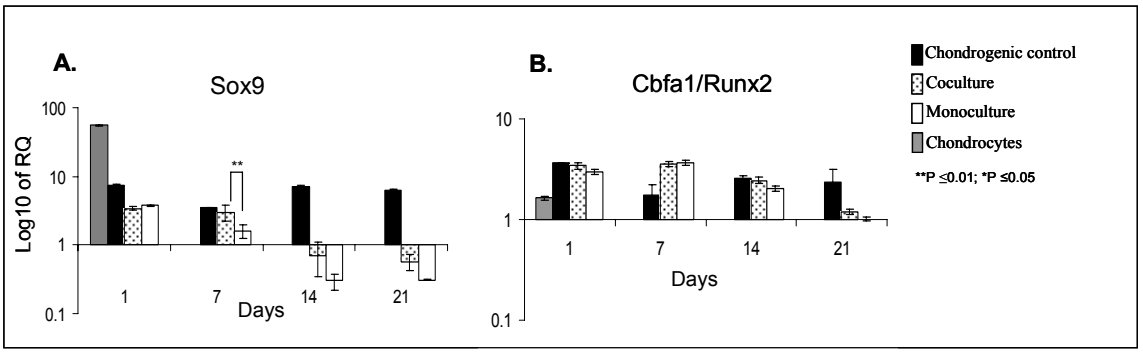


Figure 3. Relative changes in gene expression of transcription factor *Sox9* and *Cbfa1*
Relative gene expression level of *Sox 9* (A) and *Cbfa1* (B) assessed during culture time by qRT-PCR employing the ΔΔCT method using SYBR I Green dye. Target gene expression was normalized to gene expression in MSCs on day 0, which was used as a calibrator and constitutes the baseline at 1. Expression level of each gene from coculture (dotted bars) and monoculture (white bars) was compared with MSCs cultured in chondrogenic medium with addition of 10ng / ml TGFß-3 (chondrogenesis control) (black bars). Grey bar indicates relative gene expression level of freshly isolated hip articular chondrocytes used as positive control.

However, from day 14 onwards *Sox9* gene expression was reduced below the level of undifferentiated MSCs (Fig. 3A). *Cbfa-1* gene expression was induced about 3-5 fold at day 1 in alginate cultures of MSCs, remained stable until day 14 and became reduced to starting values after 21 days. The presence of cartilage did not influence relative induction of gene expression level of *Cbfa-1* (Fig. 3B). Induction of *Sox9* and *Cbfa1* gene expression did not require external TGFß-3 as the mRNA levels during the initial (Sox9) and the middle (Cbfa1) stages of the culture were equivalent to the control culture.

Effect of cartilage on gene expression profile of *Col1a1, Col2a1* and *Col10a1* and their biosynthesis

To identify the effect of cartilage explants on chondrogenesis of MSCs, expression and biosynthesis of different collagens were monitored. Altogether *Col1a1* gene expression

remained suppressed throughout the 3 weeks of culture became gradually reduced during the timeline of culture independent of cartilage (Fig. 4A).

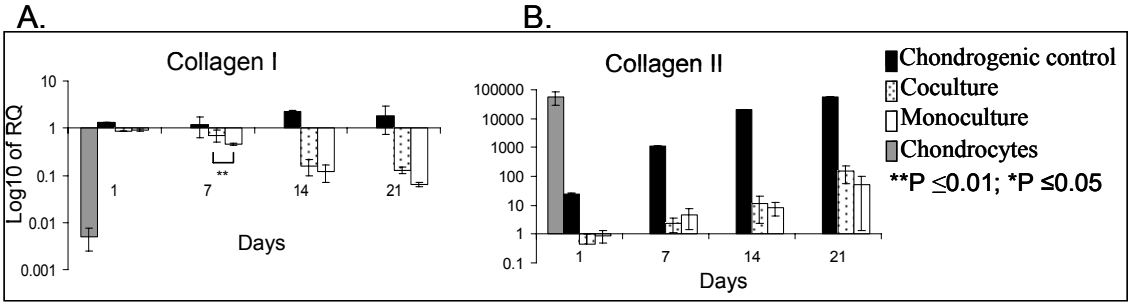
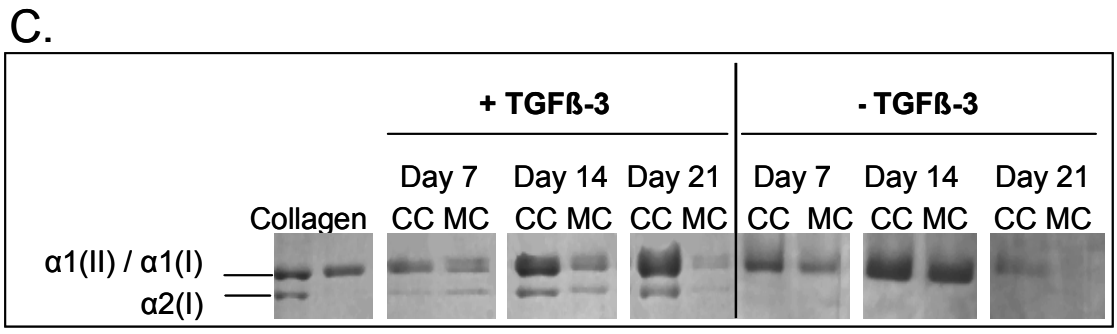
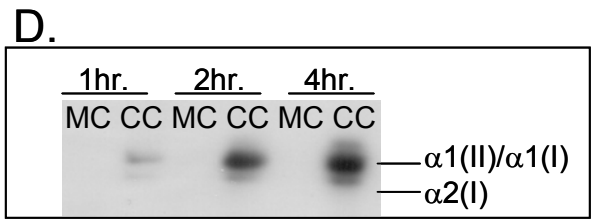


Figure 4. Relative gene expression of *Col1a1* and *Col2a1* and subsequent protein secretion Relative quantitative mRNA expression level of a marker for fibroblast-like cells, *Col1a1* (A) was compared with a marker for chondrogenesis, *Col2a1* (B) by qRT-PCR employing the ΔΔCT method using SYBR I Green dye. As calibrator gene expression of day 0 MSCs constitutes the baseline at 1. Expression level of each gene was compared between coculture (dotted bars) and monoculture (white bars). MSCs cultured in chondrogenic medium with addition of 10 ng / ml TGFß-3 were regarded as chondrogenesis control (black bars). Grey bar indicates relative gene expression level of freshly isolated hip articular chondrocytes serving as positive control. N=3.



Analysis of collagen chain secretion (C). Collagens were precipitated from culture medium, pepsin digested, separated on a 4.5%-15% gradient SDS-PAGE under reduced conditions and stained with Coomassie Blue. N=3.



Assessment of collagen secretion from mono- and cocultured MSCs by employing the pulse-chase method (D). After 21 days in presence of TGFß-3 cocultured cells were separated from cartilage explants and like monocultured cells metabolically labelled with [^{35}S]- methionine / cystein for 1h. During the chase period from 0.5h up to 4h secreted collagens were extracted from the culture supernatant. Samples were separated on a 4.5-15% gradient SDS-PAGE under reducing conditions. MC = monocultured MSCs, CC = cocultures. N=2

On the contrary, mRNA expression of *Col2a1* was progressively elevated in both mono- and cocultured MSCs similarly cartilage exhibited no influence on gene expression level of *Col2a1* (Fig. 4B). However, secretion of α1/2 (I) and α1 (II) collagen chains was positively affected by cartilage when compared with the monocultured MSCs. Notably, in absence of external TGFß-3, neither cartilage nor MSCs secreted detectable amounts of collagen I, indicated by the lack of the α2 (I) chain while addition of TGFß-3 induced secretion of collagen I (Fig. 4C). To deduce the origin of collagens detected in coculture medium, both mono and cocultured MSCs (day 21 in presence of TGFß-3) were metabolically labelled with ^{35}[S] Met/Cys in a pulse/chase regimen after removal of the cartilage explants. After 1 hour of chase newly synthesized collagens were detected in the medium of MSCs which were previously cultured as cocultures with a secretion peak at 4 hours of chase. We assume most of the collagen constitutes collagen II, because of the poor intensity of the lower α2 (I) chain compared to the strong signal of the upper α1 (II) band (Fig. 4D). Minuscule collagen secretion in monocultured MSCs corroborated the non-radioactive collagen preparation results of day 21 (Fig. 4C and D). These data suggest that the factors derived from cartilage explants influence collagen II secretion of MSCs indicating a positive influence on biosynthesis of collagen II.

Compared with freshly isolated chondrocytes undifferentiated MSCs exhibit a very low baseline expression of *Col10a1* mRNA. Induction of chondrogenic differentiation did not alter this expression pattern during the first two weeks of culture. Elevation of *Col10a1* mRNA was observed at the terminal stage of the culture (Day 21 and 28). While relative gene expression in the chondrogenic control cultures (+ TGFß-3) increased about 100x on day 28 gene expression in both, monocultured and cocultured MSCs remained suppressed. Interestingly, this strong upregulation of collagen X gene expression in MSCs under the influence of external TGFß-3 is profoundly suppressed in the presence of cartilage (Fig. 5A). Concurrently, collagen X secretion and biosynthesis was strongly suppressed in the presence of cartilage compared to the monocultured MSCs which released high amounts of collagen X into the culture supernatant. Little collagen X was detected in the cellular fraction of monocultures, however, none was found in cell layers of cocultured MSCs. (Fig. 5B). To exclude protease induced degradation of collagen X in the cell culture supernatant of cocultures ^{14}C-labelled collagen X was incubated with the cell culture supernatants of mono- and cocultured MSCs from day 21 for 7 hours at 37°C. No proteolytic activity was observed in mono- or cocultured MSCs supernatants that generated degradation products of collagen X or reduced signal intensity of the bands. Therefore, we exclude protease induced degradation

(Fig. 5C) and attribute this strong impact of cartilage derived soluble factors on suppression of collagen X secretion from MSCs to the biosynthesis level.

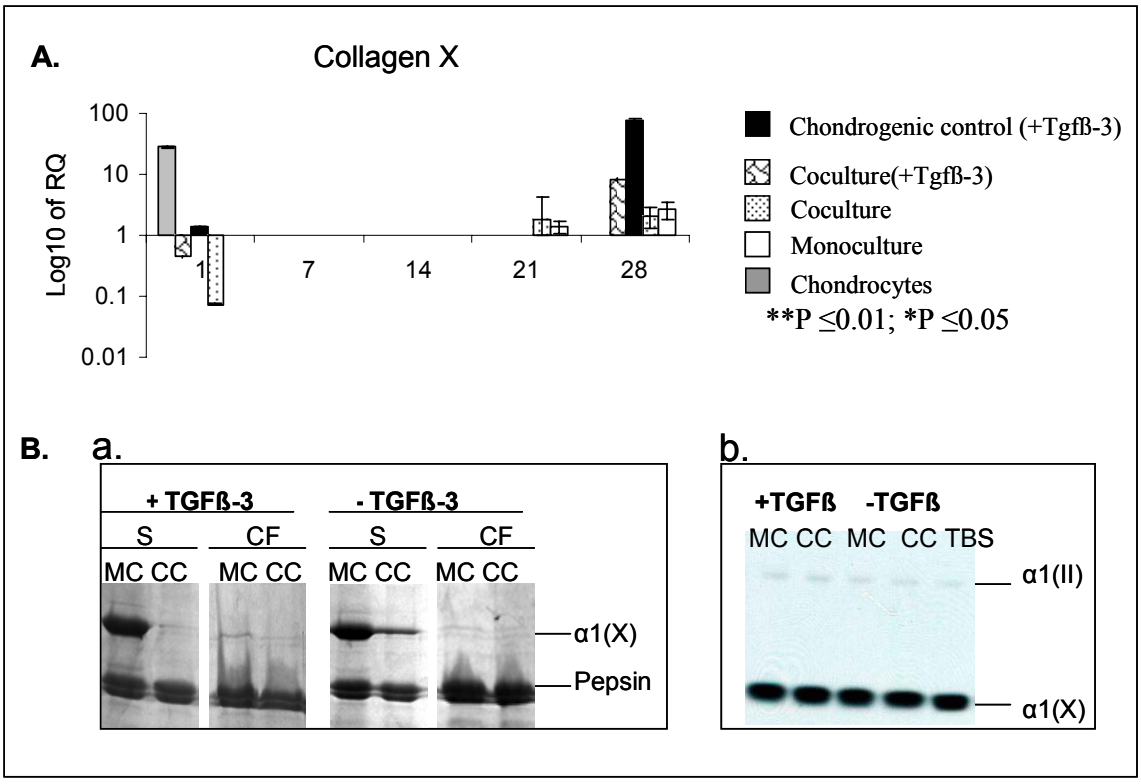


Figure 5: Relative gene expression, biosynthesis and secretion of collagen X Relative mRNA expression level of *Col10a1* is assayed by qRT-PCR employing the ΔΔCT method using SYBR I Green dye (A). Calibrator was gene expression of day 0 MSCs which constitutes the baseline at 1. Expression level of each gene was compared between coculture (dotted bars) and monoculture (white bars). Gene expression of monocultured (black bar) and cocultured (mosaic bar) MSCs cultured in chondrogenic medium with addition of 10 ng / ml TGFß-3 was determined in parallel. Grey bar indicates relative gene expression level of freshly isolated hip articular chondrocytes serving as positive control. Analysis of collagen X protein biosynthesis and secretion (B): Collagens were precipitated from the alginate beads and culture medium, pepsin digested, separated on a 4.5%-15% gradient SDS-PAGE under reduced conditions and stained with Coomassie Blue (a). Collagen X degradation assay: (^{14}C)-labelled collagen X, isolated from metabolically labelled chicken chondrocyte cultures, was incubated with supernatants from mono- and cocultured MSCs (day 21) (b). MC = monocultured MSCs, CC = cocultures, S = culture supernatant, CF = cellular fraction. N = 3

Profiling of secreted proteins

To identify soluble factors which may influence chondrogenic differentiation of MSCs cell culture supernatant was hybridized to an antibody array. Evaluation of the array containing antibodies to 19 secretory molecules resulted in identification of three molecules which exhibited differential secretion pattern dependent on the culture condition and time point of analysis. Vascular endothelial growth factor (VEGF-164α), tissue inhibitor of metalloproteases (TIMP-1) and monocyte attractant protein (MCP-1, data not shown) were observed to be secreted from MSCs and cartilage (Fig. 6).

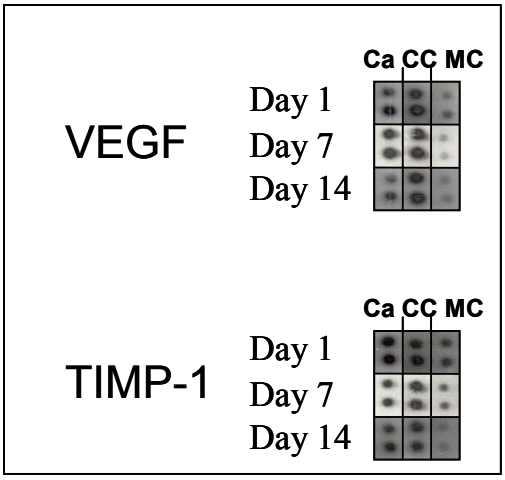


Fig. 6. Antibody array based detection of secreted proteins at day 1, 7 and 14 of the culture Culture supernatants were hybridized to nylon membrane based antibody arrays. Positive signals for TIMP-1 and VEGF-164α showed differential secretion pattern depending on the time point and condition of the culture. MC = monocultured MSCs, CC = cocultures, Ca = cartilage.

High VEGF secretion signals were observed at the onset of the culture in cocultures and cartilage explants which decrease at day 14 in cartilage supernatant. Origin of secreted VEGF cannot be attributed solely to cartilage explants because at day 14 VEGF is found mainly in the supernatant of cocultured MSCs while secretion is reduced in cartilage explant culture. However, the secretion is clearly positively influenced by cartilage in coculture because monocultured MSCs secret minuscule amounts of VEGF throughout the whole culture time. Highest secretion of TIMP-1 was observed in the cartilage explants with slight decline with time, the same was observed in cocultured MSCs. Monocultured MSCs downregulated the TIMP-1 secretion to almost negligible amount on day 14. Since cocultures supernatant did not contain more TIMP-1 compared with the cartilage explant culture, presumably MSCs and

cartilage are collectively responsible for the secretion in the cocultures. Furthermore, based on the antibody array results secretion of the following pro-inflammatory cytokines and growth factors could be excluded at any stage of the culture. These non-secreted factors included CINC-2, CNTF, Fractalkine, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, LIX, Leptin, MIP-3α, ß-NGF and TNF-α.

Analysis of secretion pattern of MMP-13 and TIMP-2 by zymography

Reverse zymography was used to detect TIMP-2 in the culture system. Monocultured MSCs did not secrete TIMP-2 at any time point; in contrast secretion of TIMP-2 by cartilage explants started at day 7 and remained detectable until the end of the culture period. Initially, less TIMP-2 secretion was detected in cocultures when compared with cartilage explant cultures however, at the end of the culture period the pattern was reversed (Fig. 7).

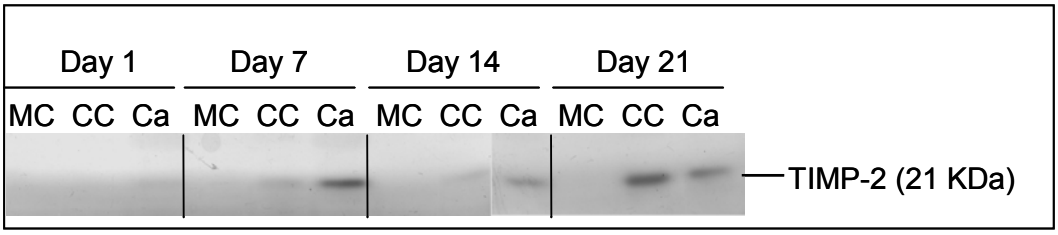


Figure 7. Reverse zymography for TIMP-2 Aliquots of culture supernatants from cells were subjected to SDS-PAGE under non-reducing conditions on a 4.5-15% gradient gel containing 1 % gelatine and 25% conditioned macrophage medium. This reverse zymographic analysis was applied for detection of TIMP-2. Ca = monocultured cartilage, MC = monocultured MSCs, CC = cocultures. N= 3

Gelatine zymogram results indicated secretion of MMP-13 and MMP-9. From the beginning of the culture cartilage and cocultures secreted increasing concentration of pro-MMP-13 without decline till day 21. In contrast, release of pro-enzyme from monocultured MSCs remained largely unchanged during the culture period. Notably, the active form of MMP-13 in coculture and cartilage culture became reduced in relation to the strongly increasing pro-form (Fig. 8A). In coculture and cartilage culture we have observed an additional gelatinolytic activity, which we assume to be pro-MMP-9 according to the molecular weight (black arrow in fig. 8A). Western blot analysis of culture supernatants confirmed the identity of MMP-13 in

both monocultured cartilage explants and in MSCs (data not shown), while MMP-2 was not detected in the culture model at any stage (Fig. 8B).

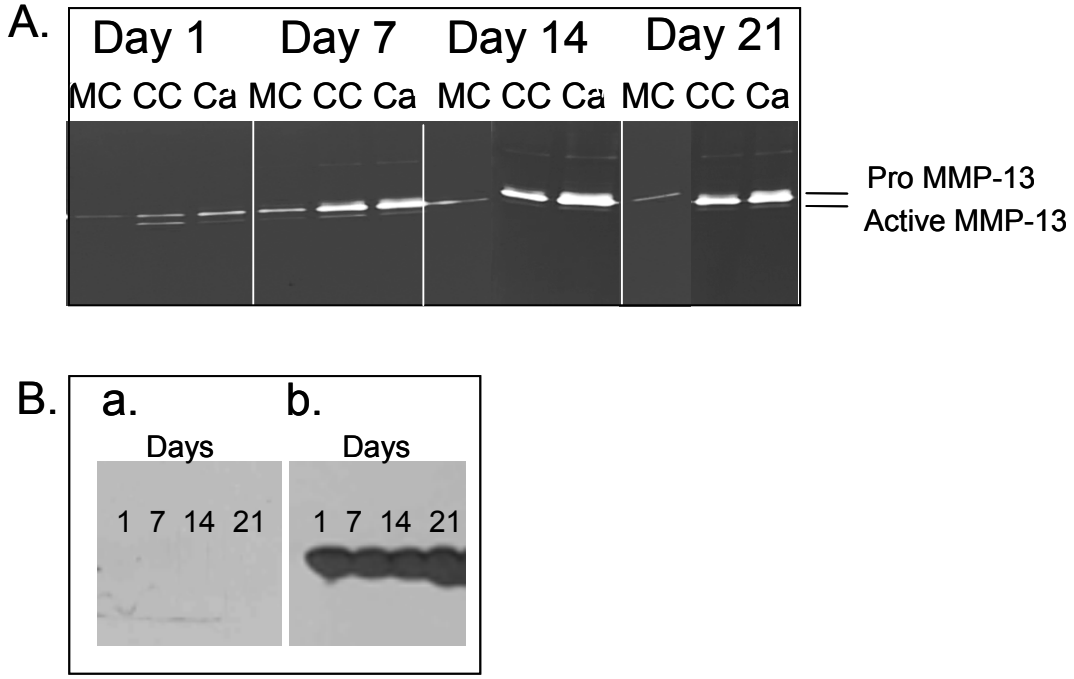


Figure 8. Gelatine zymography and western blot analysis of MMP-2 and of MMP-13
Aliquots of cell culture supernatants were subjected to SDS-PAGE under non-reducing conditions on a 4.5-15% gradient gel containing 1 % gelatine. Gelatine zymographic analysis of the culture supernatant demonstrates secretion of an approximately 60 kDa sized proteolytic activity (pro-MMP-13) and a ca. 90 kDa sized band (presumably pro-MMP-9, black arrow) into the culture supernatant (A). Selected supernatant samples of cartilage monoculture were subjected to western blotting with antibodies against MMP-2 (a) and MMP-13 (b) allowing identification of the gelatine degrading MMP as MMP-13 (B). Ca = monocultured cartilage, MC = monocultured MSCs, CC = cocultures. N=3

Discussion

Paracrine factors released from cartilage exert various catabolic and anabolic effects on neighbouring cells and tissues. To identify factors with putative regulatory effect on the chondrogenic differentiation cascade, a coculture system between articular cartilage and pluripotent undifferentiated MSCs was established. The hypothesis suggested that

differentiation of chondroprogenitor MSCs into chondrocytes is controlled and affected by soluble factors secreted by cartilage.

In the current culture system where MSCs were kept in alginate stabilized 3D environment known to induce chondrogenesis in MSCs, *Sox9* gene expression was prematurely up regulated which indicates early onset of chondrogenesis (Majumdar et al., 2000). Elevated gene expression of *Sox9* particular on day 7 under the influence of cartilage suggests involvement of chondrocyte released signalling factors in regulation of the chondrogenic inducer activity of Sox9. Several signalling pathways are involved in regulating *Sox9* and its downstream genes during cartilage differentiation. TGFß induces *Sox9* gene expression and transcriptional activity through Smad3 (Furumatsu et al., 2005) and BMP-2 enhances *Sox9* gene expression level during murine fracture healing (Uusitalo et al., 2001). TGFß, released from cartilage explants until day 14, presumably accounts in part for the observed increase in *Sox9* gene expression. On the other hand, VEGF has been described to suppress *Sox9* mRNA levels during treatment of chick embryo limb buds (Yin and Pacifici, 2001). In our system *Sox9* gene expression is reduced below initial levels after day 7. Possibly, high secretion level of VEGF from MSCs themselves plus cartilage explants induces this suppression of Sox9 in MSCs, thereby counteracting the influence of TGFß. Despite suppression of *Sox9* mRNA below baseline level, gene expression of *Col2a1* remains up regulated. This observation indicates that an active *Sox9* gene above basal level of un-induced MSCs might not be mandatory in later stages of chondrogenic differentiation of MSCs for regulation of chondrogenesis associated genes as *Col2a1*, but is imperative at initiation of culture.

The most profound effect of cartilage derived factors was observed on suppression of collagen X gene expression and protein secretion. MSCs, regardless of the presence of cartilage exhibit no collagen X secretion before day 21 corroborated by low gene expression until that time point. However, from 21 days on monocultured MSCs release high amounts of collagen X into the culture supernatant, while cocultures do not. Suppression of collagen X secretion in the presence of cartilage depicts delayed hypertrophy in our coculture model. We suggest that cartilage derived soluble factors have the ability to reduce collagen X secretion compared to a strongly elevated secretion by monocultured MSCs at that stage of culture. As we did not observe collagen X degradation products in culture supernatant we deduce that absence of collagen X secretion is due to suppression at the biosynthesis level. This suggestion is substantiated by significantly reduced gene expression in cocultured MSCs compared with the monocultured MSCs. Secondly; we did not detect collagen X protein in the cellular fraction of cocultured MSCs while small amounts were retained in monocultured MSCs.

Paracrine factors derived from surrounding chondrocytes and endothelial cells alter differentiation status of chondrocytes. Chondrocytes of normal articular cartilage do not undergo terminal differentiation whereas growth plate chondrocytes do. Articular chondrocytes in osteoarthritic joints however, express markers of terminal differentiation like collagen X and Alkaline Phosphatase (AP) (von der et al., 1992). By coculturing articular and growth plate chondrocytes Jikko *et al.* could demonstrate that soluble paracrine factors alone are responsible for inhibition of terminal differentiation. Articular chondrocytes were able to suppress calcification and AP activity in cocultured growth plate chondrocytes, indicating that articular chondrocytes produce factors which inhibit terminal differentiation (Jikko et al., 1999). Also during endochondral ossification cartilage maturation and conversion of hypertrophic cartilage to bone is delayed by paracrine signals derived from periosteal/perichondrial tissue or from the cartilage proper itself. Formation of new blood vessels and their penetration into hypertrophic cartilage appear to be essential for the elimination of this developmental barrier. Consistently with this notion, the terminal differentiation barrier in chondrocytes from the caudal part of 17-day old chick embryo sternum can be eliminated by coculturing them with endothelial cells *in-vitro*. The humoral activity secreted by endothelial cells consists of proteinases sufficient to break down the terminal differentiation barrier inherent to this chondrocyte population (Babarina et al., 2001; Bittner et al., 1998; Bohme et al., 1995a). In addition, articular chondrocytes obtained from osteoarthritic joints are known to secrete factors which activate pro-MMP-9 derived from cocultured macrophages. The stepwise activation process of pro-MMP-9 involves MMP-13, secreted from chondrocytes and MT1-MMP, also expressed by chondrocytes which converts pro-MMP-13 to its active form (Dreier et al., 2001; Dreier et al., 2004; Knauper et al., 2002).
TIMP-1, TIMP-2, VEGF-164α and MMP13 were the four prominent secreted molecules identified in the supernatant of cocultures and cartilage explants. Except VEGF-164α which seems to be mainly secreted by cocultured MSCs at least at the final culture stage, a reasonable amount of TIMP-1,-2 and MMP13 is released by differentiated chondrocytes of the cartilage explants. We observed a profound increase in pro-MMP-13 secretion in coculture and cartilage monocultures during the culture period; however with declining activation status. This effect might be due to a concurrent secretion of TIMP-1 and -2, both known as efficient inhibitors of MMP-13 activity (Will et al., 1996; Knauper et al., 1997b; Cowell et al., 1998). MMP-13 is known to degrade collagen X (Knauper et al., 1997a) however, lack of collagen X in supernatant of cocultured MSCs can not be due to proteolytic activities because collagen X degradation products or reduced signal intensities were never detected in the

cultures. Additionally, in absence of cartilage MMP-13 activation rate was not higher whereas collagen X secretion was profoundly increased. In MMP-13 knockout mice Inada *et al.* have demonstrated a profound enlargement of the anatomic domain where collagen X is expressed by hypertrophic growth plate chondrocytes and an increase of collagen X deposition in this domain. In addition to proteolytic activity of MMP-13 this protease might exert a yet unknown regulatory impact on collagen X biosynthesis (Inada et al., 2004; Stickens et al., 2004). Therefore, suppression of collagen X gene expression and accordingly biosynthesis in cocultures might be also due to a regulatory effect of MMP-13 and not to a proteolytic effect.

Multiple Cbfa1 consensus binding elements are located in the collagen X promoter region (Kim et al., 1999; Zheng et al., 2003). Despite induction of *Cbaf1* gene expression early in MSCs differentiation pathway (day 1) collagen X expression is not significantly induced before day 28 while *Cbfa1* gene expression is downregulated after 21 days prior to upregulation of the *col10a1* gene. Also, relative gene expression of *Cbfa1* is not significantly influenced by cocultured cartilage in our system during the whole culture period whereas gene expression of collagen X is. In case *Cbfa1* influences collagen X mRNA level we would not expect such a profound difference between cocultured and monocultured MSCs therefore, we cannot attribute an influence of this transcription factor on mRNA expression or eventual transcription of collagen X.

Another key player in this system might be VEGF-164α, released by cartilage and cocultured MSCs. VEGF as one of the most important mediators of angiogenesis is abundantly expressed during embryogenesis but in adults it is only found in restricted tissues and cell types, as in osteoarthritic chondrocytes, but not in resting chondrocytes (Pufe et al., 2001; Pfander et al., 2001). Interestingly, we have detected VEGF-164α in increasing amounts in cocultures and cartilage, however, main source seems to be cocultured MSCs. VEGF is known to stimulate receptor phosphorylation of its VEGF-receptor 2 (flk-1 /KDR), thereby activating the mitogen activated protein kinases ERK/1/2 inducing long-lasting activation of the transcription factor AP-1 plus expression of c-fos mRNA in dental pulp cells (Pufe et al., 2004b; Matsushita et al., 2000). As a consensus site for AP-1, a dimeric transcription factor composed of the two oncogenic products jun and fos, has been identified within the promoter of collagen X, VEGF binding to its receptor (VEGFR1) might contribute to suppression of gene expression of collagen X, while at the same time it is known to induce secretion of MMP-13 (Pufe et al., 2004a).

All four members of the TIMP-family function as inhibitors of angiogenesis by blocking MMP activity. TIMP-1 and -2, both present in cocultures, presumably account for inhibiting

generation of active MMP-13. Besides, TIMPs can exert MMP-independent functions, which might be important in our system. TIMP-2 exhibits a growth suppressive activity by interacting with the α3ß1 integrin on endothelial cells thereby inhibiting cell proliferation (Seo et al., 2003). This shows that in addition to inhibition of MMPs TIMPs also regulate cell growth and differentiation. Cell surface binding of TIMP-1 and -4 has been reported, but the identification of specific cell surface receptors is missing (Stetler-Stevenson and Seo, 2005). Therefore, TIMPs secreted by differentiated chondrocytes and MSCs may act as inducers or repressors of specific factors necessary for chondrogenic differentiation of MSCs.

Two additional molecules are described in literature with collagen X regulatory properties. PTH / PTHrP expressed in perichondrium and in resting and proliferating chondrocytes during endochondral growth (Lee et al., 1996; Iwamoto et al., 1994) strongly represses collagen X biosynthesis in chick embryonic hypertrophic chondrocytes thus inhibits matrix mineralization and apoptosis of chondrocytes (Zerega et al., 1999). It has been demonstrated that PTH / PTHrP fragments suppress collagen X gene expression *in vitro* by acting on two responsive elements within the promoter (Gebhard et al., 2004; Riemer et al., 2002). The second molecule is, osteogenic protein 1 (OP-1 or BMP-7) which belongs to the TGFß super family (Kingsley, 1994) can induce collagen X gene expression via a cell type specific response element that contains an A/T rich and a MEF-2 like sequence which react with yet unidentified nuclear proteins in response to OP-1(Harada et al., 1997).

In conclusion, we have demonstrated that the coculture system including differentiated cartilage tissue and undifferentiated MSCs is suitable for studying paracrine communication between both the components. Notably, external growth factors are not necessary for induction of chondrogenic marker genes and biosynthesis of the corresponding proteins. However, for long term maintenance of the gene expression level growth factors like TGFß are required as internal secretion by cartilage ceases from day 14 on. Cartilage derived soluble factors are sufficient to induce *Sox9* gene expression and suppress collagen X expression/secretion. MMP-13 and VEGF may have a regulatory role in collagen X expression and secretion. Besides the identified secreted proteins, MMP-13, TIMP-1 and -2 and VEGF-164α other, yet unidentified signalling molecules surely have an impact on regulating and directing the differentiation process of MSCs. It shall be kept in mind that in the coculture on one hand cartilage derived factors influence fate of MSCs; on the other hand MSCs released molecules might have an impact on cartilage metabolism as well. These putative proteins may even abrogate the intrinsic blockade which prevents hypertrophy and eventual ossification in articular cartilage. Discovering the mutual paracrine influence of

differentiated and undifferentiated cells will have direct consequences on therapeutic use of either undifferentiated or *ex vivo* differentiated MSCs for cartilage replacement. For MSCs based cartilage repair, inhibition of hypertrophy and stabilization of the cartilaginous phenotype in explants as well as in the host is a prerequisite for success and long lasting vitality.

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Chapter 5

Retrovirus based gene silencing of *Sox9*

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Abstract

Transcription factor Sox9 designated as master regulator of the chondrocytes lineage regulates the whole cascade of chondrogenic differentiation. It is also identified as the first transcription factor to be expressed during chondrogenesis. Heterozygous Sox9 knockout mice do not survive due to lack of rib cartilage and lung collapse which limits Sox9 functional studies. Many unclear aspects related to *Sox9* and its downstream differentiation specific pathways include *Sox9* gene regulation, redundancies among *Sox* family genes, feedback mechanisms, effect of level of *Sox9* expression on differentiation stage and direct and indirect impact of Sox9 activities etc. Unavailability of Sox9 homozygous knockout mice due to postnatal morbidity calls for new techniques for *Sox9* functional analysis. One option for development of a suitable model system would be *in vitro* knockdown of genes by RNA interference

(RNAi). RNAi is a post-transcriptional RNA mediated gene silencing via small interfering RNA (siRNA) or short hairpin loop RNA (shRNA). In this study we have knocked down the *Sox9* gene in murine fibroblast cell line NIH3T3 by using shRNAs specific for *Sox9* employing a retroviral gene delivery system. After 48 hours of transduction 80% knockdown at gene expression level and nearly 100% knockdown at protein level were observed. A decrease in gene expression of *Sox6* was demonstrated 48 hours after knockdown of *Sox9*. On the contrary, *Sox4* and *Tbox2* mRNA expression level remained unaffected. This pilot *in vitro* gene silencing study serves as a foundation for research on *Sox9* silenced primary mesenchymal stem cells to identify hithero unknown signalling factors, redundant genes and unidentified genes involved in chondrogenesis.

***Key words*:** rat marrow stromal cells, RNAi, Sox9, chondrogenic differentiation

Introduction

Several studies carried out in chondrocyte cell lines, mesenchymal stem cells, primary chondrocytes and on genes of chicken, mice, rats and human, have identified *Sox9* as the key player of chondrocyte phenotype induction and maintenance. Expression of most of the chondrogenesis associated genes including *Col2a1, Col9a2, Col11a2, aggrecan, COMP* and other *Sox*-family genes, is suggested to be regulated by *Sox9* (Akiyama et al., 2002; de Crombrugghe et al., 2001; Chimal-Monroy et al., 2003). *Sox9* is expressed in all chondroprogenitor cells and also in differentiated chondrocytes. The expression starts in chondroprogenitor cells, reaches a peak in resting and proliferating chondrocytes and becomes completely absent in hypertrophic chondrocytes. *Sox9* expression precedes *Col2a1* gene expression in developing chondrocytes and it is described that MSCs can not condense in absence of *Sox9* (Chimal-Monroy et al., 2003). Several other transcription factors of the Sox-family as *Sox4, L-Sox5, Sox6, Sox8, Sox9* and *Sox10* are also known to be involved in chondrogenesis. L-Sox5 and Sox-6 have redundant functions and have been specifically implied to be involved in overt chondrogenic differentiation. *L-Sox5* and *Sox6* double-null mice show virtual absence of cartilage and total arrest of chondrogenic differentiation at the stage of mesenchymal condensation. Both of them are suggested to be downstream in the *Sox9* regulatory pathway. In skeletogenesis down regulation of *Sox9* is required for transition of proliferating chondrocytes to hypertrophic chondrocytes which ultimately leads to bone formation in the process of endochondral ossification. *Cbfa1*, an integral transcription factor

of osteoblast differentiation also appears to be under direct and/ or indirect control of *Sox9* (Akiyama et al., 2002; de Crombrugghe et al., 2001). In short, Sox9 is the primary transcription factor involved in chondrocyte differentiation conserved across vertebrates, and it actively determines the fate of chondrocyte progenitor cells (Healy et al., 1999; Chimal-Monroy et al., 2003; Bi et al., 1999; de Crombrugghe et al., 2001). Attempts for *Sox9* conventional genetic studies employing transgenic mice have proven fruitless because of post natal fatality of *Sox9* heterozygous null mutants leaving no hope for generation of *Sox9* null homozygous animals. The *Sox9* heterozygous mice show malformation of cartilage primordia and premature mineralization of bones and other typical signs of the human skeletal malformation syndrome campomelic dysplasia (Bi et al., 2001). Therefore, studies to understand *Sox9* expression and activity in chondrogenesis have been limited to mouse embryo chimeras derived from *Sox9*-/- ES or on conditional inactivation of *Sox9* by using *Cre/loxP* recombination system (Bi et al., 1999; Akiyama et al., 2002). In conditional knockout study, heterozygous transgenic mice containing *loxP* flanking sites on exon 2 and 3 of Sox9 gene, which carry the DNA-binding domain, were mated with *Cre*-containing mice. The resultant *Sox9* conditional deletion indicated absolute necessity of *Sox9* for overt differentiation of chondrocytes (Akiyama et al., 2002). However, the level of Sox9 required for regulation of other genes of the chondrogenic cascade is still unresolved. Hence, use of new technologies and novel ideas for understanding Sox9 function and mode of action is vital.
RNA interference is a powerful technique for analysis of loss-of-function phenotype. It induces degradation of complementary mRNA by introducing double-stranded (ds) RNA into organisms as *C.elegans* and in eukaryotic cells (Rene F.Ketting et al., 2003). These dsRNAs are cleaved into 21-25 nucleotide duplexes of short small interfering RNAs (siRNA) which initiate sequence-specific degradation of mRNA by mimicking endogenous microRNA (miRNA).Both the endogenous miRNA and exogenous siRNA are taken up by RNA-induced silencing complex (RISC) which then attaches to the complementary mRNA resulting in gene silencing (McManus and Sharp, 2002). The role of miRNA in animals is still not identified however; they have been suggested to be involved in developmental regulation. Studies on Arabidopsis indicate transcription factors involved in patterning of development to be the targets of miRNA directed endonucleolytic cleavage of mRNA. As most of the miRNA are evolutionary conserved from worms to mammals their developmental regulation may have been a key on the route to multicellular life (Gregory J.Hannon and and Phillip D.Zamore, 2003). Short hairpin (sh)RNA based interference is a more stable RNAi approach in which ~50 bp long RNA hairpins consisting of a stem of 19-26 bp are linked to a small ~6 bp

terminal loop and ~2 bp 3´-overhangs. Inside the cytoplasm these shRNA are treated as pre-miRNA by RNaseIII enzyme called Dicer resulting in siRNA/miRNA duplexes (Paddison et al., 2002; Cullen, 2006).

In this study we have employed shRNA designed to specifically and stably knockdown *Sox9* gene expression by inducing RNAi using a retroviral based vector system.

Material and Methods

Cell Culture

NIH3T3 fibroblast cells were proliferated in DMEM F-12 with 10% FCS (Invitrogen, UK) and 1% Penicillin-Streptomycin (PAA, Germany). Cells were cultured in 6 well plates for transduction. Packaging cell line EcoPack2-293 was cultured in 10% FCS containing DMEM F-12 with 4mM L-Glutamine, 1% antibiotics and 1mM Sodium Pyruvate (Sigma, USA). For long term storage in liquid N_2 $2x10^6$ cells were suspended in 70% FCS and 10% DMSO in DMEM. Trypsin-EDTA (Invitrogen, UK; 0.25% Trypsin and 1mM EDTA) was used to detach cells from culture vessels.

Viral vector constructs:

Three individual shRNAs were selected using algorithm promoted by Clonetech, BD Bioscience (Table 1). ~69 basepair hairpin loop containing three shRNA pairs were manufactured at MWG, Germany. 100µM of top and bottom strands shRNA were each mixed in 1:1 ratio and incubated for 30 sec at 95°C and then annealed for 2 min at sequential temperature of 72°C, 37°C and 25°C. The annealed top and bottom strands of each shRNA were ligated into a shRNA expression vector (RNAi-ready pSIREN retro Q plasmid expression vector system, Clonetech, BD, Bioscience) according to the manufacturer's protocol. Briefly, 0.5µM of annealed shRNA were ligated to 25ng/µl of linearized pSIREN vector by 400/µl of T4 DNA ligase in presence of 1.5µl of 10x T4 DNA ligase buffer containing 10mg/ml BSA, reaction volume was made up to 15µl with nuclease free H_2O. After 3 h incubation at RT 2µl of the ligated vector was transformed into 50µl of *E.coli* K-12 competent cells (Fusion-Blue competent cells, Clonetech, BD Bioscience ,USA). After 30 min incubation on ice the K-12 cells were subjected to heat shock for 45 sec at 42°C in a water bath and placed back on ice; 950µl of SOC medium was added and transformation was carried out at 37°C for 1 h with shaking at 250 rpm. 50µl of the transformation mix was plated

on LB agar plates containing ampicillin and incubated overnight at 37°C. Plasmids were isolated by an endotoxin free plasmid extraction kit (NecleoBond Plasmid Maxi EF kit, Clonetech, BD Bioscience, USA) from overnight culture of bacterial colonies in 3 ml of liquid LB medium at 37°C with shaking. Positive clones were identified by restriction and sequence analysis. Restriction with *BamH*I and *EcoR*I was carried out at 37°C overnight and the restricted plasmids were resolved on a 2% agarose gel. Sequence analysis was carried out at GeneArt, Germany. Control vector containing a luciferase shRNA was handled exactly in the same way as the Sox9 shRNA containing viral constructs.

Generation of shRNA expressing retrovirus:

Endotoxin-free positively identified plasmids were transfected into EcoPak 293-2 packaging cell line with Lipofectamine2000 (Invitrogen, UK) after 24 h 6µg/ml of puromycine was added and transfected colonies were selected for ~9 days based on their puromcyin resistance. Each selected clone was transferred to a 25 cm^2 flask and cells were passaged twice in 75cm^2 flask to obtain enough plasmid harbouring transfected cells for long term storage and transduction experiments. For transduction the culture medium containing shRNA expressing virus was collected after 48 h of culture until flasks reached confluence. Virus titre was determined as advised by the manufacturer and 10^5 CFU were used for transduction.

Transduction of target cells:

15,000 of NIH3T3 cells /well were seeded in proliferation medium on a 6 well plate. After 24 hours nearly 40% confluent wells were tranduced with 1 ml of fresh filtered (0.45µm filter) virus mixed with 1 ml of fresh medium containing 6µg/ml polybrene (final concentration). After 24 h of transduction cells were harvested by trypsin digestion, pooled and used for RNA and protein analysis.

RNA Isolation and reverse transcriptase-polymerase chain reaction:

RNA was isolated by an affinity column chromatography method with Ambion's RNAqueous4-PCR kit according to the manufacturer's protocol. For removal of possible DNA contamination, isolated RNA was incubated for 1 h at 37°C in 2 units of *DNase*I enzyme (DNA-*free*, Ambion, USA). The RiboGreen RNA quantification kit (Molecular Probes, Invitrogen, UK) was used for determination of RNA concentration at 585nm wavelength. For RNA conversion to cDNA, Invitrogen´s SuperScript II reverse transcriptase kit was used with 0.5-1 µg of RNA in 20µl of total reaction volume in presence of 40units/µl

recombinant ribonuclease inhibitor (RNase OUT®). Reverse transcription was carried out with 500µg/ml of Oligo-dT primers, 10mM dNTPs and the 200 units of SuperScriptII enzyme in First-Strand Buffer and 0.1M DTT for 50 min at 42°C followed by an extension period of 15 min at 70°C.

Table 1: Sox9 shRNA sequences

shSOX9#1

Top strand

5'-gatccGGAGGAAGTCGGTGAAGAATGTTCAAGAGACATTCTTCACCGACTTCCTC CTTTTTTACGCGTg-3´

Bottom strand

5'-aattcACGCGTAAAAAAGGAGGAAGTCGGTGAAGAATGTCTCTTGAACATTCTT CACCGACTTCCTCCg-3´

shSOX9#2

Top strand

5'-gatccGCTGGTAGTCCCGTTGATATTCAAGAGATATCAACGGGACTACCAGCTTT TTTACGCGTg-3´

Bottom strand

5'- aattcACGCGTAAAAAAGCTGGTAGTCCCGTTGATATCTCTTGAATATCAAC GGGACTACCAGCg

shSOX9#3

Top strand

5'- gatccGCGGCTCCAGCAAGAACAAGTTCAAGAGACTTGTTCTTGCTGGAGCC GTTTTTTACGCGTg

Bottom strand

5'- aattcACGCGTAAAAAACGGCTCCAGCAAGAACAAGTCTCTTGAACTTGTTC TTGCTGGAGCCGCg

Quantitative PCR

Absolute and relative qPCR was carried out with SYBR Green Dye I on ABI 7000 Prism Sequence detection system (Applied Biosystems, USA) according to manufacturer's instructions. Briefly, 0.2µM of each primer was suspended in SYBR green master mix (AB Systems) and the reaction was carried out in 96 well plates with 1µl of cDNA in a 50µl final volume. For all primer pairs same amplification parameters were used and repeated for 40 cycles, denaturation at 95°C for 0.15 min and annealing at 60°C for 1 min. Triplicate values from three independent experiments were evaluated and mean relative quantification (RQ) value was plotted on a semi-logarithmic graph. RQ values were calculated by the software "RQ study application v1.1" (ABI Prism 7000 SDS software v1.1) according to the ΔΔCt method using ß-Actin as endogenous control and vector control as calibrator. For absolute quantification a standard curve of serially diluted quantified Sox9 plasmid was plotted and sample Ct values were used to determine exact copy numbers of *Sox9*. Primers were designed with either Primer3 or Primer express software supplied by Applied Biosystems and manufactured at MWG – Biotech as listed in table 2.

Table 2: Primers used for quantitative PCR

Gene / RefSeq#	Amplicon (bp)	Primer (5′ - 3′)
ß-Actin	104	forward -GTAGCCATCCAGGCTGTGTT-3′
NM_031144		reverse -CCCTCATAGATGGGCACAGT-3′
Sox4	58	forward -GGCCCATGAACGCCTTTAT-3′
XM-344594		reverse -CTGGATGAACGGGATCTTGTC-3′
Sox6	51	forward-GAAATCCATGTCCAACCAGGAG-3′
XM_215016		reverse -CGGGCCTGCTCTTCATAGTAAG-3′
Sox9	140	forward -CTGAAGGGCTACGACTGGAC-3′
XM_343981		reverse-TACTGGTCTGCCAGCTTCCT-3′
Tbox2	71	forward -GCCCACTCTCCGTT TGTATGAG-3′
XM_220810		reverse -AGGACGAGGCATCGGATTC-3′

Immunoblotting:

Western blotting was carried out with a lysate of ~10^6 cells prepared by suspending the cell pellet in 1% NP40 Buffer containing 150mM NaCl, 50mM Tris pH 8.0 and 1:10 protease inhibitors (Complete Mini, Roche, Germany) in water. Cells were heated for 5 min at 60°C immediately followed by incubation for 10 min at 100°C. Cell debris was precipitated by centrifugation at 14000xg for 30 min at 4°C and the concentration of protein containing supernatant was determined. A protein aliquot of 5µg was suspended in equal volume of 0.1M Tris/HCl buffer (pH 6.8) loading buffer containing 4% SDS, 20% glycerine, 5% ß-Mercaptoethanol and 0.01% bromophenol blue and subjected to 4.5-15% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransfered to a nitrocellulose membrane for 3 h at 4°C. To reduce unspecific binding the membrane was blocked for 1h in blocking buffer (5% dry milk in PBS containing 0.1% Tween 20). Blots were incubated overnight with 1:500 diluted polyclonal antibodies against Sox9 (AB5535 Chemicon, USA). Detection was performed using horseradish-peroxidase coupled secondary antibodies against rabbit (A2074, Sigma, USA; 1:50000). Signals were visualized with ECL reagent (Pierce, USA) and the membrane was exposed to autoradiography film (Pierce, USA). For dot blot analysis, 5µg of protein was spotted and air dried on a nitrocellulose membrane. After blocking for 1h the membrane was incubated overnight with an antibody against ß-Actin (AB978, Chemicon, USA; 1:200) and developed as above.

Statistical Analysis: one way ANOVA and student t test were employed for quantitative PCR experiments performed in triplicate. The level of significance was determined by Turkey´s test with 2 group comparison.

Results and Discussion:

Cloning and generation of virus particles: One of the preferred gene delivery systems used for mammalian cells is retroviral transduction. High transduction efficiency achieved with difficult to transfect primary cells as stem cells parallel to long lasting genetic stability are the biggest advantages. The annealed *Sox9* shRNAs were subcloned into low copy number RNAi-ready pSIREN retro Q plasmid expression vector system (BD, Bioscience.) suitable for long term studies up to several weeks (Fig 1).

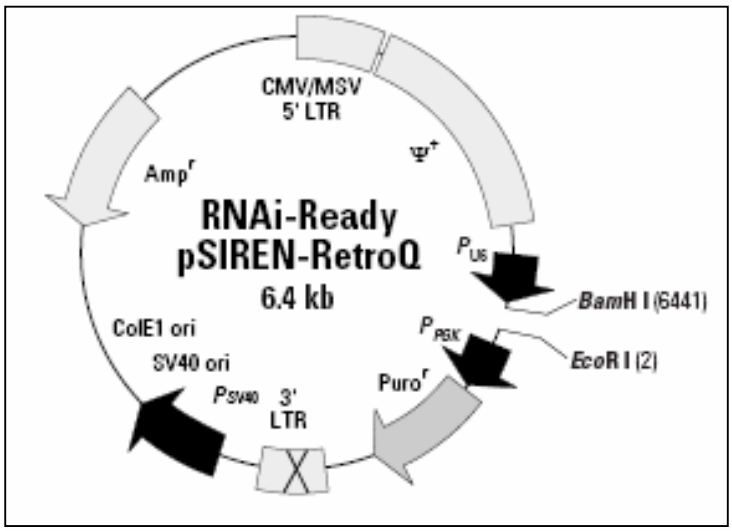

Figure 1. RNAi-Ready pSIREN-RetroQ vector was linearized by *BamH*I and *EcoR*I digestion and shRNA containing *BamH*I and *EcoR*I overhangs were subsequently ligated. The shRNA containing vector was transformed into competent K-15 E. coli cells and positive clones were selected by ampicillin resistance.

This modified retroviral gene expression system is a two face system where the vector itself is replication incompetent and contains necessary viral RNA processing elements as the LTR promoter, packaging signal (Psi^+) and tRNA primer binding site along with bacterial origin of replication (ColE1 ori). The structural genes *gag-pro-pol* required for particle formation and replication are integrated in an ecotropic EcoPak-293 packaging cell line. Once the replication incompetent shRNA from the vector becomes stably integrated into the packaging cell genome, the infectious retroviral particles containing the shRNA are produced. The expression vector relies on an U6 promoter to drive the expression of shRNA in mammalian cells.

The retroviral particles produced from the 293 cells were used to infect the murine NIH3T3 fibroblast cell line to transmit the shRNA. *E.coli* ampicillin resistance gene is necessary for selection and propagation of positive clones in bacteria while puromycin resistance permitted selection and enrichment of mammalian cells which had taken up the plasmid. The cloned plasmids screened by *BamH*I and *EcoR*I double digestion which produced a 6.4 Kb linearized vector and 69 bp shRNA (data not shown). Positive colonies were used for DNA sequencing which showed proper alignment and the correct shRNA sequence. (Fig 2).

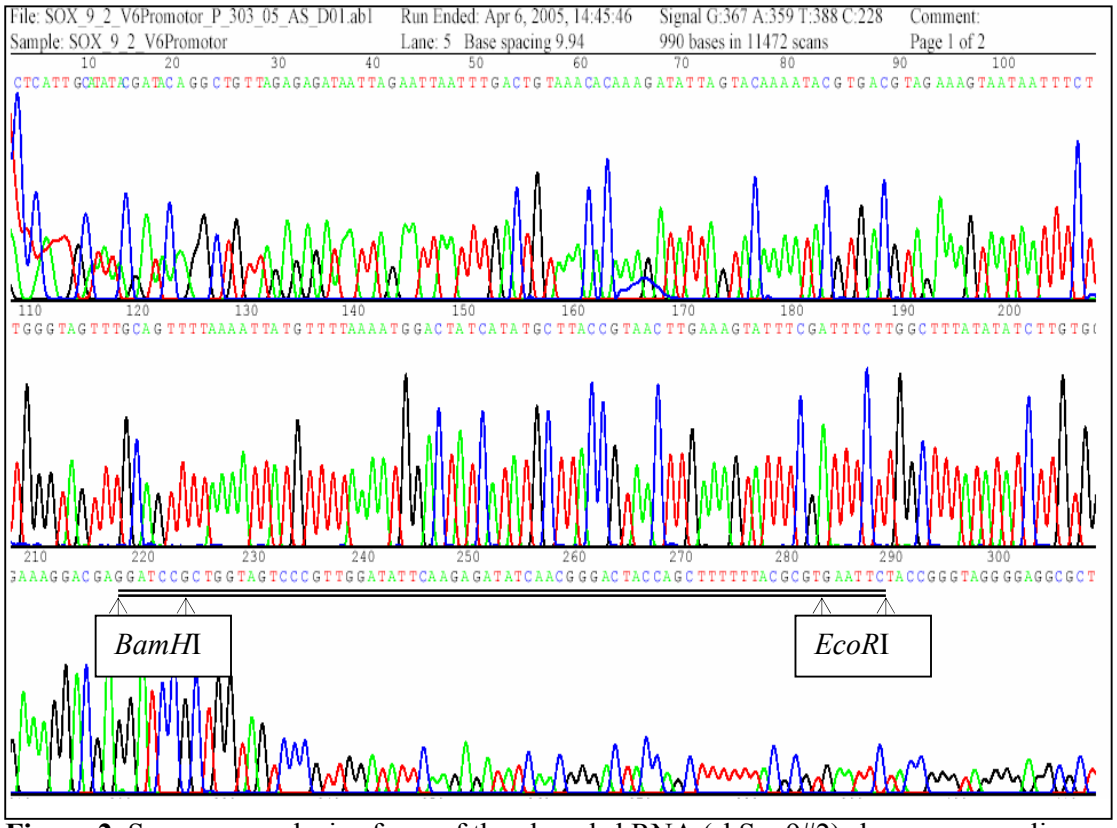

Figure 2. Sequence analysis of one of the cloned shRNA (shSox9#2) shows proper alignment with the plasmid construct. Arrows show *BamH*I and *EcoR*I restriction overhangs integrated to the shSox9 sequence for linear ligation into the vector.

Transduction and evaluation of the knockdown efficiency

For the evaluation of transfection efficiency cells were harvested after 48 hours of transduction. Readout for all tested parameters was the gene expression level of *Sox9* normalized to endogenous control ß-actin and analysed by quantitative PCR. 80% knockdown of *Sox9* was observed in comparison with the control cells which were transduced with viral particles containing an unrelated luciferase shRNA. Evaluation of the knockdown was performed at the protein level with western blot and dot blot analysis. Nearly 100% absence of Sox9 protein confirmed the knockdown effect. Housekeeping protein ß-actin remained unaffected indicating *Sox9* gene silencing to be specific and not due to RNAi provoked unspecific silencing or viral interferon mediated “off target” mechanism (Fig 3).

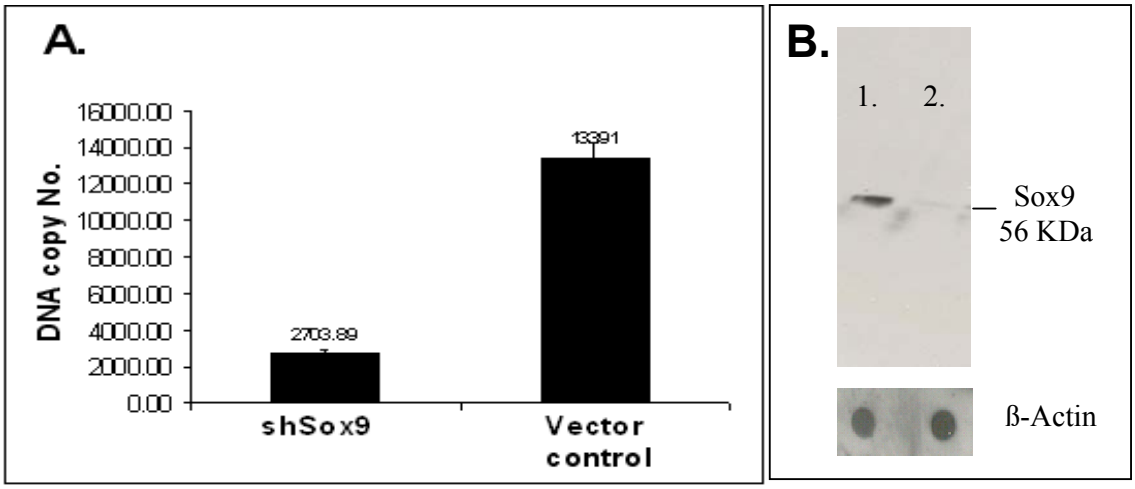

Figure 3. Knockdown of *Sox9* gene expression performed at mRNA level with qPCR shows 81% lower *Sox9* gene expression in the transduced cells in comparison with the control cells harbouring the luciferase shRNA vector (A). Nearly 100% reduction was observed at protein level (lane 2) in the shSox9 transduced cells compared with the controls (lane 1), while ß-Actin remained unaffected as observed in the dot blot indicating a Sox9 specific knockdown (B).

Downstream gene expression analysis

After successful *Sox9* knockdown downstream effect of the silencing was analysed on 3 transcription factors. Among them *Sox6* is a known member of *Sox9* regulated chondrogenic pathway (Lefebvre et al., 2001), while the role of *Sox4* in chondrogenic regulation is not clear though it is expressed in respiratory tract of embryonic cartilage (Reppe et al., 2000). The role of *Tbox2* or *Brachyury 2* in chondrogenesis is not evident, however Hoffmann *et al* suggest its gene expression to be independent of *Sox9* during the chondrogenic cascade (Hoffmann et al., 2002). Therefore, we evaluated relative mRNA expression of these three transcription factors after 48 hours of Sox9 knockdown and compared it with the expression in control cells.

The results showed a clear down regulation of *Sox6* while *Tbox2* (1.4 folds) and *Sox4* (1.8 folds) were increased below significant level and thus appeared relatively unaffected by the absence of *Sox9* (Fig 4). Down regulation of *Sox6* in *Sox9* knocked down cells is in consort with earlier studies which place *Sox6* along with its redundant partner *Sox5* into the regulatory pathway of Sox9 (Lefebvre et al., 1998). Thus its gene expression level in *Sox9*-silenced cells could be used as an indicator of a successful *Sox9* knockdown exerting an overt effect on other genes. It was also observed that 48 hours of gene silencing is sufficient to display an effect on downstream regulated genes as half life of *Sox9* mRNA, like for most of the

transcription factors, is relatively short and has been reported to be between 1.2- 2.5 h (Sekiya et al., 2001; Kinkel and Horton, Jr., 2003).

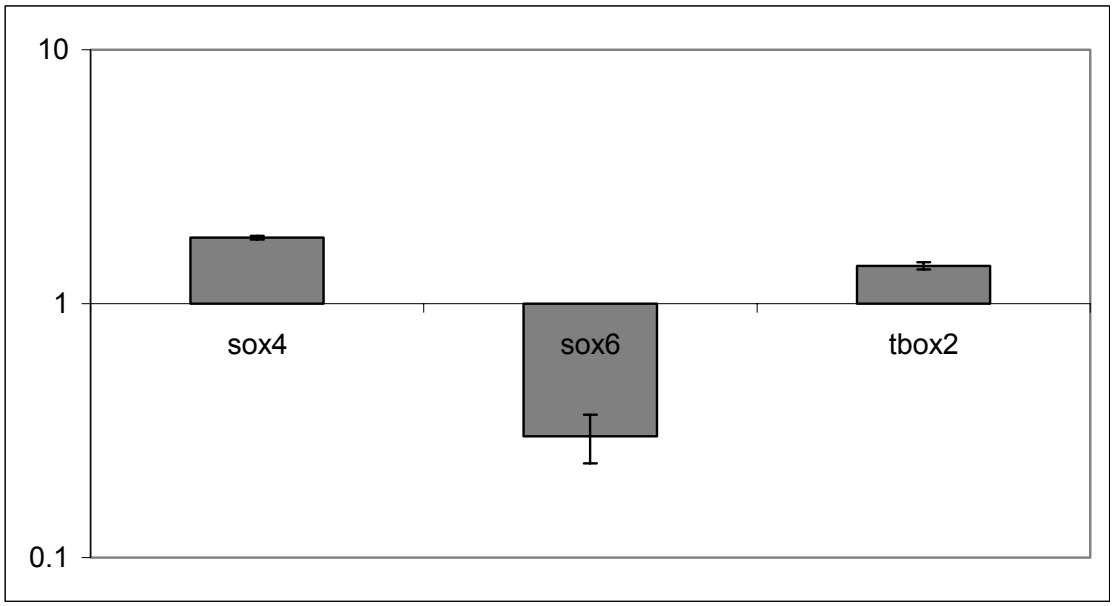

Figure 4. Relative qPCR analysis after 48 hours of Sox9 knockdown shows reduction in Sox6 mRNA level while Tbox2 and Sox4 expression became not significantly elevated.

Sox4 is known to be expressed in osteoblast cell lines and has been localized to the zone of hypertrophic chondrocytes in the growth plates of developing foetal mouse hind limbs. This suggests an integral role of Sox4 in maturation of osteoblasts (Reppe et al., 2000). Here we indicate for the first time that Sox4 gene expression is not influenced by absence of Sox9. Tbox2 is a transcription factor containing a T-box DNA binding domain. This family of transcription factors is implicated in regulation of the FGF receptor 3 signalling pathway which is involved in induction of chondrogenesis in MSCs. In addition it contains receptors which bind to FGFs and TGFß, both of these growth factors are known to influence skeletal development. On these grounds *Hoffman et al* have argued a role for *Tbox2* in the chondrogenic differentiation pathway independent or even prior to *Sox9* (Hoffmann et al., 2002). Our results are in consort with this study which places Tbox2 not in the Sox9 regulatory cascade but rather parallel to it.

In conclusion, we have successfully silenced chondrogenic master regulator Sox9 in a mouse fibroblast cell line by inducing RNAi employing shRNA placed into a retroviral gene delivery vector. We could demonstrate effect on *Sox6* gene expression level, one of the major regulatory transcription factors downstream of Sox9. We also suggest *Sox4* and *Tbox2* not to be directly regulated by Sox9. This study provides a gene silencing system for future studies on regulation of chondrogenesis and early stages of osteogenesis. The system was designed to knockdown *Sox9* in MSCs to study possible redundancies in *Sox9* driven chondrogenic gene cascade. At a later stage the system is to be adapted to an inducible knockdown system where *Sox9* gene expression will be knocked down during different stages of chondrogenesis to identify the role of Sox9 in maintenance of the chondrogenic phenotype. An inducible i*n vitro* knockdown system for *Sox9* has a wide application for osteo- chondrogenic regulation studies.

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Chapter 6

Conclusions

Arguably one of the most challenging complex tissues for regenerative medicine is articular cartilage. It's avascular nature, dense extracellular matrix, few cells, and no known specific progenitor cells are but a few issues (Cancedda et al., 2003; Hunziker, 2002). For *ex vivo* cartilage regeneration multipotent adult stromal MSCs are the tool of choice because of their *in vitro* pliability and retention of differentiation potential (Barry, 2003). In addition, for cell based cartilage therapy use of MSCs instead of chondrocytes as cell source also avoids donor site morbidity (Cancedda et al., 2003). However, production of tissue engineered functional cartilage with articular characteristics is still a challenge. To effectively address this problem employment of the two elements of tissue engineering, the scaffolding material and the cells, are needed to be optimized. Thus, studies aiming at better understanding of the chondrogenic differentiation cascade and the molecular regulatory mechanisms are essential.

This thesis concentrated on external regulation and influence on chondrogenesis in MSCs. Indisputable effects of microenvironment on proliferation, growth and differentiation of MSCs were the intellectual background. The experimental approach was coculture of different cell types to explore the impact of microenvironment on chondrogenically differentiating MSCs *in vitro*. Regulatory effects of both the native and artificial environment were reported after analyzing the gene expression and protein translation data. The study was carried out in a rat model system which is not well characterized for MSCs based differentiation studies; therefore, pre-screening studies were a prerequisite. Often in literature it has been pointed out that MSCs change their antigenic profile and subsequently loose plasticity upon *in vitro* expansion (Bianco and Robey, 2001). Thus, prior to differentiation studies, MSCs were characterized upon their antigenic profile and CFU-F forming capability in **chapter 2**. The colonogenic cells were expanded and found to be predominantly positive for the known positive markers of cells of stromal origin as CD71, CD106, CD49a and D7Fib (Pittenger et al., 1999). However magnetic and fluorescence sorting of the cell population also revealed presence of 20-30% CD45-positive cells of haematopoietic origin **(chapter 2, 3)**. The

expanded MSCs were screened for osteo-chondro related basal gene expression and secretion profiles of cytokines, growth factors and proteases and the differences among this basal profile were compared with that of the differentiated cells. *Col1a1* was identified as the most highly expressed gene in undifferentiated MSCs which could be used as one of the markers of marrow derived MSCs. The high expression of master regulators of chondrogenesis and osteogenesis, *Sox9* and *Cbfa1* respectively, in the un-induced MSCs could be an indicator of commitment to differentiation towards osteo-/chondrogenic lineages (Bianco et al., 2001). These cells also showed retained osteogenic and chondrogenic potential as demonstrated by histology. This comprehensive screening of rat marrow derived MSCs serves as a continual reference point for differentiation and gene regulation studies.

In **chapter 3** the effect of bone marrow native environment was determined on chondrogenically differentiating MSCs *in vitro*. Bone marrow microenvironment is a complex structure where HSCs are retained until they mature and are released into circulation. The structural support to these cells is provided by stroma and stromal cells as MSCs, known to be precursors of osteoblasts, chondrocytes, fibroblasts, adipocytes, endothelial cells and myocytes (Pittenger et al., 1999). Osteogenesis and heamtopoiesis occurs in close proximity; osteoblast progenitors influence haematopoiesis and haematopoietic precursor cells generate osteoclasts essential for bone resorption (Begg et al., 1993; Yin and Li, 2006). Therefore, the *in vitro* chondrogenic capacity of the mixed population of MSCs (CD45-negative) and HCs (CD45-positive) was compared with that of the pure MSCs population to determine the effect of CD45- positive bone marrow cells on CD45-negative stromal cells or MSCs. The gene expression analysis revealed an up regulation in mRNA level of chondrogenic marker genes as *Sox9*, *col2a1 col10a1* and *COMP* in the mixed population. The results indicate that CD45-positive cells may create a microenvironment which helps in promotion of chondrogenesis under specified culture conditions and thus support chondrogenesis in MSCs *in vitro*. Stem cells are regulated by environmental signals and intrinsic regulatory mechanisms; this *in vitro* study indicates potential involvement of HSCs/ HCs in regulation of chondrogenesis.

Chapter 4 dealt with articular cartilage released factors and their putative paracrine effect on early and late chondrogenic cascade in MSCs. Articular cartilage is a known paracrine organ as it maintains its differentiation status via continual regulation through extracellular signals. Here, it was shown that the articular cartilage when cultured in the vicinity of MSCs influences chondrogenesis in MSCs. The cartilage explant and MSCs were cocultured in absence of external growth factors in chambers separated by a 1µm porous membrane which limited the contact to paracrine level. The most curious and exciting discovery was

suppression of collagen X expression and release from the MSCs under the influence of cartilage. Collagen X is accepted as beacon of hypertrophy (Reddi, 1994; Goldring et al., 2006) its lack indicates delayed hypertrophy due to signalling factors secreted by cartilage. Appearance of collagen X is indicative of progressing endochondral ossification while maintenance of stable chondrogenic phenotype requires suppression of collagen X. For MSCs based *ex vivo* regeneration of articular cartilage with sustained phenotype, it is vital to understand and control collagen X regulation via extrinsic factors. The identified soluble signaling molecules including MMP-13, TIMP-2 and VEGFα are proposed to be partially responsible for this delay by effecting the gene regulation and/ or biosynthesis. MMP-13 has a dual role during hypertrophy, first is the conventional role of the collagenase involved in matrix turnover secondly, it is indicated to be a regulator of collagen X gene expression (Inada et al., 2004). VEGFα can also activate collagen X inhibiting DNA binding proteins; additionally it indirectly promotes MMP-13. The identified molecules may create a chain of events causing overt collagen X suppression. Interestingly expression of *Sox9*, the main transcriptional factor of chondrogenesis, was found to be stimulated by the factors released by cartilage tissue. This effect was observed in absence of external growth factors required for chondrogenic differentiation *in vitro*. Most likely growth factor Tgfß-3, secreted by cartilage is responsible for this early up regulation. After one week of culture an overall decline in *Sox9* mRNA was observed irrespective of Tgfß-3, however, this decline did not hinder the progressive up regulation of pro cartilage structural genes. Sox9 is integral for induction of chondrogenic differentiation, however, its role in sustenance of chondrogenic phenotype in our system remained ambiguous (Lefebvre and Smits, 2005). To find out true hierarchy of Sox9 in chondrogenesis in **chapter 5** a method was developed to knock down *Sox9* by RNA interference. The silencing of *Sox9* was successfully established in the NIH3T3 fibroblast cell line by shRNA delivered by a retroviral expression system. The initial effect on downstream regulatory genes indicated a decline in mRNA of *Sox6* while *Sox4* and *Tbox2* remained uninfluenced. The establishment of this system proves that the major chondrogenesis regulator transcription factor *Sox9* could be knocked down in cell culture. The importance of this system is reflected by the fact that *Sox9* homozygous knockout mouse system is unachievable due to prenatal mortality (Akiyama et al., 2002). Studies have also indicated that over-expression of *Sox9* does not directly relate to over induction of chondrogenesis, therefore involvement of other signalling molecules is likely (Furumatsu et al., 2005). This knockdown system will be of valuable help in future to identify direct and indirect role of *Sox9* in chondrogenic regulation.

In conclusion, this study on paracrine effects of microenvironment during MSCs commitment and differentiation demonstrated that the chondrogenic differentiation pathway of rat bone marrow derived MSCs is influenced by the microenvironment (Fig 1). The observation of the paracrine effect of cartilage tissue on collagen X is a foundation for future studies on the identified soluble factors to discover a direct correlation between the effect and the putative effectors. The presented paracrine coculture system between cartilage and MSCs and the coculture of different bone marrow populations could be used for gene and protein regulation studies in future. Identification of signalling molecules responsible for cell fate determination are empirical for cartilage regenerative medicine.

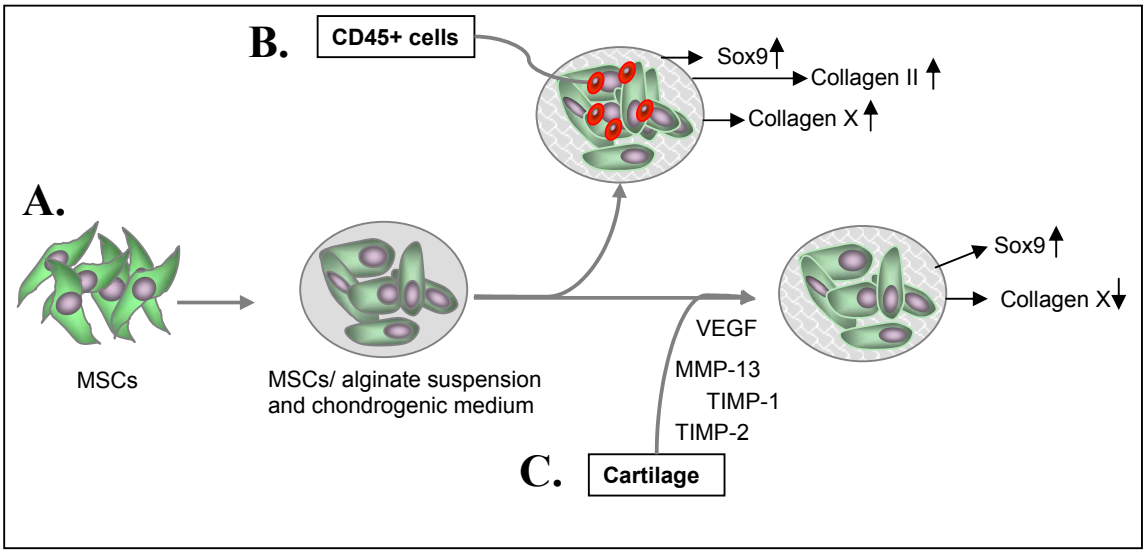

Figure 1: 'Influencing chondrogenesis' in a nutshell: A summarized schematic diagram of main results acquired during this study shows chondrogenic differentiation of rat bone marrow derived MSCs upon suspension in alginate and culture in defined chondrogenic medium (A) **(Chapters 2 and 3)**. The up regulatory effect of CD45-positive cells on chondrogenic differentiation of MSCs (B) **(Chapter 3)**. Early induction and delayed hypertrophy induced on differentiating MSCs via soluble factors secreted by cartilage tissue (C) **(Chapter 4)**.

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Abstract

Cartilage damaging injuries or degenerative diseases like osteoarthritis are restrictive and painful for normal mobility. Damaged articular cartilage has limited regeneration capacity mainly due to lack of chondroprogenitor cells. Chondrocytes are the only cell type of cartilage and pluripotent mesenchymal stem cells (MSCs) are the only known adult chondrocyte progenitor cells. MSCs have shown promise for tissue engineered therapeutic approach for focal defects in articular cartilage. However, production of functional cartilage with articular characteristics is still a challenge. Therefore, it is important to identify the molecular events and regulatory mechanisms characterizing chondrogenesis in MSCs.

This thesis has addressed external regulation of chondrogenesis in MSCs. Paracrine signaling factors from the surrounding cells are known to modulate differentiation and proliferation in surrounding cells, thus, the impact of native and acquired microenvironment on differentiation of MSCs was investigated *in vitro*. Two interesting observations concerning chondrogenesis were reported. Firstly the major population of bone marrow the haematopoietic cells support chondrogenesis in MSCs *in vitro*. Gene expression of all the chondrogenesis related genes was found to be many folds increased when the MSCs were cocultured with 20-30% CD45-positive haematopoietic cells. Secondly we found out that articular cartilage when cultured in the vicinity of MSCs also exerts affect on chondrogenesis in MSCs. Both of the findings indicate that signaling molecules originating from differentiated cells influence chondrogenesis. We also identified some of the soluble signaling molecules including MMP-13 TIMP-1, TIMP-2, Tgfß-3 and VEGFα. Interestingly *Sox9*, the main transcriptional factor of chondrogenesis, was found to be stimulated by factors released from cartilage. The most curious observation was suppression of collagen X secretion from MSCs under the influence of cartilage. The identified soluble molecules like MMP-13, VEGFA and TIMP-2 may have a role to play in suppression of collagen X while Tgfß-3 can be cause of induction of *Sox9*.

The presented paracrine coculture system between cartilage and MSCs and the coculture of different bone marrow populations could be used for gene and protein regulation studies in future. Identification of signalling molecules responsible for cell fate determination and molecular control are empirical for cartilage regenerative medicine.

Zusammenfassung

Degenerative Erkrankungen des Knorpels wie Osteoarthritis oder Verletzungen im Gelenk sind sehr schmerzhaft und schränken die normale Beweglichkeit und Lebensqualität ein. Die natürliche Regenerationskapazität geschädigten Gelenkknorpels ist jedoch aufgrund des Mangels an Knorpelvorläuferzellen stark eingeschränkt. Knorpel wird nur von Chondrozyten aufgebaut und pluripotente mesenchymale Stammzellen (MSCs) sind die einzigen bekannten adulten Vorläuferzellen von Chondrozyten. MSCs bilden einen vielversprechenden therapeutischen Ansatz um mit Hilfe des Tissue Engineering Defekte im artikulären Knorpel zu behandeln. Es stellt jedoch eine große Herausforderung dar, funktionellen Knorpel mit artikulärer Charakteristik herzustellen. Deshalb ist es von großer Bedeutung, Abläufe auf molekularer Ebene und Regulationsmechanismen der Chondrogenese zu identifizieren.

Diese Arbeit beschäftigt sich mit der externen Reglulation der Chondrogenese in MSCs. Es ist bekannt, dass parakrine Faktoren der umgebenden Zellen die Differenzierung beeinflussen; darum wurde die Rolle von nativem Knorpel und dessen Mikoumgebung in der Differenzierung von MSCs *in vitro* untersucht. Dabei wurden zwei interessante Beobachtungen gemacht: 1. Den Großteil der Zellpopulation des Knochenmarks bilden hämatopoetische Zellen welche die Chondrogenese in MSCs *in vitro* unterstützen. Die Expression aller Gene, die in Zusammenhang mit der Chrondrogenese stehen, wurde durch die Kokultur mit 20-30% CD45-positiven hämatopoetischen Zellen um ein vielfaches erhöht.

2. Ausserdem fanden wir heraus, dass artikulärer Knorpel in Kokultur mit MSCs einen positiven Effekt auf die Chondrogenese der MSCs ausübt. Diese beiden Resultate zeigen, dass Faktoren, die von differenzierten Zellen stammen, auf die Chondrogenese Einfluss nehmen. Wir konnten des Weiteren einige lösliche Signalmoleküle identifizieren wie z.B. MMP-13, TIMP-1, TIMP-2, Tgfß-3 and VEGFα. Interessanterweise wird *Sox9*, der wichtigste Transkriptionsfaktor der Chondrogenese durch von Knorpel sezernierte Faktoren stimuliert. Eine aussergewöhnliche Beobachtung war, dass in der Anwesenheit von Knorpel die Kollagen X Sekretion durch MSCs unterdrückt wurde. Die löslichen Faktoren wie MMP-13, VEGFα und TIMP-2 spielen möglicherweise eine Rolle in der Suppression von Kollagen X, während Tgfß-3 *Sox9* induzieren kann.

Das dargestellte parakrine Kokulturmodell zwischen Knorpel und MSCs und der Kokultur verschiedener Knochenmarks-Zellpopulationen kann in der Zukunft für Gen- und Protein-Regulationsstudien verwendet werden.

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Papers:

Ahmed N., Grifka J. and Grassel S. (2006) Cytokine secretion and gene expression profile of rat chondro-progenitor marrow stromal cells. *Submitted: Cell and tissue research*

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Ahmed N and Ahmed M. Innervation pattern in the capsule of club foot. 14th International Orthopaedic Conf. Peshawar, Pakistan, 6th -9th Nov, 1999

Poster presentations:

Ahmed N., Dreier R., Grifka J. and Grassel S.Role of cartilage derived factors in chondrogenic differentiation of mesenchymal stem cells. XXth FECTS- Federation of European Connective Tissue Society meeting. Oulu, Finland, 1st- 5th July, 2006

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