TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 938

MEDICA - ODONTOLOGICA

FACTORS REGULATING CHONDROGENIC DIFFERENTIATION

by

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TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2010

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ISBN 978-951-29-4481-1 (PRINT)
ISBN 978-951-29-4482-8 (PDF)
ISSN 0355-9483
Painosalama Oy - Turku, Finland 2010

To my family

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Factors Regulating Chondrogenic Differentiation
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ABSTRACT

Chondrogenesis is a co-ordinated differentiation process in which mesenchymal cells condensate, differentiate into chondrocytes and begin to secrete molecules that form the extracellular matrix. It is regulated in a spatio-temporal manner by cellular interactions and growth and differentiation factors that modulate cellular signalling pathways and transcription of specific genes. Moreover, post-transcriptional regulation by microRNAs (miRNAs) has appeared to play a central role in diverse biological processes, but their role in skeletal development is not fully understood.

Mesenchymal stromal cells (MSCs) are multipotent cells present in a variety of adult tissues, including bone marrow and adipose tissue. They can be isolated, expanded and, under defined conditions, induced to differentiate into multiple cell lineages including chondrocytes, osteoblasts and adipocytes *in vitro* and *in vivo.* Owing to their intrinsic capability to self-renew and differentiate into functional cell types, MSCs provide a promising source for cell-based therapeutic strategies for various degenerative diseases, such as osteoarthritis (OA). Due to the potential therapeutic applications, it is of importance to better understand the MSC biology and the regulatory mechanisms of their differentiation.

In this study, an *in vitro* assay for chondrogenic differentiation of mouse MSCs (mMSCs) was developed for the screening of various factors for their chondrogenic potential. Conditions were optimized for pellet cultures by inducing mMSC with different bone morphogenetic proteins (BMPs) that were selected based on their known chondrogenic relevance. Characterization of the surface epitope profile, differentiation capacity and molecular signature of mMSCs illustrated the importance of cell population composition and the interaction between different populations in the cell fate determination and differentiation of MSCs. Regulation of Wnt signalling activity by Wnt antagonist sFRP-1 was elucidated as a potential modulator of lineage commitment. Delta-like 1 (dlk1), a factor regulating adipogenesis and osteogenesis, was shown to exhibit stage-specific expression during embryonic chondrogenesis and identified as a novel regulator of chondrogenesis, possibly through mediating the effect of TGF-β1. Moreover, miRNA profiling demonstrated that MSCs differentiating into a certain lineage exhibit a specific miRNA expression profile. The complex regulatory network between miRNAs and transcription factors is suggested to play a crucial role in fine-tuning the differentiation of MSCs.

These results demonstrate that commitment of mesenchymal stromal cells and further differentiation into specific lineages is regulated by interactions between MSCs, various growth and transcription factors, and miRNA-mediated translational repression of lineage-specific genes.

Keywords: Mesenchymal stromal cell, chondrogenesis, dlk1, signalling pathway, microRNA

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Ruston erilaistumista säätelevät tekijät
Lääketieteellinen biokemia ja genetiikka, Biolääketieteen laitos, Lääketieteellinen tiedekunta, Turun yliopisto ja Turun biolääketieteellinen tutkijakoulu, Turku
Annales Universitatis Turkuensis, Medica-Odontologica, 2010

TIIVISTELMÄ

Mesenkyymisolut ovat monikykyisiä kantasoluja tai niiden pidemmälle erilaistuneita esiasteita, joita voidaan eristää mm. luuytimestä ja rasvakudoksesta. Soluja voidaan viljellä ja erilaistaa useiksi solutyypeiksi kuten luu-, rusto- ja rasvasoluiksi. Solujen kyky jakaantua ja erilaistua toiminnallisiksi solutyypeiksi on herättänyt runsaasti kiinnostusta ja toiveita niiden mahdollisesta käytöstä useiden rappeumatautien, kuten nivelrikon hoidossa. Jotta mesenkyymisoluja voitaisiin hyödyntää kantasolusiirre-teknologiassa, on tärkeää tuntea niiden biologiset ominaisuudet ja erilaistumiseen vaikuttavat biokemialliset ja molekylaariset mekanismit.
Raajankehitykseen liittyvä ruston erilaistuminen on tarkoin säädelty tapahtuma, jossa mesenkyymisolut kondensoituvat, erilaistuvat rustosoluiksi ja alkavat tuottaa soluväliaineen molekyylejä. Solujen välinen vuorovaikutus keskenään ja soluväliaineen kanssa sekä erilaiset kasvutekijät säätelevät solulinjalle ominaisten signaalinvälitysreittien toimintaa ja tärkeiden geenien ilmenemistä. Lisäksi mikroRNAt, RNA-molekyylit, jotka estävät proteiinisynteesin kiinnittymällä tietyn geenin lähettiRNAhan, ovat osoittautuneet avaintekijöiksi useiden biologisten toimintojen säätelyssä. Niiden merkitys luuston kehityksessä on vielä osittain tuntematon.
Työ perustuu viiteen osatyöhön, joissa tutkittiin mesenkyymisolujen erilaistumiseen eri tasoilla vaikuttavia tekijöitä. Aluksi kehitettiin soluviljelymalli mesenkyymisolujen erilaistamiseksi rustosoluiksi, jota hyödynnettiin muissa osatöissä rustosoluiksi erilaistumiseen vaikuttavien tekijöiden tutkimisessa. Solupopulaation koostumus vaikutti solujen kykyyn erilaistua rustosoluiksi. Vähemmän valikoitu solupopulaatio erilaistui nopeasti, mutta mineralisoitui, kun taas alkeellisempia mesenkyymisoluja sisältävä populaatio erilaistui hitaasti, mutta vältti epäedullisen mineralisaation. Kahta mesenkyymisolulinjaa vertailemalla havaittiin, että Wnt -signaalireitin vaimentaminen sFRP-1 -proteiinin välityksellä ohjasi soluja erilaistumaan rasvasoluiksi, kun taas Wnt-signalointi oli tärkeää luusolujen ja rustosolujen suuntaan ohjaavassa erilaistumisessa. Työssä havaittiin myös, että mesenkyymisolujen erilaistumista rasva- ja luusoluiksi säätelevää delta-like 1 (dlk1) -proteiinia tuotetaan myös kehittyvän ruston kypsyvissä ja jakaantuvissa soluissa, mutta ei lainkaan lopullisesti erilaistuneissa rustosoluissa. Sen havaittiin säätelevän ruston varhaiskehitystä mahdollisesti TGF-β -signalointireitin välityksellä. Lisäksi tiettyjen mikroRNA-molekyylien, transkriptiotekijöiden ja muiden geenien välinen verkosto on mahdollisesti yksi tärkeistä mesenkyymisolujen erilaistumista säätelevistä tekijöistä.
Työssä esitettyjen tulosten perusteella mesenkyymisolujen erilaistumista säädellään usealla tasolla. Solujen väliset vuorovaikukset, kasvu- ja transkriptiotekijät ja niiden väliset vuorovaikutukset sekä mikroRNA –molekyylien aikaansaama proteiini-synteesin säätely ovat tärkeitä solujen erilaistumista sääteleviä tekijöitä.
Avainsanat: Mesenkyymisolu, ruston erilaistuminen, signaalinvälitysreitti, dlk1, mikroRNA

TABLE OF CONTENTS

ABBREVIATIONS

Acan	The murine gene for aggrecan
ALK	Activin receptor-like kinase
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancing binding protein
D-MEM	Dulbecco's modified Eagle's medium
Col1a1	The murine gene for proα1(I) collagen
Col2a1	The murine gene for proα2(II) collagen
COMP	Cartilage oligomeric matrix protein
dlk1/Pref-1/FA1	delta-like 1/preadipocyte factor-1/ fetal antigen 1
ESC	Embryonic stem cell
ECM	Extracellular matrix
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GDF	Growth and differentiation factor
HRP	Horseradish peroxide
IGF	Insulin-like growth factor
Ihh	Indian hedgehog
iPSC	Induced pluripotent stem cell
KO	Knock out
LEF	Lymphoid enhancer binding factor
LTS	Long-term selected
LRP	Low lipoprotein receptor-related protein
MEF	Mouse embryonic fibroblast
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal cell
OA	Osteoarthritis
PTHrP	Parathyroid hormone-related peptide
PPARγ	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Quantitative real time polymerase chain reaction
RISC	RNA-induced silencing complex
Runx2	Runt family transcription factor 2
Sox	Sex-determining region Y–type high motility group box
TG	Transgenic
TGF-β	Transforming growth factor β
Sca-1	Stem cell antigen-1
sFRP-1	Secreted frizzled-related protein-1
STS	Short-term selected
TCF	T-cell factor
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WT	Wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-V:

I **Taipaleenmäki H**, Suomi S, Hentunen T, Laitala-Leinonen T, Säämänen A-M. Impact of stromal cell composition on BMP-induced chondrogenic differentiation of mouse bone marrow derived mesenchymal cells. *Experimental Cell Research* 314(13): 2400-10, 2008.

II **Taipaleenmäki H**, Abdallah BM, Aldahmash A, Säämänen A-M, Kassem M. Wnt signalling mediates the cross-talk between bone marrow derived pre-adipocytic and pre-osteoblastic cell populations. Submitted.

III Harkness LM, **Taipaleenmäki H**, Mahmood A, Frandsen U, Säämänen A-M, Kassem M, Abdallah BM. Isolation and differentiation of Chondrocytic Cells Derived from Human Embryonic Stem Cells Using dlk1/FA1 as a Novel Surface Marker. *Stem Cell Reviews and Reports* 5(4):353-68, 2009.

IV **Taipaleenmäki H***, Harkness LM*, Hauberg K, Säämänen A-M, Kassem M, Abdallah BM. Characterization and regulation of dlk1 expression during *in vitro* chondrogenesis. Submitted. *Equal contribution. Submitted.

V Suomi S*, **Taipaleenmäki H***, Seppänen A, Ripatti T, Väänänen K, Hentunen T, Säämänen A-M, Laitala-Leinonen T. MicroRNAs regulate osteogenesis and chondrogenesis of mouse bone marrow stromal cells. G*ene Regulation and Systems Biology* 2 177-191, 2008. *Equal contribution.

In addition, some unpublished data are presented in this thesis.

1. INTRODUCTION

Degenerative cartilage diseases, such as osteoarthritis (OA), affect millions of people worldwide and thus have enormous social and economic consequences. Osteoarthritis is characterized by progressive destruction of articular cartilage and eventually the entire joint, associated with variable degrees of local inflammation, motional pain, swelling of the damaged tissue and thickening of the joints. The internal repair capacity of articular cartilage is poor due to several unique characteristics of the tissue, including slow turnover of cartilage collagen, lack of vascularization and the low number of stem cells that could contribute to the repair. In mature articular cartilage, chondrocytes have little intrinsic potential for repair. Repair may occur by an extrinsic mechanism, which depends on mesenchymal stromal cells (MSCs) in adjacent connective tissue. Several treatment options have been used for cartilage repair in cartilage lesions, including soft tissue crafts, and chondrocyte and osteochondral transplantation. Autologous chondrocyte transplantation studies have shown promising results, but the technique remains controversial and is limited to small lesions. New approaches for treatment are needed and transplantation using MSCs offers a promising novel technique.

MSCs are present in a variety of adult tissues, such as bone marrow and adipose tissue. They are maintained in protected stem cell niches containing various cell types, including haematopoietic stem cells and committed MSC progenitors. MSCs are multipotent cells that have the ability to proliferate extensively and differentiate into multiple cell lineages *in vitro* and *in vivo*, including chondrocytes, osteoblasts and adipocytes. Additionally, their immunosuppressive and paracrine factor-secreting properties make MSCs ideal candidates for therapeutic approaches for treating a variety of degenerative and age-related diseases for which no effective treatment is currently available. As chondrogenic differentiation rarely occurs spontaneously, a better understanding of MSC biology, lineage commitment and regulatory mechanism of chondrogenic differentiation will provide new insights for the improvement of repair therapies.

In vivo, chondrogenesis is initiated by sonic hedgehog signalling, which induces signalling of bone morphogenic proteins (BMPs) and directs MSC differentiation into the chondrogenic lineage. Sox9, a key transcription factor in chondrocyte differentiation, activates expression of several cartilage-specific genes, including *Col2a1, Acan* and *Comp*. In addition, several other transcription factors, such as L-Sox5, Sox6 and Runx2, and growth factors, such as TGF-β and BMPs, are involved in the development of chondrocyte phenotype. Phenotypic changes in the extracellular matrix deposition are well characterized in differentiating chondrocytes. Differentiated chondrocytes express type II collagen, aggrecan and cartilage oligomeric matrix protein (COMP), while hypertrophic chondrocytes synthesize type X collagen. *In vitro,* chondrogenesis is typically carried out in the pellet culture system, which allows cell-cell interactions similar to those occurring in prechondrogenic condensations during

embryonic development. Several growth factors that promote chondrogenesis *in vivo* have been also demonstrated to enhance chondrogenesis of MSC *in vitro*.

Dlk1 is a transmembrane protein of the Notch/Delta/Serrata family. It is an important modulator of cell fate decisions during embryogenesis, and is involved in many differentiation processes in post-natal organisms. Its expression is mainly restricted to embryonic and early postnatal development. Dlk1 has been identified as a negative regulator of MSC differentiation into osteoblasts and adipocytes. Recently, it was shown to play a dual role in chondrogenesis, but the regulatory mechanisms in chondrocyte maturation and hypertrophy have not been clarified.

MicroRNAs (miRNAs) are small single-stranded RNA molecules that bind to the 3' untranslated region of mRNAs and induce translational repression or mRNA degradation. MicroRNAs act as key regulators in diverse biological processes, such as early development, cell proliferation, differentiation, apoptosis and cancer. Several studies indicate that tissues in developing and mature organisms are characterized by unique profiles of miRNA expression. The miRNA profile during MSC differentiation and the specific miRNAs that contribute to the regulation of their differentiation into osteoblasts and chondrocytes still remain to be identified.

The aim of this study was to understand the mechanisms of stem cell differentiation into mesenchymal, particularly chondrogenic, lineages and to identify novel chondrogenic factors functioning at the transcriptional and translational level that affect embryonic and adult stem cell differentiation into chondrocytes.

2. REVIEW OF THE LITERATURE

2.1 Synovial joints

Synovial joints connect weight-bearing bones to each other in the vertebrate skeleton and facilitate mobility by allowing articulation of the bones. Synovial joints develop from the mesenchymal cells and are composed of spezialized connective tissues including cartilage, bone, synovium, tendon and ligaments (Khan *et al*., 2007). Articular cartilage, a thin layer of hyaline cartilage, covers the articular surfaces of the bones. Fibrocartilaginous menisci provide additional support to the joint. A highly vascularized synovium supplies the joint with synovial fluid that nourishes the joint cartilage. A fibrous joint capsule encapsulates the joint and is surrounded by muscles and tendons that connect muscles to adjacent bones. Ligaments provide additional stabilization to the synovial joint by binding the skeletal elements together (Horton 1993).
Interactions between the tissues in the joint determine the balance of molecular signals that regulate homeostasis, damage and remodelling. A finely-tuned balance is required for a healthy joint, and the maintenance of homeostasis in a damaged joint can be impaired by different factors. Inflammatory factors released from the bone and cartilage have been suggested to trigger a non-specific inflammation in the synovium. Accumulation of interstitial fluid, leukocytes and synovial macrophages in the joint further increases pain and loss of function of the synovial joint. Moreover, cytokines and tissue destructive enzymes produced by the synovium can contribute to cartilage destruction (Lories, 2008).

2.2 Cartilage

Cartilage is a an avascular, aneural and alymphatic connective tissue present in many sites of the vertebrate body, including the articulating joints between bones, rib cage, ear, nose, bronchial tubes and intervertebral discs. It is essential for breathing, hearing, articulation and locomotion. During embryogenesis, cartilage anlagen develop before bone and provide the first skeleton of the embryo. Development of cartilage is needed for body growth and it provides structural templates for most bones in the skeleton (Olsen *et al*., 2000). Cartilage is classified into three subtypes based on its morphology and the composition of the extracellular matrix (ECM); hyaline, elastic and fibrocartilage. The most abundant of these is hyaline cartilage which is present in long bones, on articular surfaces, in the nose and in the respiratory tract. It is composed of type II collagen and chondrotin sulfate and is the only cartilage subtype that can sustain mechanical strain.

Unlike other connective tissues, cartilage does not contain blood vessels. The chondrocytes in articular cartilage are nourished and their waste products are removed by diffusion from and to the surrounding perichondrium, epiphyseal plate capillaries, and via synovial fluid to the synovial membrane. Cartilage is repaired at a slower rate than other connective tissues. This is due to the lack of the three-step wound healing

process present in other tissues, which consist of three phases; inflammation, proliferation and tissue remodelling. Several unique properties of the cartilage tissue, which are discussed in more detail in the other chapters, contribute to its poor wound healing and repair processes (Table 1.)

Table 1. Unique properties of cartilage

Feature/structure	Property
Cells	Relatively acellular tissue (2-10% of tissue volume) Only one specialized cell type, chondrocytes →Highly differentiated →Limited proliferation capacity
Collagen fibrils	Half-life of collagen is long → Mature collagen does not renew →Integration of the repair tissue into the surrounding tissue is problematic
Lack of blood vessels, lymphatic vessels and nerves	Nutrients diffuse from the surrounding tissues → Low oxygen pressure, anaerobic metabolism No bleeding, lymphatics or macrophages → Removal of debris and damaged tissue is difficult → Lack of would healing No pain or other symptoms until the injury has reached other tissues

2.2.1 Articular cartilage

Articular cartilage is hyaline cartilage that covers bone heads in a diarthrodial joint and is underlined by a subchondral bone plate (Poole *et al*., 2001). It has an articulating surface that abuts the synovial joint cavity. Articular cartilage, with the synovial fluid, provides an almost frictionless articulation in the joint and serves to absorb and dissipate the loads applied by the body weight and locomotion.

The articular cartilage is nonmineralized and is traditionally divided into three pseudostratified zones (the superficial, intermediate and deep zone) that have a distinct cell density and organization of collagen fibrils (Figure 1). Collagen fibrils are arranged to run tangential to the joint surface in the superficial zone, randomly in the intermediate zone, and radially to the articular surface in the deepest zone. Collagen content is highest in the superficial zone while proteoglycan content is highest in the deep zone. The articular cartilage is separated by a tidemark from the calcified cartilage that lies next to the subchondral bone plate. The matrix surrounding the chondrocytes also has a regional organization. The chondrocytes are surrounded by a proteoglycan-rich pericellular matrix forming a structural, functional and metabolic unit of cartilage called the ‘chondron’ (Poole, 1997).

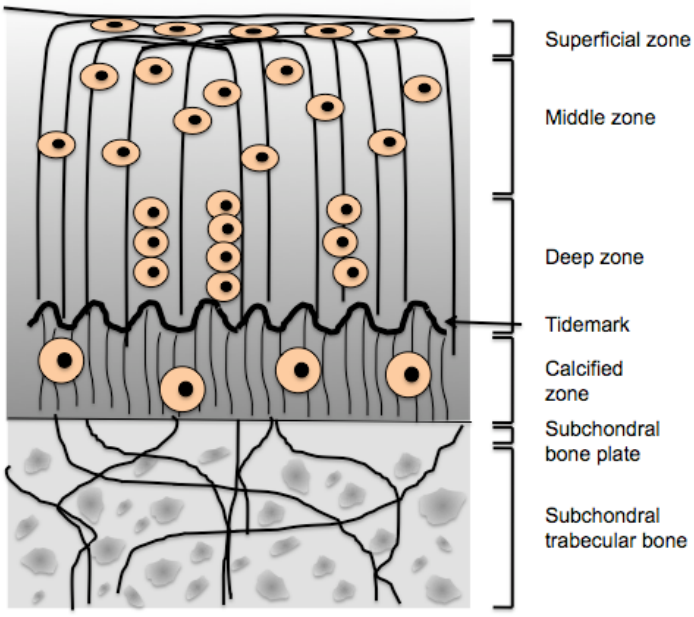


Figure 1. A schematic representation of the structure of articular cartilage. Different zones and regions of the articular cartilage and subchondral bone are shown. Modified from Säämänen *et al.* (2010).

Articular cartilage in adults is a comparatively acellular tissue in which chondrocytes form only about 2-10 % of the tissue volume (Mitrovic *et al.*, 1983). The majority of the cartilage consists of an extracellular matrix, which is responsible for the biomechanical properties of the tissue. These properties result from the interplay between the two major components of the extracellular matrix, collagens and proteoglycans, and their interactions with water.

Collagen is the most abundant protein in mammals. Collagen molecules form fibrillar elements that are present in the extracellular space of connective tissues. Twentynine different types of collagen composed of at least 46 distinct polypeptide chains have been identified thus far. Collagen molecules are composed of three parallel polypeptide strands in a left-handed, polyproline II-type helical conformation (Shoulders and Raines, 2009). Type II collagen is the most abundant collagen in cartilage. It associates with the type XI and type IX collagens to form co-polymeric bundles that are organized into a fibrous network. This fibrous network restricts overswelling of the hydrophilic proteoglycan substance and is responsible for the tensile strength of the tissue. Minor quantities of other types of collagen, including types VI, XII, and XIV, are found in the cartilage (Eyre, 2002). Additionally, type XXVII collagen, a developmentally regulated collagen, is present in cartilage (Jenkins *et al.*, 2005). Type X collagen is produced exclusively by prehyperthophic and hyperthrophic chondrocytes. Type I and type III collagens are expressed in degenerating cartilage.

Hydrophilic proteoglycans create the elastic component of the tissue. Proteoglycans are macromolecules containing glycosaminoglycans (GAGs), long, unbranched polysaccharide side chains attached to a linear core protein. The most abundant proteoglycan in cartilage is aggrecan. It contains two types of GAGs, keratan sulphates and chondroitin sulphates, which are composed of repeating disaccharide units carrying negatively-charged sulphate and carboxyl groups that attract sodium and other cations. This causes osmotic pressure and swelling of the proteoglycans, thus providing the elastic properties of the tissue. Aggrecan molecules form large aggregates by binding non-covalently to hyaluronans and link-proteins with a molecular weight reaching millions of Daltons.

The abundance of glycoproteins such as COMP, chondroadherin, chondromodulin, matrilin 1, perlecan, versican, and small leucine-rich proteoglycans (fibromodulin, lumican, biglycan and decorin) vary according to the type of cartilage and the regions of the cartilage elements involved (Heinegard, 2009). They have various functions, including regulation of the collagen fibril assembly and organization. Additionally, they bind growth factors, thereby modulating their tissue distribution and activity. Regulation of growth factor organization and presentation by proteoglycans has an important function in the maintenance of stem cell niches in various tissues (Bi *et al*., 2007).

2.2.2 Epiphyseal growth plate

Epiphyseal growth plates develop at the ends of long bones and are responsible for the longitudinal growth of the long bones (Figure 2). They consist of a continuum of three zones; resting, proliferating and hypertrophic zones, named according to the morphology, maturation stage of the chondrocytes and the composition of the ECM. These zones can be detected during embryonic development, but the growth plate becomes more evident after birth when the secondary ossification centre develops and separates it from the developing articular cartilage. The resting (periarticular) zone of the growth plate is hyaline cartilage and located closest to the bone end. It is responsible for providing chondrocytes to the differentiation pathway. The chondrocytes in the resting zone are spherical, randomly arranged and relatively quiescent (Hunziker, 1994). Dividing chondrocytes in the proliferating zone are responsible for the bone growth. The chondrocytes are flat, arranged in longitudinal columns parallel to the axis of growth and produce large amounts of ECM components. In the prehypertrophic zone, mature chondrocytes stop dividing, enlarge and continue to terminal differentiation in the hypertrophic zone (Cowell *et al*., 1987). Hypertrophic chondrocytes in the osteochondral junction synthesize the vascular endothelial growth factor (VEGF), which is released from the matrix and results in invasion of blood capillaries (Ferrara *et al.*, 2003). This is followed by chondrocyte apoptosis or transdifferentiation into osteoblasts (Cancedda *et al.*, 1995). Most of the cartilaginous matrix is degraded by osteoclasts/chondroclasts (Karsenty, 1999).

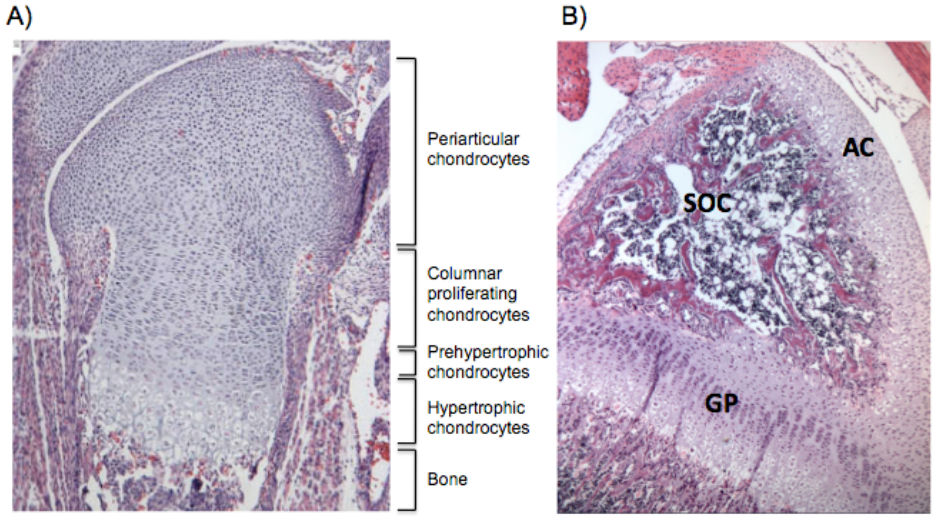


Figure 2. Cellular organization in the developing epiphyseal head of A) a newborn and B) a 10-day-old mouse distal femur. A) Different zones and chondrocyte morphologies present in the newborn epiphysis are demonstrated by histological staining with haematoxylin and eosin. B) The growth plate (GP) and developing articular cartilage (AC) are separated by the secondary ossification centres (SOC) that appear by day 10 in mouse knee epiphyses.

2.3 Chondrogenesis

Chondrogenesis is the earliest phase of skeletal development, involving recruitment and condensation of mesenchymal cells followed by their differentiation into chondrocytes, chondrocyte maturation, and production of ECM (Figure 3). Chondrogenesis depends on signals initiated by cell-cell and cell-matrix interactions, and is associated with increased cell adhesion and formation of cap junctions (Goldring *et al*., 2006). The process is controlled by cellular interactions with the surrounding matrix, growth and differentiation factors and other environmental factors that initiate or suppress cellular signalling pathways and transcription of specific genes in a spatio-temporal manner. Additionally, a number of cytokines trigger intracellular signalling pathways during chondrogenic differentiation (Erlebacher *et al.*, 1995; Mundlos and Olsen, 1997).

The continuum of chondrocyte differentiation can be obtained first in the developing primary ossification centres in the middle diaphysis of the cartilage anlagen of the long bones, but later also surrounding the secondary ossification centres when the articular cartilage and growth plate develop at the bone epiphyses (Figure 2). In these sites, chondrogenesis leads to endochondral ossification, a process in which chondrocytes undergo hypertrophy, subsequent apoptosis and become replaced by bone-forming osteoblasts (Cancedda *et al*., 1995). Each step in the differentiation pathway is characterized by specific histological features, gene expression profiles and cellular activities (Figure 3, 4).

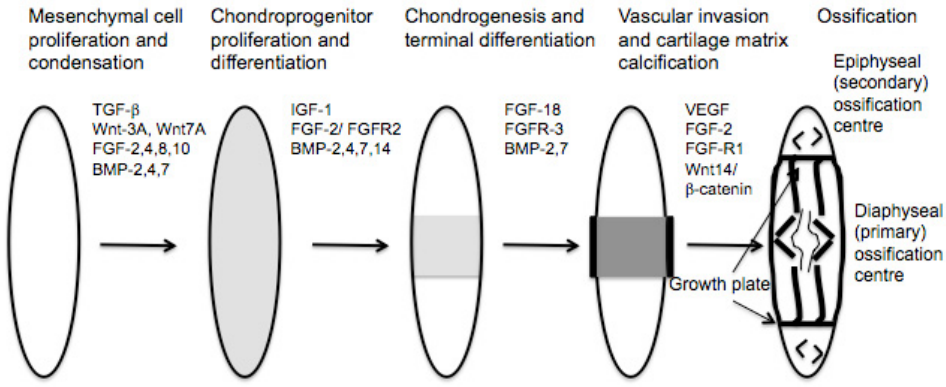


Figure 3. Temporal pattern of chondrogenesis. A schematic representation of secreted factors associated with the development of long bones. Modified from Goldring *et al*. (2006).

2.3.1 Transcriptional regulation of chondrogenesis

In vivo, chondrogenesis in the developing limb bud is initiated by sonic hedgehog signalling, which induces BMPs and directs mesenchymal cell differentiation into the chondrogenic lineage (Figure 4). Sox9 is one of the earliest markers expressed in the mesenchymal condensations and is the key transcription factor in chondrocyte differentiation (Lefebvre *et al.*, 1998). Skeletal precursors also express *Runx2*, a Runt domain transcription factor required for osteoblast differentiation. Sox9 ensures chondrogenesis over osteogenesis by negatively regulating Runx2. It inhibits the Runx2 activity directly by binding to it and indirectly through transcriptional repression mediated by transcription factor Nkx3.2/Bapx1 (Lengner *et al.*, 2005; Yamashita *et al.*, 2009; Zhou *et al*., 2006). Two other Sox family members, L-Sox5 and Sox6, are regulated by and co-expressed with Sox9 and their activity is required for Sox9-mediated transformation of cells into early chondroblasts (Han and Lefebvre, 2008; Lefebvre *et al.*, 2001). Sox9, in concert with L-Sox5 and Sox6, regulates cartilage formation and maintains the chondrocyte phenotype in the mature cartilage by activating expression of several cartilage-specific genes, including genes for the types II, IX and XI collagen (*Col2a1, Col9a1 and Col11a1*), aggrecan (*Acan*) and COMP (*Comp*) (Huang *et al.*, 2001; Ng *et al.*, 1997). The proliferative capacity is maintained by transcription factors, such as members of the activating transcription factor (ATF)/cyclic AMP response element binding protein (CREB) family (Long *et al*., 2001a; Wang *et al.*, 1992).

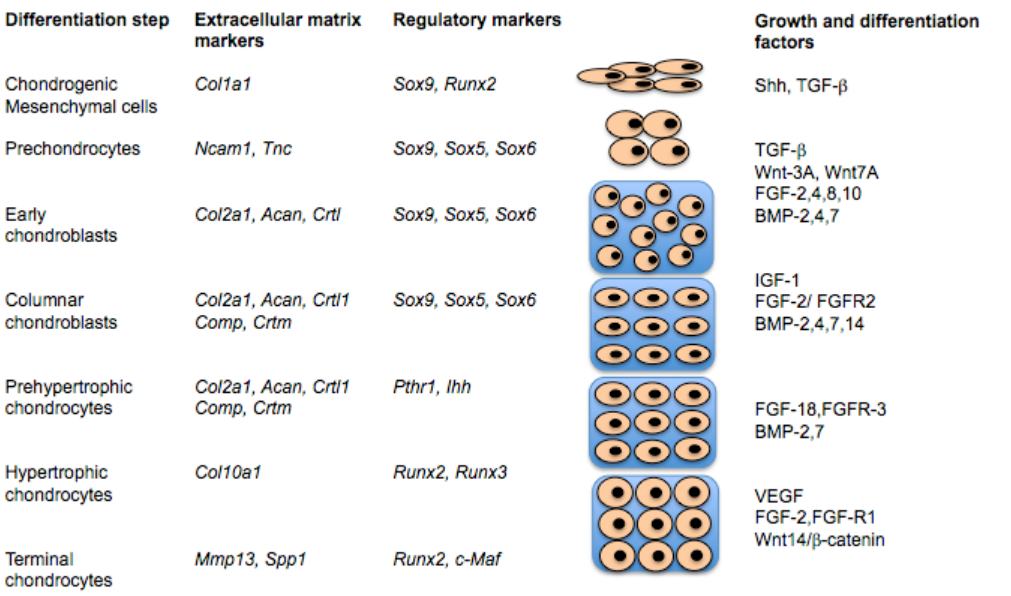


Figure 4. Phases of chondrogenic differentiation. A schematic representation of the different stages of chondrogenesis showing the temporal pattern of extracellular matrix markers, transcription factors, and growth and differentiation factors. Modified from Lefebvre and Smits (2005).

The maturation and hypertrophy of proliferating chondroblasts are under the tight control of negative and positive regulators. Sox9 negatively regulates the maturation process through Nkx3.2-mediated repression of *Runx2* and modulation of nuclear β-catenin levels, and Wnt signalling (Topol *et al.*, 2009). L-Sox5 and Sox6 prevent premature prehypertrophy by down-regulating the *Runx2, Ihh* and *Fgfr3* expression (Lefebvre and Smits, 2005). Thus, the down-regulation of Sox proteins is required for chondrocyte hypertrophy. *Runx2* is expressed in prehypertrophic chondrocytes and is crucial for chondrocyte hypertrophy (Enomoto *et al*., 2000; Enomoto-Iwamoto *et al.*, 2001; Otto *et al.*, 1997; Ueta *et al.*, 2001). It is induced by BMPs and up-regulates matrix metalloproteinase (MMP)-13 (Inada *et al*., 1999; Leboy *et al.*, 2001). Additional transcription factors that positively control chondrocyte maturation and hypertrophy include Runx1, Runx3, Msx2, Fra2 and the basic helix-loop-helix transcription factors Mef2c and Mef3d (Arnold *et al.*, 2007; Karreth *et al.*, 2004; Satokata *et al.*, 2000; Yoshida *et al.*, 2004).

In the growth plate, chondrocyte proliferation and prehypertrophy are controlled by a local negative feedback loop involving parathyroid-related peptide (PTHrP) and Indian hedgehog (Ihh). *PTHrP* is expressed in perichondrial cells in the distal zone of the growth plate. It maintains chondrocyte proliferation and prevents premature hypertrophy via its receptor, which is expressed in the periarticular chondrocytes (Lanske *et al*., 1996). *Ihh* is expressed in the prehypertrophic zone (Bitgood and McMahon, 1995). It accelerates the differentiation of round proliferative chondrocytes

into flat proliferating chondrocytes, increases the rate of proliferation of adjacent chondrocytes, and directs the differentiation of perichondrial cells to osteoblasts (Long *et al.*, 2001b; Vortkamp *et al.*, 1996). Additionally, Ihh increases the expression of *PTHrP* and by the resulting feedback loop maintains chondrocyte proliferation. The chondrocytes located at a sufficient distance from the source of PTHrP stop proliferating, only synthesize Ihh and enter into endochondral ossification (St-Jacques *et al.*, 1999). Ihh also exerts its function independently of PTHrP to promote chondrocyte hypertrophy and regulate the growth plate length. It is expressed in the prehypertrophic chondrocytes as they exit the proliferative phase and begin to express type X collagen (*Col10a1*) and alkaline phosphatase (*Alpl*) (Kobayashi *et al.*, 2005).

Terminal differentiation of the hypertrophic chondrocytes is essential for the final remodelling of the cartilage into bone. This step is controlled by cMaf, a basic leucine zipper protein that is expressed in the late hypertrophic chondrocytes (MacLean *et al.*, 2003). The hypertrophic chondrocytes secrete angiogenic factors, including VEGF, that induce sprouting of angiogenesis from the perichondrium (Gerber *et al.*, 1999). Primary ossification centres are formed as a result of vascular invasion with osteoblasts, osteoclasts and haematopoietic cells. Within the ossification centres, the hypertrophic cartilage matrix is degraded, chondrocytes undergo apoptosis, osteoblasts replace the disappearing cartilage with trabecular bone, and bone marrow is formed (Colnot, 2005). Ablation of VEGF or its receptors results in a defect in the replacement of cartilage by bone, indicating that vascular invasion is required for normal ossification to occur (Gerber *et al.*, 1999; Maes *et al.*, 2002).

The transcriptional control of articular chondroblast and chondrocyte differentiation is yet to be clarified in detail. Articular chondrocytes develop from prechondrocytes that line joint cavities during joint development. Differentiating articular chondroblasts activate the lubricin gene (*Prg4*), and at the end of postnatal development, differentiate into articular chondrocytes (Rhee *et al.*, 2005). The articular chondrocytes express *Sox5, Sox6* and *Sox9* and maintain a high expression of *Agc1* and *Prg4* and low expression of *Col2a1*. Chondrocyte hyperthophy occurs only in the tidemark chondrocytes, which express *Col10a1* and *Runx2* and induce mineralization of the cartilage matrix (Girkontaite *et al.*, 1996). In contrast to the metabolically active growth plate chondrocytes, chondrocytes in the mature articular cartilage have little intrinsic potential for replication. The normal articular chondrocytes rarely divide and never undergo hypertrophy. However, in OA, the articular chondrocytes can revert to an immature chondroblastic stage and undergo hypertrophy accompanied by terminal maturation, indicating that their differentiation stage is normally permanent but not terminal (Lefebvre and Smits, 2005).

Regulation of the growth plate chondrogenesis is trophic and promotes chondrocyte proliferation and differentiation. In contrast, in articular cartilage tight regulation is required to maintain and control the chondrocyte phenotype. It has been suggested that the qualitative and quantitative differences between the regulation and gene expression profiles of growth plate and articular chondrocytes reflect the differences in the

function of the two types of cartilage by maintaining proliferation, differentiation and maturation in the growth plate and preventing chondrocyte hypertrophy in the articular cartilage (Lefebvre and Smits, 2005).

The extracellular matrix plays a crucial role in mesenchymal cell fate determination, chondrocyte development and endochondral ossification (Bi *et al*., 2005). The expression of the cartilage ECM genes is under strict control and ECM, in turn, regulates the signalling pathways to co-ordinate cartilage and bone formation. Absence of ECM proteins, such as type II collagen, type XI collagen, aggrecan, perlecan and link-protein, results in severe skeletal malformations (Arikawa-Hirasawa *et al.*, 1999; Li *et al.*, 1995; Li *et al.*, 2001; Watanabe *et al.*, 1994; Watanabe and Yamada, 1999). However, the final phenotype has been shown to be often a consequence of abnormal ECM rather than the lack of a single molecule, demonstrating the importance of ECM signalling network (So *et al.*, 2001; Wai *et al.*, 1998). ECM integrates several signalling pathways, including the fibroblast growth factor (FGF), BMP and IHH pathways (Arikawa-Hirasawa *et al.*, 1999; Cortes *et al.*, 2009; Yoon *et al.*, 2005c). In addition, the bioactivities of transforming growth factor β (TGF-β), tumor necrosis factor α (TNFα), platelet-derived growth factor and other growth factors are modulated by ECM components that activate or inactivate them by proteolytic processing or directly binding to the cytokine receptors (Gleizes *et al.*, 1997; Hildebrand *et al.*, 1994; Nili *et al.*, 2003; Santra *et al.*, 2002; Tufvesson and Westergren-Thorsson, 2002;). Bone ECM proteins, such as osteocalcin and osteopontin, function in cell-matrix interactions during endochondral ossification. The ECM remodelling is regulated by MMPs including MMP-9, MMP-10, MMP-13 and MMP-14 (Ortega *et al.*, 2004).

The development of the chondrocyte phenotype is regulated by a combination of growth factors. These secreted molecules include TGF-β, BMPs and FGFs, and their action is controlled at several levels both intra- and extracellularily (de Crombrugghe *et al.*, 2000; Watanabe *et al.*, 2001). The balance of FGF and BMP signalling pathways determines the pace of differentiation in each step of the cascade by regulating chondroblast proliferation, maturation and hypertrophy (Minina *et al.*, 2002; Ornitz, 2005). Additionally, the spatio-temporal expression of the Wnt molecules and the activity of the Wnt signalling pathway control the differentiation cascade (Day *et al.*, 2005b).

2.3.2 Transforming growth factor-β (TGF-β) superfamily

The transforming growth factor beta (TGF-β) superfamily consists of signalling molecules, including TGF-β, BMPs, activins, inhibins, and growth and differentiation factors (GDFs), that are important regulators of embryonic development, postnatal tissue repair and homeostasis (Table 2) (Hogan, 1996). These molecules have been demonstrated to have a wide range of effects in various cellular processes, including cell growth and differentiation, extracellular matrix production, pattern formation and tissue specification (Ganan *et al*., 1996; Kingsley, 1994). TGF-β members direct the

mesenchymal stem cell fate into the chondrogenic and osteogenic direction but inhibit myogenesis and adipogenesis (Kingsley, 1994).

Table 2. Members of the TGF-β superfamily, their receptors, co-factors and inhibitors

TGF-β superfamily ligand	Type II receptor	Type I receptor	R-smad	Co-Smad	Ligand inhibitor
GDF1	ACVRIIA	ACVRIB (ALK4)	SMAD2, SMAD3	SMAD4	
GDF11	ACVRIIB	ACVRIB (ALK4), TGF-βRI (ALK5)	SMAD2, SMAD3	SMAD4	
BMP2/4 group	BMPRII	BMPRIA (ALK3), BMPRIB (ALK6)	SMAD1, SMAD5, SMAD8	SMAD4	Noggin, Chordin
The osteogenic protein 1 group (OP1, BMP-7) BMP-5, BMP-6, BMP-7, BMP-8	BMPRII	BMPRIB (ALK6) ALK2	SMAD1, SMAD5, SMAD8	SMAD4	Noggin, Chordin
GDF5 group GDF5, GDF6 (BMP13), DGF7 (BMP12)	BMPRII	BMPRIB (ALK6)	SMAD1, SMAD5, SMAD8	SMAD4	
TGF-β	TGF-βRII	TGF-βRI	SMAD2, SMAD3	SMAD4	Lefty

TGF-β molecules signal through a heteromeric cell surface serine/theorine kinase receptor complex consisting of dimeric type I and type II receptors (Figure 5) (Attisano and Wrana, 2002; Heldin *et al.*, 1997). The type I receptors are termed activin receptor-like kinases (ALKs). Binding of the ligand to a type II receptor activates the type I receptor and results in phosphorylation of the cytoplasmic receptor-regulated Smad molecules (R-Smads) by a type I receptor. Activated R-Smads are released from the receptor complex, associate with Co-Smad (Smad4) in the cytosol and translocate into the nucleus where they recruit distinct transcription co-factors and directly influence gene expression (Feng *et al*., 1998; Lagna *et al*., 1996). Inhibitory Smads (I-Smad) antagonize the signalling by R-Smads and Co-Smads (Derynck and Zhang, 2003).

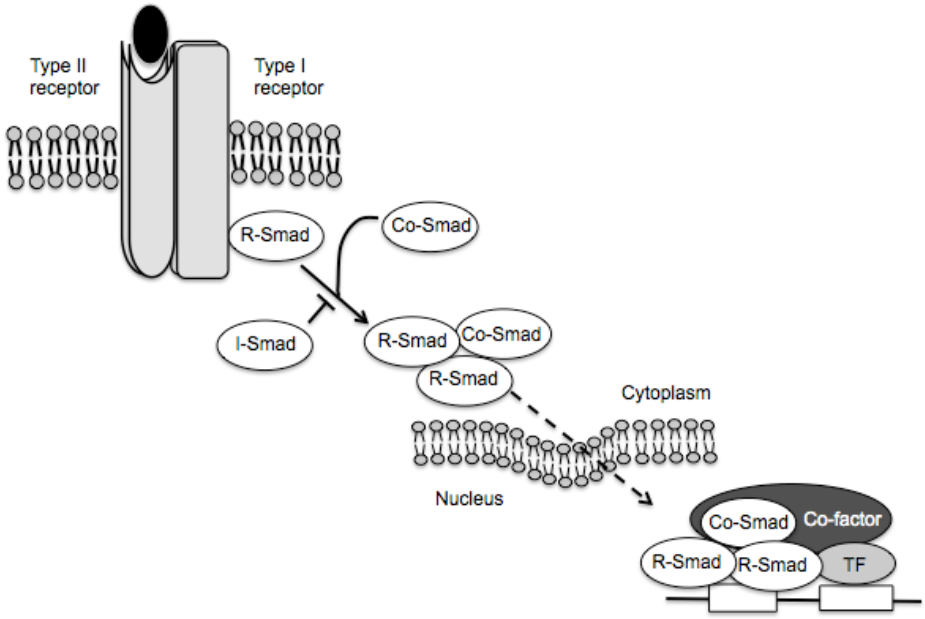


Figure 5. Signalling of the TGF-β superfamily members. Members of the TGF-β superfamily bind to a type II receptor, which activates a type I receptor. Activation of the type I receptor results in phosphorylation of receptor regulated Smads (R-Smads). The activated R-Smads bind to Co-Smad and this complex translocates to the nucleus where it recruits specific transcription factors (TF) and co-factors to regulate gene expression. Inhibitory Smads (I-Smad) antagonize signalling.

The interaction of the TGF-β pathway with other signalling pathways has been shown to be crucial for the development and tissue homeostasis. BMPs are downstream targets of growth factors such as FGF-2 (Choi *et al*., 2005; Yoon *et al.*, 2006) and act in conjunction with other growth factors, including insulin-like growth factor (IGF)-1 and IGF-II (Canalis and Gabbitas, 1994). In certain cell models, the IGF-1 stimulated PI3K/Akt pathway is required for BMP-induced osteogenesis (Canalis and Gabbitas, 1994; Ghosh-Choudhury *et al.*, 2002). The convergence of the BMP and MAP kinase signalling pathways has been extensively studied and ERK has been demonstrated to stimulate BMP signalling (Aubin *et al.*, 2004). Conversely, by activating Ras, BMP-2 stimulates ERK and p38 that differentially mediate BMP-2 function in osteoblasts by activating osteoblast factors (Weston *et al.*, 2002). TGF-β mediator Smad1 mediates the cross-talk with the FAK and EGF-signalling pathways (Kretzschmar *et al*., 1997; Tamura *et al.*, 2001). Additionally, TGF-β and Wnt signalling converge to synergistically regulate chondrogenesis and osteogenesis (Gaur *et al.*, 2005; Mbalaviele *et al*., 2005). The TGF-β family is divided into two general branches, whose members have diverse but often complementary effects: the TGF-β/Activin/Nodal branch and the BMP/GDF branch.

2.3.2.1 TGF-β signalling

TGF-β is synthesized as a precursor proprotein, which is cleaved during secretion (Gray and Mason, 1990; Lawrence, 1991). The mature TGF-β is a disulfide-bonded dimer that remains non-covalently associated with the N-terminal propeptide, latency-associated protein (LAP). This complex is referred to as small latent TGF-β, and it prevents mature TGF-β from binding to its receptor. The large latent complex is formed when the latent TGF-β-binding protein (LTBP) binds to LAP. LTPB mediates the deposition of the latent complex to the extracellular matrix and has a central role in the processing and secretion of TGF-β (Taipale *et al.*, 1994). Release of the mature TGF-β from the latent complex *in vivo* is a tightly regulated process that can be accomplished by different mechanisms, such as proteolytic cleavage of LAP by plasmin, deglycosylation of LAP or the interaction with trombospondin or integrin $\alpha_v\beta_6$ (Koli *et al.*, 2001).

The TGF-β1 receptor complex consists of two type I and two type II receptors (Attisano and Wrana, 2002; Heldin *et al.*, 1997) (Figure 5). An activated TGF-β type II receptor recruits the TGFβ type I receptor, ALK5 or ALK1 in chondrocytes (Heldin *et al.*, 1997). Truncated type II receptors and specific inhibitors of type II receptors have been used to block TGF-β1 signalling *in vitro* and *in vivo* (Chen *et al.*, 1993; Derynck *et al.*, 1998; Tang *et al.*, 2009). Activation of ALK5 is followed by Smad2 and Smad3 phosphorylation. In addition to Smad-mediated transcription, TGF-β has also been demonstrated to activate other signalling cascades, including the ERK, JNK and p38 MAPK kinase pathways (de Caestecker *et al.*, 1998; Engel *et al.*, 1999; Funaba *et al.*, 2002). Some of these pathways regulate Smad activation, but others may induce responses unrelated to Smad (Yue and Mulder, 2000; Yu *et al.*, 2002).

Numerous reports have demonstrated the important role of Smads, in particular the Smad3-mediated TGF-β signalling in chondrogenesis. TGF-β1 promotes chondrocyte proliferation through the activation of β-catenin signalling, and primary chondrogenesis through Smad3 and chromatin remodelling (Furumatsu *et al.*, 2009; Li *et al.*, 2006). Smad3 stimulates transcriptional activity of *Sox9* in a TGF-β1-dependent manner and, together with Smad2, Smad4 and Sox9, forms a transcriptional complex in the enhancer region of *Col2a1* to activate its expression (Furumatsu *et al.*, 2009; Furumatsu *et al.*, 2005). However, the Smad2/3 pathway inhibits chondrocyte maturation and hypetrophy *in vitro* and *in vivo* (Ferguson *et al.*, 2000; Ferguson *et al.*, 2004; Hayamizu *et al.*, 1991; Zhang *et al.*, 2004). A deficiency of Smad3 leads to premature chondrocyte hypertrophy, and mutant mice develop a degenerative joint disease resembling OA (Yang *et al.*, 2001). Similarly, the expression of kinase defective TGF-βII in the skeletal tissue promotes chondrocyte terminal differentiation and OA (Serra *et al.*, 1997).

TGF-β isoforms are expressed in the mouse and human in the condensing mesenchyme during the early stages of chondrocyte differentiation, while no expression is observed in the hypertrophic cells. (Millan *et al.*, 1991; Pelton *et al.*, 1990). Four TGF-β

isoforms (TGF-β1, TGF-β2, TGF-β3 and TGF-β5) display a differential response to chondrogenesis of mesenchymal cells cultured in micromass conditions, depending on the embryonic stage (Chimal-Monroy *et al.*, 1996; Chimal-Monroy and Diaz de Leon, 1997). TGF-β1 has been reported to promote the *in vitro* chondrogenesis in chondroblasts, mesenchymal cells, MSC, periosteum and perichondrium (Johnstone *et al.*, 1998; Lorda-Diez *et al.*, 2009). Injection of TGF-β1 into the periosteum of a femur induces chondrocyte differentiation and cartilage formation (Chimal-Monroy and Diaz de Leon, 1997; Joyce *et al.*, 1990). In contrast, TGF-β1 negatively regulates chondrocyte maturation by slowing their rate of maturation and inhibiting hypertrophy in cell culture, and in chick embryonic limb development *in vivo* (Ferguson *et al.*, 2000; Ferguson *et al.*, 2004; Hayamizu *et al.*, 1991). Loss of TGF-β1 function results in premature chondrocyte maturation (Yang *et al.*, 2001).

2.3.2.2 Bone morphogenetic proteins

BMPs are secreted growth factors. They are synthesized as large precursors, which are processed and proteolytically cleaved to yield a mature protein containing seven highly conserved cysteins in the carboxy-terminal region (Wozney *et al.*, 1988). Functional BMPs are dimeric proteins with a single interchain disulfide bond. The dimeric conformation is required for the biological activity of BMPs as reduction of the intermolecular bond results in the loss of activity (Eimon and Harland, 1999). More than 20 BMP-related proteins have been characterized and they can be divided into three subgroups based on the sequence homology: the BMP2/4 group, the osteogenic protein 1 (OP1, BMP7) group and the growth and differentiation factor 5 (GDF5) group (Miyazono *et al.*, 2005). BMPs signal through receptor complexes which determine the specificity of the intracellular signals (Liu *et al.*, 1995; Yamashita *et al.*, 1996). Three type II receptors (the BMP type II receptor (BMPRII), Activin type IIA receptor (ActIIA) and Activin type IIB receptor (AcrIIB)) and three type I receptors (ALK2, ALK3 (BMPRIA) and ALK6 (BMPRIB)) have been identified for BMP signalling. BMP-2 and BMP-4 preferentially bind to ALK3 and ALK6 while members of the OP1 group bind to ALK2 and ALK6 (Miyazono *et al.*, 2005). *BmprIA* and *BmprIB* are expressed differentially during development, and functional ALK3 and ALK6 are essential for chondrogenesis and maintenance of the chondrocyte phenotype (Chen *et al.*, 1998; Kawakami *et al.*, 1996; Yoon *et al.*, 2005b; Zhang *et al.*, 2003a; Zou *et al.*, 1997).

BMPs were originally discovered in the bone matrix and identified as molecules that induce ectopic endochondral ossification (Chen *et al.*, 2004; Urist, 1965). Particularly BMP-2, BMP-4 and BMP-7 have been demonstrated to promote bone formation and bone repair in several mouse models (Reddi, 1998; Wozney and Rosen, 1998). The enhancement of osteoblast differentiation is mediated by the activation of Runx2 and other transcription factors, such as Dlx5 (Banerjee *et al.*, 2001; Lee *et al.*, 2000; Miyama *et al.*, 1999). BMP-2 can be regulated by other BMPs in osteoblasts, and BMP-2/4 promoter contains Runx2-binding sequences, implying a positive feedback loop for the regulation of BMP signalling in osteogenesis (Ghosh-Choudhury *et al.*,

2002; Helvering *et al.*, 2000). BMPs play a role in many stages of chondrogenic differentiation, initiating chondroprogenitor cell determination and differentiation of precursors into chondrocytes, and also at the stage of chondrocyte maturation and terminal differentiation (Pizette and Niswander, 2000; Retting *et al.*, 2009; Wozney *et al.*, 1988). In addition, signalling through the BMP receptors is required for the maintenance of the articular cartilage in post-natal organisms (Rountree *et al.*, 2004). Moreover, BMPs promote cell death and apoptosis of chondrocytes (Zou and Niswander, 1996).

The members of the BMP family have distinct expression patterns and biological functions due to their ability to bind receptors in different combinations and with different affinities (McCullough *et al.*, 2007). BMP-2, -4 and -7 co-ordinately regulate the limb patterning depending on the spatial and temporal expression of the BMP receptors and antagonists, noggin and chordin (Niswander, 2002; Tickle, 2002; Yoon and Lyons, 2004; Yoon *et al.*, 2005a). BMP-2, -4, -6, -7, -9 and -13 have been shown to induce chondrogenesis *in vitro* (Majumdar *et al.*, 2001; Schmitt *et al.*, 1999; Sekiya *et al.*, 2005) However, the reported relative potencies of BMPs to induce chondrogenesis have been contradictory, partially due to variation in the model systems and culture conditions used. Additionally, MSCs from different species and tissue sources vary in their BMP responsiveness, partly due to their distinct receptor repertoires (Hennig *et al.*, 2007; Osyczka *et al.*, 2004).

2.3.3 Wnt signalling

The Wnt signalling pathway plays an important role in a variety of cellular activities, including cell fate determination, proliferation, migration, polarity, and gene expression (Moon *et al.*, 2002a). The canonical Wnt/β-catenin pathway is initiated through the binding of a Wnt ligand to its Frizzled receptor and the low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) co-receptors (Figure 6). In the absence of the Wnt signal, cytosolic β-catenin is phosphorylated by the glycogen synthase kinase (GSK-3β) and degraded by the ubiquitin/proteasome pathway (Aberle *et al.*, 1997). Upon the interaction of the Wnt ligand and its receptor, GSK-3β is inhibited resulting in the stabilization of β-catenin. β-Catenin translocates to the nucleus where it forms complexes with transcription factors, such as the T-cell factor/lymphoid enhancer binding factor (TCF/LEF) family, and regulates downstream gene expression (Behrens *et al.*, 1996; Reya and Clevers, 2005).

The canonical Wnt/β-catenin signalling has emerged as a key regulator of mesenchymal progenitor differentiation into various lineages. The activated Wnt/β-catenin signalling promotes osteogenesis and inhibits adipogenesis (Nuttall and Gimble, 2000; Qiu *et al.*, 2007). Several possible molecular mechanisms can explain the enhancement of osteogenesis by β-catenin, for instance the stimulation of *Runx2* (Gaur *et al.*, 2005). The Wnt signalling pathway inhibits adipogenesis through an inhibition of adipogenic transcription factors CCAAT/enhancing binding protein (C/EBPα) and peroxisome proliferator activated receptor gamma (PPARγ) (Ross *et al.*,

2000). An appropriate level of the canonical Wnt signalling is crucial for chondrogenesis, demonstrated by the abnormal growth plate phenotype in mice harbouring inactivated *β-catenin* in chondrocytes (Ryu *et al.*, 2002). β-Catenin is highly expressed in mesenchymal cells committed to the chondrocytic lineage but down-regulated at the stage of early chondrogenic differentiation, upon up-regulation of Sox9 (Akiyama *et al.*, 2004; Ryu *et al.*, 2002). Sox9 interacts with β-catenin and enhances its phosphorylation and subsequent degradation or competes with it and inhibits the activity of β-catenin-Tcf/Lef by binding to the Tcf/Lef-binding site of β-catenin (Akiyama *et al.*, 2004; Topol *et al.*, 2009). Wnt signalling is again up-regulated during hypertrophy and promotes chondrocyte hypertrophy and endochondral ossification (Day *et al.*, 2005a; Hill *et al.*, 2005).

Wnt signalling activity is controlled by many extracellular and intracellular molecules (Logan and Nusse, 2004). Among the extracellular proteins are Wnt inhibitors such as secreted frizzled-related proteins (sFRPs) and WIF (Wnt inhibitory factor)-1 that primarily bind to Wnt proteins as well as dickkopfs (DKKs) and SOST/Sclerostin that block canonical Wnt signalling through LRP5/6 (Kawano and Kypta, 2003). Studies with the antagonists of Wnt signalling have confirmed the important role of active Wnt signalling in osteogenic and chondrogenic differentiation processes (Bodine *et al.*, 2005; Bodine *et al.*, 2009a; Gaur *et al.*, 2006a; Gaur *et al.*, 2009).

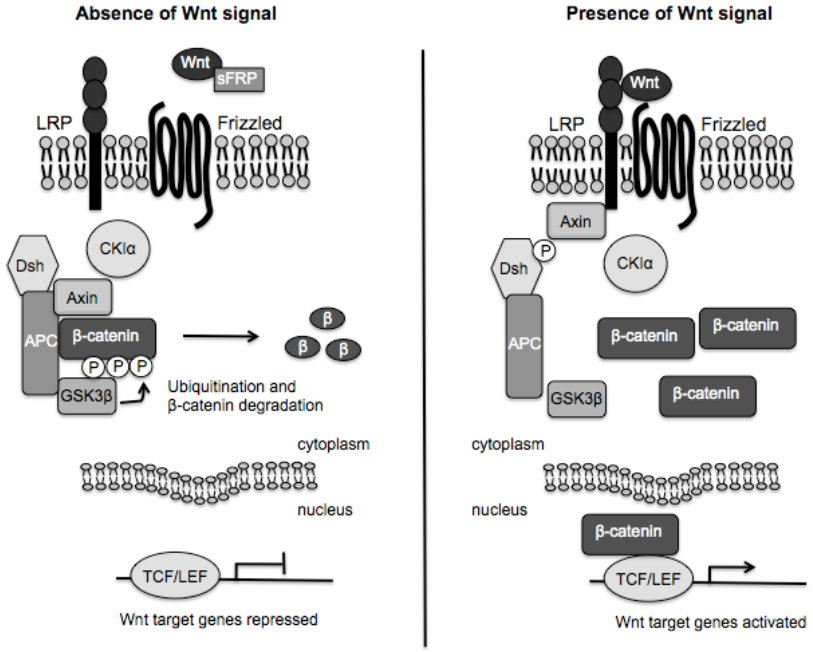


Figure 6. The canonical Wnt signalling pathway. The action of the pathway in the presence of a Wnt antagonist (sFRP) and absence of a receptor-binding Wnt (left panel), and in the presence of an active Wnt signal (right panel). LRP= low density lipoprotein receptor-related protein; CKI = casein kinase I; GSK3β = glycogen synthase kinase; APC = adenomatous polyposis coli; TCF = T-cell factor; LEF = lymphoid enhancer binding factor.

2.3.3.1 Secreted frizzled-related protein-1 (sFRP-1)

The secreted frizzled-related protein family consists of five secreted glycoproteins in humans (sFRP1, sFRP2, sFRP3, sFRP4, sFRP5) that act as extracellular signalling ligands. Secreted frizzled-related protein-1 (sFRP1) is a 36 kD protein containing a cysteine-rich domain (CRD) that shares 30-50% sequence homology with the CRD of Frizzled (Fz) receptors (Rattner *et al.*, 1997). sFRP-1 antagonises potentially both the canonical and non-canonical Wnt signalling by competing with the extracellular Wnt for binding to the Frizzled receptor and by binding directly to Wnt proteins (Bodine *et al.*, 2004b).

Expression of sFRP-1 has been reported in the brain, skeleton, kidney, eye, spleen, abdomen, heart and somites in early embryos (Trevant *et al.*, 2008b). Several studies have demonstrated the importance of sFRP-1 in osteoblast differentiation and skeletal development. *In vitro*, a deletion of sFRP-1 results in increased osteoblastic differentiation and decreased osteoblast and osteocyte apoptosis (Bodine *et al.*, 2005). sFRP-1-deficient mice display increased trabecular bone formation and bone mass without any apparent non-skeletal phenotypic changes, and enhanced fracture healing due to increased intramembranous ossification in the absence of sFRP-1 and in the presence of active Wnt signalling (Bodine *et al.*, 2004a; Gaur *et al.*, 2009; Trevant *et al.*, 2008a). Recently, overexpression of sFRP-1 was shown to inhibit bone formation *in vivo* (Yao et al., 2009). Furthermore, sFRP-1-deficient mice exhibit accelerated hypertrophic chondrocyte maturation *in vivo* and *in vitro*, indicating that sFRP-1 acts as a negative regulator of chondrocyte maturation (Gaur *et al.*, 2006b).

2.4 Stem cells

Stem cells are defined as undifferentiated cells capable of proliferation, self-renewal and differentiation into one or more specialised cell types. During fetal development, embryonic stem cells differentiate into the tissues of the body, and, later, stem cells participate in the cellular homeostasis and in tissue maintenance and repair (Alison *et al.*, 2002). Stem cells are classified as 1) totipotent stem cells, which are able to differentiate into all cell types, including extra-embryonic tissues, 2) pluripotent stem cells that can differentiate into the cells of all the three germ layers, and 3) multipotent stem cells, capable of differentiation into a limited range of cell types.

Generally, stem cells are also divided into embryonic and adult stem cells. Embryonic stem cells (ESCs) are cells isolated from the inner cell mass of the blastocyst. ESCs can extensively replicate via mitotic division while retaining their undifferentiated state. ESCs are pluripotent cells that have the ability to differentiate into any mesodermal, ectodermal and endodermal cell type (Doss *et al.*, 2004). Adult or somatic stem cells can be extracted from various tissues. They have a more limited differentiation potential, preferentially differentiating into mature cell types of the tissue of their origin. However, several studies have demonstrated a plasticity of adult

stem cells where the cells have been successfully induced to differentiate into cell types of another lineage (Jiang *et al.*, 2002; Zhao *et al.*, 2002).

A novel type of stem cell was introduced in 2006, when two groups independently reprogrammed adults cells into induced pluripotent stem cells (iPSCs) by viral transduction of four transcription factors, Oct 3/4 and Sox2 in combination with Klf4, and c-Myc or Nanog and Lin-28 (Takahashi and Yamanaka, 2006; Yu *et al.*, 2007). These factors were demonstrated to induce nuclear reprogramming of differentiated adult cells into cells with many of the characteristics of pluripotent ESCs, including the differentiation potential into any adult cell type. Since this discovery, iPSCs have been induced from various somatic cell types by using different combinations of transcription factors (Okita *et al.*, 2007).

2.4.1 Mesenchymal stromal cells (MSCs)

Mesenchymal stromal cells (MSCs) were originally isolated from the bone marrow and described as non-haematopoietic, spindle shaped, colony forming cells (Friedenstein *et al.*, 1968; Friedenstein *et al.*, 1970). Following their discovery, these multipotent cells have been shown to be present in practically all adult tissues, including the periosteum, synovium, adipose tissue, tendon, muscle, umbilical cord blood, amniotic fluid and peripheral blood (Bi *et al*., 2007; da Silva Meirelles *et al*., 2006; De Bari *et al*., 2001; De Coppi *et al*., 2007; ; Kuznetsov *et al*., 2001; Rosada *et al*., 2003; Zuk *et al*., 2001). Small populations of multipotent progenitor cells have been identified even in articular cartilage (reviewed in Säämänen *et al.,* 2010). MSCs isolated from various tissues share some common properties and surface markers but exhibit differences in their differentiation capacities and gene expression profiles (Sakaguchi *et al.*, 2005; Wagner *et al.*, 2005).

In the bone marrow, MSCs represent a small percentage, less than 0.01% of the total population of the nucleated cells (Caplan, 1991). The bone marrow MSCs provide a supportive stroma for growth and differentiation of haematopoetic stem cells (HSCs) and exert immunoregulatory functions (Dexter *et al.*, 1979; Le Blanc, 2003; Le Blanc *et al.*, 2003). MSCs reside in areas called stem cell niches at specific sites of the tissue. Stem cell niches have been characterized, for instance, in the tendon and articular cartilage, where proteoglycans regulate the growth factor activity and thus have an important role in maintaining and organizing the niches (Bi *et al.*, 2007; Karlsson *et al.*, 2009). MSCs secrete several growth factors and cytokines, and the subsequent cross-talk between the cells plays an important role in MSC maintenance and differentiation. Another fundamental function of this bidirectional regulation system in which cells secrete and respond to bioactive factors is to maintain tissue homeostasis, remodelling and repair (Caplan and Dennis, 2006).

MSCs have been commonly termed “mesenchymal stem cells” or “skeletal stem cells”. As MSCs represent a rather heterogeneous cell population consisting mostly of a mixture of various mesenchymal progenitor cells rather than true stem cells, the International Society for Cellular Theraphy (ISCT) has recommended the use of a broader term “multipotent mesenchymal stromal cells” for this cell population (Horwitz *et al.*, 2005). The suggested minimum criteria for human MSCs are 1) plastic adherence under standard culture conditions; 2) expression of CD105, CD73 and CD90 and the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface markers; and 3) the differentiation capacity to osteogenic, adipogenic and chondrogenic lineages (Dominici *et al.*, 2006). Single cell clonal analyses have revealed that among the bone marrow cells that fulfil the first two MSC criteria, only around 20-50% represent multipotent MSCs (Kuznetsov *et al.*, 1997). Remaining cell populations have been suggested to represent a mixture of bipotential and unipotential MSC (Muraglia *et al.*, 2000).

MSCs from human and rat bone marrow have been extensively characterized for their morphology, surface epitope profile and differentiation capacity (Colter *et al.*, 2001; Javazon *et al.*, 2001; Sekiya *et al.*, 2002). For several reasons, isolation, culturing and differentiation of mouse MSCs (mMSCs) is more difficult. Contamination by haematopoietic progenitors has appeared as one of the pitfalls as there is no single surface marker that can be used for the isolation of mMSCs (Phinney *et al.*, 1999a). Hence, negative selection has been used to remove haematopoietic cells, but the remaining cells have a reduced proliferation capacity due to down-regulation of the genes involved in proliferation and the cell cycle (Baddoo *et al.*, 2003; Hachisuka *et al.*, 2007). Other complicating factors include the variation in yield, growth, surface epitope profile and differentiation capacity between mouse strains (Peister *et al.*, 2004; Phinney *et al.*, 1999b).

2.4.1.1 Differentiation of MSCs

MSCs lack tissue specific characteristics, but under defined conditions the cells are, *in vitro* and *in vivo*, able to differentiate into multiple mesenchymal cell lineages including chondrocytes, osteoblasts, adipocytes and non-mesodermal cells, such as neuronal-like cells and hepatocytes (Figure 7) (Dezawa *et al.*, 2004; Jiang *et al.*, 2002; Luk *et al.*, 2005; Pittenger *et al.*, 1999; Prockop, 1997). Recently, bone marrow-derived MSCs were reported to be able to differentiate into cells with the characteristics of all three germ layers *in vitro* and *in vivo*, suggesting that also adult cell populations contain distinctly pluripotent cells (Kuroda *et al.*, 2010). The differentiation of MSCs is tightly regulated via interactions with specific extracellular mediators. Several signalling pathways, including Wnt, TGF-β and functional cross-talk between the signalling cascades, play important roles in the fate decision and differentiation of MSC (Ling *et al.*, 2009). *In vitro* differentiation of MSCs into a desired differentiation lineage requires a precisely defined culture medium and controlled culture conditions.

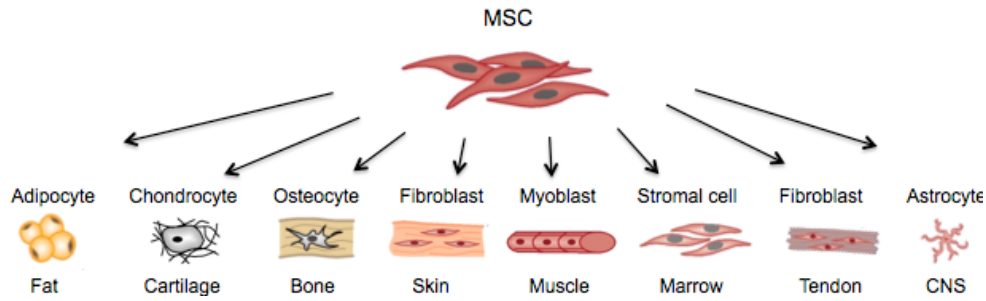


Figure 7. The multilineage potential of adult mesenchymal stromal cells (MSCs). Mesenchymal stromal cells have the ability to differentiate into multiple lineages, such as adipocytes, chondrocytes, osteoblasts, fibroblasts, myoblasts and astrocytes, which in turn give rise to specified tissues. CNS = Central nervous system. Modified from Säämänen *et al*., 2010.

2.4.1.2 Osteogenic and adipogenic differentiation of MSCs

Osteogenesis and adipogenesis are strongly associated processes and the interplay between the cell types and commitment to differentiate into either of the lineages is intensively studied (Gimble *et al.*, 2006). *In vivo* studies have revealed that in many bone loss states, such as aging, osteoporosis and undergoing glucocorticoid therapy, there is an inhibition of bone formation and increased bone marrow adipogenesis (Burkhardt *et al.*, 1987; Justesen *et al.*, 2001; Meunier *et al.*, 1971; Rozman *et al.*, 1989; Wang *et al.*, 1977). The inverse relationship between osteogenesis and adipogenesis has been explained by the selective differentiation of MSCs into either the osteogenic or adipogenic lineage at the expense of the alternative lineage. This hypothesis has been supported by *in vitro* studies showing that factors inducing adipogenic differentiation (e.g. dexamethasone) inhibit osteogenesis (Beresford *et al.*, 1992; Dorheim *et al.*, 1993; Falconi *et al.*, 2007), and vice versa, osteoblastic differentiation inducing factors, e.g. BMP-2, inhibit adipogenesis (Gimble *et al.*, 1995).

The osteogenic differentiation of MSCs is a co-ordinated process, defined by four stages; cellular commitment, proliferation, matrix maturation and mineralization. The chemically-defined culture conditions usually contain dexamethasone, ascorbic acid and β-glycerophosphate that are needed for the lineage commitment, collagen fibril formation and as a source for phosphate groups, respectively (Beresford *et al.*, 1993). The cells committed to osteoblast lineage change their morphology and express alkaline phosphatase, an early marker of osteoblast differentiation, bone matrix molecules including type I collagen, and non-collagenous proteins, such as osteocalcin, osteopontin and bone sialoprotein (Christenson, 1997; Huang *et al.*, 2004; Wolf, 1996).

Osteoblast differentiation is tightly regulated by hormones such as parathyroid hormone (PTH) and by local growth factors including BMPs, IGF and FGFs (Giustina *et al.*, 2008; Heino and Hentunen, 2008; Locklin *et al.*, 1999; Okamoto *et al.*, 2006;). These factors activate specific intracellular pathways that trigger the expression of several osteoblast-specific transcription factors. Runx2 is an essential transcription factor for the differentiation of MSCs into the osteogenic lineage and for bone

formation (Ducy *et al.*, 1997; Komori *et al.*, 1997). It regulates the expression of several other osteoblastic genes, such as *COL1A1*, *ALPL* (alkaline phosphatase), *IBSP* (bone sialoprotein), *SPP1* (osteopontin*)* and *BGLAP* (osteocalcin) (Ducy *et al.*, 1999; Karsenty *et al.*, 1999). Osterix is another crucial transcription factor required for the differentiation of preosteoblasts into fully-functioning osteoblasts (Nakashima *et al.*, 2002). Other important transcription factors involved in osteoblast differentiation include activating transcription factor 4 (ATF4), transcriptional modulator (TAZ), TWIST and homeodomain proteins Msx1, Msx2, Dlx5 and Dlx6 (Komori, 2006; Marie, 2008; Nakashima and de Crombrugghe, 2003).

Adipogenic differentiation is characterized by morphological changes in which MSCs lose their fibroblastic shape and turn into round cells accompanied by small perinuclear granules that fuse to form cytoplasmic lipid vacuoles. Adipogenesis is commonly induced using insulin, dexamethasone and 3-isobutyl-1-methylxantine (IBMX) which trigger the expression of adipogenic factors (Gregory *et al.*, 2005). The transcriptional network regulating adipogenesis involves activation of three classes of transcription factors that directly induce adipogenic differentiation; PPARγ, C/EBP and adipocyte determination and differentiation factor 1/ sterol regulatory element binding protein-1 (ADD1/SREBP1) (Farmer, 2006; Rosen *et al.*, 2000). In response to an adipogenic stimulus, C/EBPβ and C/EBPδ induce the expression of PPARγ, a key regulator of adipocyte differentiation and glucose homeostasis (Gesta *et al.*, 2007; Tontonoz *et al.*, 1994). PPARγ activates C/EBPα, which acts synergistically with PPARγ to co-ordinate the adipocyte differentiation cascade (Tontonoz *et al.*, 1995). The cross-regulation between PPARγ and C/EBPα is a key component of the transcriptional control of the adipogenic cell lineage and important in maintaining their differentiated state (Wu *et al.*, 1999). ADD1/SREBP1 activates PPARγ by inducing its expression and by promoting the production of PPARγ ligands (Farmer, 2006).

2.4.1.3 Chondrogenic differentiation of MSCs

The conditions that induce the MSC differentiation into the osteogenic lineage are well understood. The chondrogenic differentiation appears to be more problematic. Particularly challenging is the generation of a functional extracellular matrix. *In vitro* chondrogenesis is typically carried out in a micromass pellet culture system which allows cell-cell interactions recapitulating prechondrogenic condensations during embryonic development (Barry *et al.*, 2001a). Chondrogenic differentiation has been shown to occur when MSCs are grown under conditions that include a 3D culture format, a serum-free nutrient medium and an addition of dexamethasone and one or more members of the TGF-β superfamily, preferentially TGF-β1 or TGF-β3 (Johnstone *et al.*, 1998; Mackay *et al.*, 1998; Tanaka *et al.*, 2004). In addition, several growth factors that promote chondrogenesis *in vivo* have been demonstrated to enhance chondrogenesis of MSCs also *in vitro* (Sekiya *et al.*, 2001; Sekiya *et al.*, 2005). Bone morphogenetic proteins have a synergistic effect with TGF-β on promoting MSCs into hyaline-like cartilage tissue (Toh *et al.*, 2005). BMP-2, BMP-4 and BMP-6 enhance the gene expression of cartilage-specific genes and proteoglycans in hMSCs (Sekiya *et al.*,

2001; Sekiya *et al.*, 2005). Additionally, FGF-2 and IGF promote the MSC chondroprogenitor proliferation and chondrogenic potential (Mastrogiacomo *et al.*, 2001; Solchaga *et al.*, 2010).

Under specific conditions, cells undergo a sequence of cellular and molecular events and begin to express cartilage-specific matrix components in a differentiation stage-dependent manner. During differentiation, MSCs undergo a series of morphological changes (Sekiya *et al.*, 2002). During the first day of induction, the spindle-shaped MSCs aggregate and form junctional complexes. After a week, the cell pellets consist of three layers: the superficial zone containing fibroblast-like, elongated cells with junctional complexes, the middle zone with apoptotic cells and the deep zone occupied by matrix-producing chondrocyte-like cells. In two weeks, the middle zone disappears, and by three weeks the spindle-shaped cells begin to disappear. The chondrocyte-like cells in the deep zone are covered by matrix containing chondroitin sulphate and type I, II and X collagens (Ichinose *et al.*, 2005). Despite the marked increase in the pellet size, there is a progressive loss of cells due to apoptosis (Sekiya *et al.*, 2002).

The ECM molecules, cadherins, fibromodulin and COMP, are expressed early in the differentiation. The intermediate stage is defined by an increase in the aggrecan, versican core protein, decorin and biglycan expression, followed by the synthesis of type II collagen and chondroadherin (Barry *et al.*, 2001a; Sekiya *et al.*, 2002). The expression of L-Sox5, and Sox6 and -9, fibroblast growth factor receptor (FGFR-2) and parathyroid hormone-related peptide receptor (PTHrPR) continuously increase during the course of differentiation. Additionally, the *Col10a1* gene, predominantly a marker for chondrocyte hypertrophy, is expressed in MSC cultures simultaneously or preceding the expression of *Col2a1*. However, the type X collagen protein seems to remain absent in the MSC pellet cultures (Barry *et al.*, 2001b; Mwale *et al.*, 2006; Nelea *et al.*, 2005; Sekiya *et al.*, 2002).

2.4.1.4 MSC-based tissue engineering applications for skeletal repair

Due to their ability to expand and differentiate into various cell types, stem cells have generated a great deal of interest concerning their use in regenerative medicine and tissue engineering. Although embryonic stem cells provide a powerful tool for regeneration, their use is impeded by a plethora of ethical and immunological issues. As the cells derived from ESCs are allogeneic, their use is likely to require the co-administration of immunosuppressive agents, which carry their own substantial risks. Additionally, the undirected growth and potentially pathological differentiation after transplantation creates a risk of teratoma, a complication that can occur late after administration. Using iPSCs derived from the patient's own cells has been introduced as an alternative technique to overcome the ethical and immunological concerns of ESCs. However, the use of the iPSC technique in clinical use remains controversial due to the safety issues and inefficient methods in producing the iPSCs (Okita *et al.*, 2007). Compared to the pluripotent ESCs and iPSCs, MSCs have many advantages for developing cellular therapy (Le Blanc and Pittenger, 2005). They can be easily isolated

and maintain their genetic stability during an *in vitro* expansion (Abdallah and Kassem, 2009). Moreover, they are hypoimmunogenic, allowing allogenic transplantation, and are capable of homing to injured tissue (Humphreys and Bonventre, 2008; Penna *et al.*, 2008). Modification of the CD44 surface antigen has been shown to improve MSC homing to bone but the detailed mechanisms for the MSC homing capacity are still poorly understood (Sackstein *et al.*, 2008).

In addition to their presumptive plasticity, the immunomodulatory properties of MSCs are considered a major advantage for regenerative purposes. MSCs are suggested to modulate the inflammatory response through a synergistic down-regulation of proinflammatory cytokines and an up-regulation of both pro-survival and anti-inflammatory factors. MSCs are capable of suppressing T-cell and natural killer (NK) cell functions and the maturation and proinflammatory potential of dentritic cells (Salem and Thiemermann, 2010). The mechanisms underlying the immunomodulatory properties are not known in detail. While the initial cell-to-cell contact phase seems to be required, the ultimate signal is suggested to be mediated by soluble factors. Due to the anti-inflammatory and immunosuppressive properties, MSCs have successfully been exploited in a number of disease models including rheumatoid arthritis (Chen and Tuan, 2008; Muller *et al.*, 2008).

Transplantation techniques using MSCs have been successfully used in pre-clinical animal models in treatment of skeletal defects (Ohgushi *et al.*, 1989; Xian and Foster, 2006). In addition, clinical case reports have demonstrated the potentiality of autologous MSCs for bone and cartilage repair (Hesse *et al.*, 2010; Kuroda *et al.*, 2007; Ohgushi *et al.*, 2005; Wakitani *et al.*, 2007; Wakitani *et al.*, 2010). Despite the promising pre-clinical and clinical results, MSC therapy has its limitations. The regeneration of a three-dimensional tissue is a challenging process and often requires a scaffold to provide the required structural and mechanical support. While several scaffolds have been reported to promote regeneration, the safety and biocompatibility issues still require further investigation. Additionally, *ex vivo* culturing of MSCs may influence their *in vivo* proliferation and differentiation capacity. The precise mechanism of action of MSCs needs to be elucidated, as well as their potential risk of becoming tumorigenic (Arthur *et al*., 2009).

MSCs can be transplanted into patients through local implantation or systemic infusion. Stem cell therapy can also be combined with tissue engineering techniques and gene therapy (Kassem *et al.*, 2004). The use of MSCs as a vehicle for genes, such as transcription factors and morphogens that promote chondrogenesis, growth factors that stimulate matrix synthesis and receptor antagonists that inhibit responses to catabolic cytokines, has offered alternative approaches for skeletal repair (Grande *et al.*, 2003). The adenovirus-mediated gene transfer using chondrogenic factors, such as TGF-β1, BMP-2 and over-expression of *Sox9* of in MSCs, has provided promising results as a novel gene therapy strategy to induce chondrogenesis (Noel *et al.*, 2004; Palmer *et al*., 2005; Tsuchiya *et al.*, 2003).

2.5 Delta-like 1/Preadipocyte factor-1/ Fetal antigen 1 (dlk1/Pref-1/FA1)

Delta-like 1 (dlk1) is an EGF repeat-containing protein of the Notch/Delta/Serrata family (Baladron *et al.*, 2005a). It is synthesized as a transmebrane protein containing an extracellular domain with six EGF-like repeats, a juxtamembrane region, a single transmembrane domain and a short cytoplasmic tail (Smas and Sul, 1993b). The extracellular domain of dlk1 is proteolytically cleaved by ADAMT17/TACE to produce a soluble bioactive FA1 fragment corresponding to the extracellular domain (Wang and Sul, 2006). A membrane-bound form of dlk1 is not able to mediate the biological function of dlk1, indicating that the cleavage is required for its activity (Mei *et al.*, 2002). The nomenclature of the protein varies between laboratories. Here, the membrane bound protein is exclusively referred to as dlk1 and the soluble form of dlk1 as fetal antigen 1 (FA1). Dlk1 is also called preadipocyte factor-1 (Pref-1) (Smas and Sul, 1993b).

Dlk1 is a paternally-expressed, imprinted gene located in human chromosome 14 and mouse chromosome 12. Patients with maternal uniparental disomy 14 (UPD14), a human syndrome in which *DLK1* is silenced, exhibit obesity, premature puberty, macrocephaly, short stature and small hands (Berends *et al.*, 1999). Similarly, dysfunction of *Dlk1* in mice results in a variety of developmental defects, including growth retardation, obesity, skeletal malformations and abnormal haematopoiesis, illustrating the importance of dlk1 in development (Manzoni *et al.*, 2000; Moon *et al.*, 2002b; Sakajiri *et al.*, 2005b).

Dlk1 is widely expressed in most embryonic tissues, while in post-natal organisms its expression is limited to hormone-producing cells in the pituitary gland, pancreatic islets, adreanal glands, testes and monoaminergic neurons in the central nervous system (Floridon *et al.*, 2000; Jensen *et al.*, 2001; Tornehave *et al.*, 1993; Yevtodiyenko and Schmidt, 2006a). Additionally, dlk1 is found in the progenitor cell compartment in many cell types during regeneration, suggesting a role in maintaining cells in an undifferentiated state during differentiation (Sul, 2009).

2.5.1 Dlk1 in differentiation

Dlk1 was originally identified as a negative regulator of adipocyte differentiation, and the inhibitory role of dlk1 on adipogenesis is well established, both *in vitro* and *in vivo* (Smas and Sul, 1993a; Laborda, 2000a). Over-expression of dlk1 or treatment with soluble FA1 inhibits adipocyte differentiation, and, conversely, inhibition of dlk1 results in enhanced adipogenesis (Mei *et al.*, 2002; Smas *et al.*, 1997; Smas *et al.*, 1998). Similarly, mice lacking *Dlk1* exhibit increased adipogenesis, while transgenic mice overexpressing soluble FA1 in the adipose tissue or liver display decreased adipocity (Lee *et al.*, 2003; Moon *et al.*, 2002c; Villena *et al.*, 2008). Recent data suggest that dlk1 has a role in many other differentiation processes during embryogenesis and in post-natal organisms, including haematopoiesis (Sakajiri *et al.*,

2005a), neurogenesis (Costaglioli *et al.*, 2001), myogenesis (Crameri *et al.*, 2004) and osteogenesis (Abdallah *et al.*, 2004a; Abdallah *et al.*, 2007).

Dlk1 has been reported to negatively regulate the osteoblastic and adipocytic differentiation of MSCs. It is highly expressed in preadipocytes and preosteoblasts; the expression is decreased during the differentiation and is absent in mature adipocytes and osteoblasts (Abdallah *et al.*, 2004b; Smas *et al.*, 1997; Wang *et al.*, 2006). Recently, it was demonstrated to promote the early commitment of MSCs to the chondrocytic lineage but to prevent chondrocyte maturation and hypertrophy (Wang and Sul, 2009a).

2.5.2 Dlk1 signalling

In contrast to other members of Notch/Delta/Serrata family, dlk1 lacks the DSL (Delta/Serrata/LAG-2) domain, which is conserved in all classical Notch ligands to mediate receptor-ligand interaction (Gordon *et al.*, 2008; Laborda, 2000b). It is thus not known whether dlk1 functions as a receptor or a ligand and the signalling pathways for dlk1 have not been fully elucidated. Dlk1 has been shown to influence the composition of the MSC microenvironment by modulating the expression of pro-inflammatory cytokines, and thereby to inhibit osteoblast and adipocyte differentiation (Abdallah *et al.*, 2007). Contradictory observations have been reported on the involvement of Notch in the dlk1 function; dlk1 either antagonizes or enhances Notch (; Baladron *et al.*, 2005b; Bray *et al.*, 2008; Nueda *et al.*, 2007). Moreover, dlk1 has been suggested to interact with IGF-1 and to regulate the IGF-1 receptor-mediated p42/p44 mitogen-activated protein kinase (MAPK) activation (Nueda *et al.*, 2008; Zhang *et al.*, 2003b). Similarly, other pathways have also been implicated as being affected by dlk1, including the MAPK and MEK/ERK pathways (Kim *et al.*, 2007; Ruiz-Hidalgo *et al.*, 2002). Dlk1 has been demonstrated to dose-dependently increase the phosphorylation of ERK. Furthermore, it was recently shown to interact with fibronectin and to activate integrin downstream signalling and the MEK/ERK pathway to subsequently inhibit adipogenesis (Wang *et al.*, 2010).

Recently, *Sox9* was identified as a dlk1 target that mediates mesenchymal cell commitment and differentiation (Wang and Sul, 2009a). Dlk1 was shown to up-regulate *Sox9* through the MEK/ERK activation. By preventing the down-regulation of *Sox9*, dlk1 inhibited the expression of *Runx2* and thereby osteoblast differentiation. Similarly, Sox9 was shown to suppress the activity of C/EBPβ and C/EBPδ, resulting in the inhibition of adipogenesis. In addition, it was suggested that by inducing *Sox9* dlk1 promoted early chondrogenesis but inhibited chondrocyte maturation.

2.6 MicroRNAs (miRNAs)

Post-transcriptional regulation by non-coding RNA molecules has been discovered to be an important epigenetic mechanism to control cellular differentiation. Epigenetic modifications are inherited changes in the cellular phenotype, caused by a mechanism other than changes in the underlying DNA sequence (Bird, 2007). The post-translational modifications (methylation, acetylation, phosphorylation and ubiquitination) of nucleosomal histone proteins and methylation of gene promoters in the 5-position of cytosine in CpG nucleotides represent additional and extensively characterized epigenetic mechanisms that regulate gene expression and influence the cellular phenotype (Zaidi *et al.*, 2010a). Epigenetic events have been suggested to control numerous biological processes, including differentiation, development and ageing. Additionally, genetic reprogramming is an important feature in several diseases, such as cancer and the pathogenesis of OA (Zaidi *et al.*, 2010b).

2.6.1 MicroRNA biogenesis and function

MicroRNAs (miRNAs) are evolutionarily conserved, small, single-stranded RNA molecules that are processed through a series of post-transcriptional biogenesis steps (Figure 8) (Winter *et al.*, 2009). Most miRNA genes are located in regions distant from annotated genes, suggesting that they derive from independent transcription units (Lagos-Quintana *et al.*, 2001; Lee and Ambros, 2001). A minority of miRNA genes are in the introns of protein coding genes, preferentially in the same orientation as the mRNA, suggesting that they are processed from the introns rather than transcribed from their own promoters (Lagos-Quintana *et al.*, 2003; Lai *et al.*, 2003). A significant number of miRNA genes are clustered in the genome and transcribed as a multi-cistronic primary transcript. The miRNAs within a cluster are often related to each other (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001). Similarly to protein-coding genes, miRNA genes are transcribed by RNA polymerase II as a long primary miRNA (pri-miRNA) (Lee *et al.*, 2004). Pri-miRNA is a hairpin structure that is recognized by a microprocessor complex consisting of nuclear RNAse III endonuclease Drosha and its cofactor DGCR8 (Lee *et al.*, 2003). Drosha cleaves the RNA duplex into a ~70 nt-long stem loop structure called precursor miRNA (pre-miRNA), which is then actively exported from the nucleus to the cytoplasm via export receptor Exportin-5 and Ran-GTP (Lund *et al.*, 2004; Yi *et al.*, 2003; Yi *et al.*, 2005). In the cytoplasm, the pre-miRNA is loaded into a complex of RNAse III endonuclease Dicer and TRBP/Loquacious (Hutvagner *et al.*, 2001). This complex cleaves the loop from the pre-miRNA to produce a double-stranded structure composed of the miRNA and antisense miRNA*. The miRNA* strand is typically degraded, and the mature ~ 22 nt long miRNA strand is incorporated into the Argonaute protein (Ago 2)-containing ribonucleoprotein complex known as RISC (RNA-induced silencing complex) (Hutvagner and Simard, 2008). The miRNA guides the RISC complex to the 3' untranslated region (UTR) of its target mRNA (Bartel, 2009; Lai, 2002). The seed sequence (2-8 nt) of miRNA is important for the targeting and silencing of specific mRNA (Lewis *et al.*, 2005). Association of miRNA with its target results in mRNA cleavage if the miRNA is perfectly complementary to the mRNA, or repression of

translation if the miRNA is partially bound to its target (Cullen, 2003; Giraldez *et al.*, 2006; Pillai *et al.*, 2005; Zeng and). Repressed mRNAs are translocated into the cytoplasmic P-bodies, which are known sites of mRNA destabilization (Bruno and Wilkinson, 2006).

Hundreds of unique miRNAs have been identified in man, and each is predicted to regulate several target genes (Lewis *et al.*, 2003; Lim *et al.*, 2005). Computational predictions indicate that over 35% of all human protein-coding genes may be regulated by miRNAs (Friedman *et al.*, 2009).

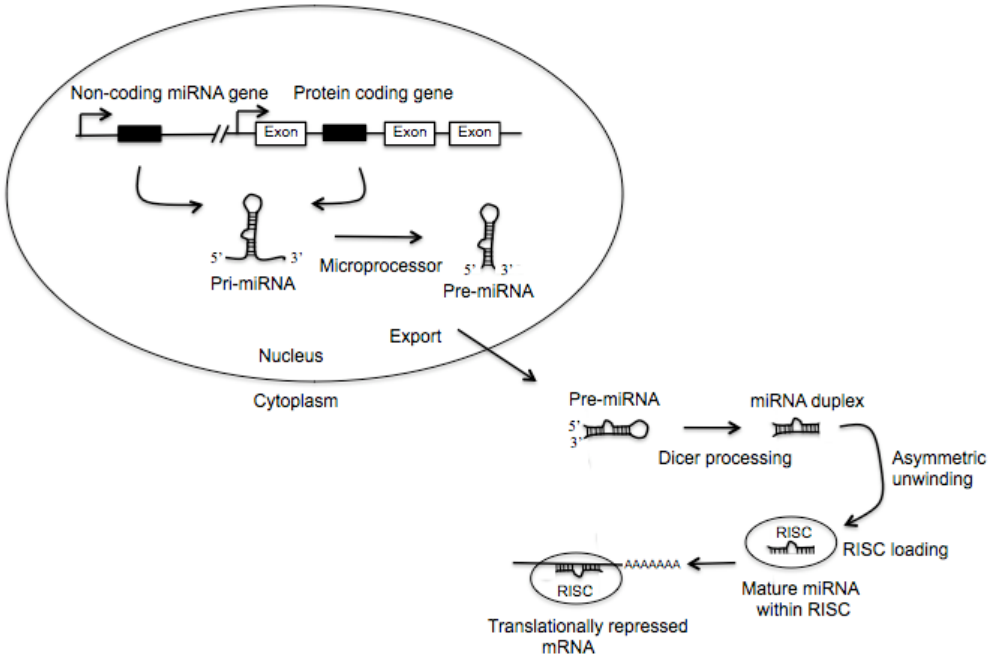


Figure 8. MicroRNA biogenesis and function. miRNAs are transcribed as long precursors (pri-miRNAs) that are cleaved by the microprocessor complex to yield stem-loop pre-miRNAs. The pre-miRNAs are translocated from the nucleus to the cytoplasm by the Exportin5 complex. In the cytoplasm, pre-miRNAs are processed by Dicer into around 22-nucleotide-long mature miRNAs. These are incorporated into the RNA-induced silencing complex (RISC), which guides the miRNAs to their target mRNAs, resulting in mRNA cleavage or repression of translation.

2.6.2 Regulation of differentiation by microRNAs

MicroRNAs were originally discovered in *Caenorhabditis elegans* in which they regulated the developmental timing (Lee *et al.*, 1993), and further described in vertebrates and invertebrates in 2001 (Lagos-Quintana *et al.*, 2001). Since their first discovery, miRNAs have been intensively studied and are reported to act as key regulators in processes as diverse as early development, cell proliferation and cell death, apoptosis, and cell differentiation; furthermore, miRNA perturbations have been associated with numerous diseases, including cancer (Chen *et al.*, 2004; Garzon *et al.*, 2006; Inui *et al.*, 2010; Kloosterman and Plasterk, 2006; Stefani and Slack, 2008).

Functional miRNAs are essential for vertebrate development, proven by studies demonstrating that universal disruption of *Dicer* in mice results in embryonic lethality and depletion of pluripotent stem cells, and a deficiency of Argonaute in severe developmental defects (Bernstein *et al.*, 2003; Liu *et al.*, 2004). Tissue-specific disruption of *Dicer* leads to diverse developmental defects, and in most of the examined tissues, to increased cell death, indicating that miRNAs play a crucial role in the development of various tissues including the heart, brain, muscle, lungs, limbs and T cells among others (Gaur *et al.*, 2010; Kloosterman and Plasterk, 2006; Kobayashi *et al.*, 2008). Several studies have indicated that tissues in developing and mature organisms are characterised by unique profiles of miRNA expression (Li *et al.*, 2008; Krichevsky *et al.*, 2003).

2.6.2.1 Regulation of skeletal development by microRNAs

The importance of functional miRNAs in skeletal development has been addressed by generating conditional limb mesenchyme and osteoblast-specific *Dicer* knockout (KO) mice. The disruption of *Dicer* in chondrocytes results in an abnormal cartilage phenotype with impaired chondrocyte proliferation and accelerated maturation, indicating that miRNAs play a crucial role in chondrogenesis by maintaining chondrocyte proliferation and inhibiting their premature differentiation into hypertrophic chondrocytes (Kobayashi *et al.*, 2008). During bone formation, miRNAs are important in two periods of the process; in promoting the osteoblast differentiation and in controlling bone accrual in a post-natal organism (Gaur *et al.*, 2010). An increasing number of miRNAs have been identified to regulate osteoblast differentiation and bone formation positively by targeting negative regulators of osteogenesis or negatively by targeting important osteogenic factors (Table 3).

Table 3. MicroRNAs involved in skeletal development

miRNA	Target	Reference
Osteogenesis		
Negative regulators		
miR-26a	SMAD1	(Luzi *et al.*, 2008)
miR-133, miR-204	Runx2	(Li *et al.*, 2008; Huang *et al.*, 2010)
miR-135	SMAD5	(Li *et al.*, 2008)
miR-141, miR-200a	Dlx5	(Itoh *et al.*, 2009)
miR-196a	Hoxc8	(Kim *et al.*, 2009)
miR-206	Cx43	(Inose *et al*., 2009)
Positive regulators		
miR-29a	Dkk1, Kremen, sFRP-2	(Kapinas *et al.*, 2009; Kapinas *et al.*, 2010)
miR-29b	HDAC4,TGF-β3, Acvr2a, Ctnnbip1, Dusp2	(Li *et al.*, 2009)
miR-2861	HDAC5	(Li *et al.*, 2009)
Chondrogenesis		
Negative regulators		
miR-140	ADAMTS5	(Miyaki *et al.*, 2009; 2010)
miR-199*	Smad1	(Lin *et al.*, 2009)
Positive regulators		
miR-675		(Dudek *et al.*, 2010)

Only a few miRNAs have been characterized as specifically regulating chondrogenesis and cartilage homeostasis. miR-199* negatively regulates chondrogenesis by targeting *Smad1*, while miR-675, whose expression is upregulated by Sox9, positively regulates *Col2a1*, thereby promoting chondrogenesis (Dudek *et al.*, 2010; Lin *et al.*, 2009). Recently, miRNAs were implicated as playing a role in the pathogenesis of OA (Akhtar *et al.*, 2010; Yamasaki *et al.*, 2009). miR-140 is highly expressed in normal human articular cartilage, and the expression is reduced in OA (Miyaki *et al.*, 2009). Disruption of miR-140 predisposes to age-related OA and, conversely, its over-expression in chondrocytes protects from OA indicating that miR-140 prevents the development of the disease by a mechanism that could at least to some extent involve regulation of ADAMTS5 (Miyaki *et al.*, 2010). These results suggest that miRNAs play an important role in the articular cartilage pathology and homeostasis *in vivo* and thus provide a novel insight into therapeutical implications for OA.

3. AIMS OF THE STUDY

Chondrogenesis is a tightly controlled process in which several factors regulate discrete stages of the differentiation programme. Understanding the mechanisms of cell commitment and further differentiation into a particular lineage is of importance in the development of therapeutic treatments of skeletal lesions based on MSC transplantation techniques. This research aimed to screen the factors that affect chondrogenic differentiation and function at different levels of the regulation of embryonic and adult cell differentiation. The objective was to identify novel regulatory factors and mechanisms that may ultimately have potential as tools for studies on chondrogenesis and for the development of therapeutic approaches using transplanted MSCs in order to repair cartilage lesions.

Factors regulating chondrogenesis at the cellular, transcriptional (mRNA) and translational (miRNA) level were studied, and the specific aims of this thesis project were:

- To optimize an *in vitro* differentiation assay for chondrogenesis and to compare the ability of different growth factors to induce proliferation and chondrogenesis of MSCs.

- To determine the role of the MSC population in chondrogenic differentiation at the level of primary and selected heterogeneous populations and committed clonal cell lines.

- To study the mechanisms behind the commitment and interactions between committed MSC progenitors.

- To study the expression of dlk1 in limb chondrogenesis and mature cartilage, and characterize its role in chondrogenesis *in vitro* and *in vivo*.

- To characterize the role of miRNAs during *in vitro* chondrogenesis and osteogenesis by studying the expression profile of a set of miRNAs and their targets.

4. MATERIALS AND METHODS

4.1 Study animals

Mouse MSCs (I, V) were isolated from C57/bl × DBA hybrid mice maintained at the Animal Core Facility of the University of Turku, Finland (Salminen *et al.*, 2001). $mMSC^{Adipo}$ cells and $mMSC^{Bone}$ cells (II) were obtained from the β-actin-eGFP+ transgenic apolipoprotein-E homozygote knockout C57BL/6 mice (β-actin-GFP+-ApoE) (Post *et al.*, 2008). mMSCs, mouse embryonic fibroblasts (MEFs) and limb bud cells (IV) were isolated from wild type (WT) and *Dlk1*-/- mice of the SvJ129/C57Bl background (Raghunandan *et al.*, 2008). The study protocols for the experiments were approved by the local institutional committees for animal welfare.

4.2 Isolation and culturing of cells

4.2.1 Mouse mesenchymal stromal cells (mMSCs) (I, II, IV, V)

Mouse mesenchymal stromal cells (mMSCs) were isolated from the bone marrow of 8-10 week old male mice. Mice were sacrificed by cervical dislocation, the femur and tibia were removed, and cells were flushed from the bone marrow with isolation medium (RPMI-1640 (Gibco Invitrogen) supplemented with 12% fetal bovine serum (FBS; Gibco Invitrogen), 100 U/ml penicillin (Gibco Invitrogen) + 100 μg/ml streptomycin (1% P/S) (Gibco Invitrogen) and 12 μM L-glutamine (Gibco Invitrogen)) using a syringe and a needle. The cells were incubated on plastic for 2 hours at 37°C to remove fibroblasts and other rapidly adhering cells, and the non-attached cells were plated at a density of 1×10^6 cells/cm^2 in expansion medium (D-MEM; Gibco Invitrogen, 12% FBS and 1% P/S). After 48 hours, non-adherent cells were discarded, adherent cells were washed with PBS and fresh medium was added.

To obtain short-term selected (STS) MSCs, the plastic adherent cells were cultured in expansion medium for 1 week with a medium change after 4 days, and lifted by incubation in 0.25% trypsin/1 mM EDTA for 5 min at 37°C. For long-term selected (LTS) MSCs, the adherent cells were cultured in isolation medium for 4 weeks with a medium change after every 3 to 4 days, lifted with trypsin, and replated at a density of 10 000 cells/cm^2 in isolation medium. After 1– 2 weeks, cells were lifted by trypsinization, plated at a density of 1000 cells/cm^2, and cultured in expansion medium until confluent (1–2 weeks) for two more passages.

The isolation and culture of the clonal mouse bone marrow-derived $mMSC^{Adipo}$ and $mMSC^{Bone}$ cells was performed as described by Post and co-workers using a method developed by Peister and others (Peister *et al.*, 2004; Post *et al.*, 2008). The cells were cultured in expansion medium (see above). To obtain conditioned medium (mMSC-CM), the cells were cultured in the absence of FBS and CM was collected after 48 hours.

4.2.2 Mouse mesenchymal ST2 cells (II)

The mouse ST2 mesenchymal cells were originally purchased from Riken Cell Bank, Tsukuba Science City, Japan. Cells were cultured in D-MEM supplemented with 10% FBS and 1% P/S.

4.2.3 Mouse pre-osteoblastic calvaria cells (II)

Primary calvaria cells were isolated from 3-day-old mice by sequental digestion. The calvaria were cleaned, diced into small pieces and digested in 4 mg/ml of collagenase type II (Worthington Biochemical Corporation) in PBS for 10 min at 37°C. The initial digestions were discarded. Supernatants from the second and third sequential digestions at 37°C were collected. Calvaria cells were cultured in D-MEM supplemented with 20% FBS (Biochrom) and 1% P/S.

4.2.4 Mouse embryonic fibroblasts (MEFs) (IV)

Mouse embryonic fibroblasts were isolated from E13.5 old mouse embryos. An entire mouse was minced and trypsinized with 0.25% trypsin for 5 min at 37°C. The cells were plated in D-MEM + 10% FBS + 1% P/S and cultured until confluent. The confluent MEFs were trypsinized for 5 min at 37°C and replated at a density of 6000 cells/ cm^2. Cells from passage 2 or 3 were used for the differentiation studies.

4.2.5 Mouse limb bud cells (IV)

Limb bud cells were isolated from E11.5 WT embryos. Limbs were dissected and digested with 3mg/ml Dispase (Invitrogen) for 20 min at 37^{o}C. The cells were suspended at a density of $2x10^7$ cells/ml in D-MEM/F12 + 10% FBS and plated as a micromass in 10µl of medium. The cells were incubated for 2 h at 37^{o}C prior to the addition of 500 µl of the medium (D-MEM/F12 supplemented with 10% FBS), 50 µg/ml ascorbic acid (Sigma-Aldrich) and 10 nm β-glycerophosphate (Sigma-Aldrich). The medium was changed every second day.

4.2.6 Human embryonic stem cells (hESCs) (III)

The undifferentiated hESC line HUES9 was obtained from the Howard Hughes Medical Institute, Harvard University (Cowan *et al.*, 2004). The KMEB2 and KMEB3 hESC lines were derived and characterized according to previously described procedures (Harkness *et al.*, 2010). Undifferentiated hESCs were maintained on a MEF feeder layer in Knockout Dulbecco's Modified Eagle's Medium (KO-D-MEM, Invitrogen) supplemented with 15% Knockout Serum Replacement (KSR; Invitrogen), 0.5% human serum albumin (ZLB Behring), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1% MEM non-essential amino acids solution (Invitrogen), 2 mM glutamine as Glutamax-I (Invitrogen), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen), and 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Invitrogen). Subconfluent ESC cultures were passaged every four to seven days by trypsinization.

4.3 Differentiation of cells

4.3.1 Chondrogenic differentiation of MSCs, MEFs and ESCs (I, II, III, IV, V)

Chondrogenic differentiation was induced in pellet cultures (Johnstone *et al.*, 1998) in which 200 000 cells were placed in a 15ml polypropylene tube (Falcon™ BD Biosciences) and centrifuged into a pellet at 500 ×g for 6 min. The pellets were cultured at 37°C in 5% CO_2 in 0.5 ml of chondrogenic medium: high-glucose D-MEM (Gibco Invitrogen) supplemented with 10 ng/ml of TGF-β3 (R&D Systems), 100 nM dexamethasone (Sigma-Aldrich), 50 μg/ml of L-ascorbate-2-phosphate (Sigma-Aldrich), 40 μg /ml of L-proline (Sigma-Aldrich), 100 μg/ml of sodium pyruvate (Sigma-Aldrich) and 50 mg/ml of ITS + Premix (BD Biosciences). Chondrogenic medium was supplemented with 500 ng/ml rhBMP-2 for mMSC and 100 ng/ml for MEF cultures. To compare the effects of BMPs on MSC chondrogenesis (I), the chondrogenic medium was supplemented with 500 ng/ml of rhBMP-2, rhBMP-4, rhBMP-5, rhBMP-6, rhBMP-7 or the rhBMP-2/7 heterodimer (R&D Systems). The medium was replaced every three to four days.

4.3.2 Osteogenic differentiation of MSCs and calvaria cells (I, II, V)

For osteogenic differentiation, the cells were cultured at a density of 10 000 cells/cm^2 and induced with osteogenic medium containing α-MEM (Gibco Invitrogen) or D-MEM supplemented with 12% FBS, 1% PS, 10 mM Na-β-glycerophosphate (Fluka BioChemika) and 50 μg/ml ascorbic acid. The medium was changed every three to four days. To investigate the role of sFRP-1 in osteogenesis (II), the medium was supplemented with recombinant sFRP-1 (100 ng/ml, 500 ng/ml; R&D Systems).

4.3.3 Adipogenic differentiation of MSCs (I, II, V)

For adipogenesis, mMSCs were plated at a density of 10 000 cells/cm^2 and incubated in D-MEM supplemented with 12% FBS, 1% P/S, 5 μg/ml insulin (Sigma-Aldrich), 50 μM indomethacin (Sigma-Aldrich) and 1 μM dexamethasone with a medium change every three to four days. To induce adipogenesis in the ST2 mesenchymal cells, the cells were cultured in D-MEM supplemented with 10% FBS, 450 μM IBMX, 250 nM dexamethasone, 1μM BRL (BRL49653; kindly provided by Novo Nordisk) and 5μg/ml insulin. After 2 days of induction, medium was changed to D-MEM containing 10 % FBS, 1% PS and 5 μg/ml insulin. For the evaluation of the effect of sFRP-1 on adipogenesis, sFRP-1 was added to the differentiation medium (100 ng/ml, 500 ng/ml).

4.3.4 Embryoic body (hEB) formation and the outgrowth culture of hEBs (III)

For hEBs, $12x10^6$ hESCs were placed into an ultralow adhesion Petri dish (Corning) and cultured in a suspension in KO-D-MEM supplemented with 15% KSR, 0.5% human serum albumin, 1% MEM non-essential amino acids solution, 2 mM glutamine and 1% P/S, or in a serum-free, chemically defined medium (CDM; Invitrogen). After

48 h, the hEBs were separated from single cells by gravity sedimentation and further cultured in a suspension for 10 or 25 days with a medium change every three days.

For the outgrowth culture, day 10 hEBs were collected by centrifugation, and 30-50 EBs were plated on a fibronectin-coated well of a six-well plate. Outgrowths were cultured in serum-free CDM supplemented with 10 ng/ml Activin B or 1 μM SB431542 with a medium change every three days.

4.3.5 In vivo *differentiation and teratoma formation of hESCs (III)*

Four million hESCs were mixed with the matrigel basement membrane matrix (1:1) (Becton Dickinson) and injected subcutaneously into the dorso-lateral area of NOD-SCID mice (NOD/LtSz-Prkdcscid). After 8 weeks of implantation, teratomas were removed and processed for histology.

4.4 Histological and immunochemical methods

4.4.1 Preparation of paraffin sections (I, II, III, IV, IV, V)

Prior to histochemistry and immunohistochemistry, the chondrocyte pellets and hESC implants were fixed in 4% PFA for 15 minutes, dehydrated and embedded in paraffin. Mouse embryonic samples were collected on days E11.5 to 18.5, fixed in 4% PFA for from 3 hours to overnight and embedded in paraffin. The hind limbs of newborn, 10-day, 20-day and 2-month-old mice were dissected free of skin and muscle, decalcified in 10% EDTA, 0.1 M sodium phosphate buffer (pH 7.0) for 1 to 4 weeks, and embedded in paraffin. The paraffin embedded samples were cut into 5 μm sections.

4.4.2 Histochemical stainings (I, II, III, IV, V)

Proteoglycans were demonstrated by Toluidine blue (1% in H_2O) or Alcian blue (1% in 3% acetic acid, pH 2.5) staining in rehydrated paraffin embedded sections after a chondrogenic induction. Both stainings were performed for 5 minutes at room temperature.

To detect alkaline phosphatase, cells were fixed with an acetone: citrate buffer (pH 4.2), 2:1 (v/v) for 5 min at RT. Naphtol-AS-TR-phosphate (Sigma-Aldrich) was diluted 1:5 in H_2O, and Fast Red TR (Sigma-Aldrich) diluted 1:1.2 in 0.1 M Tris buffer (pH 9.0; OUH Pharmacy). The solutions were mixed 1:1 (v/v) and used as a substrate for alkaline phosphatase staining for 1 h at room temperature. Cells were counterstained with Mayers-Haematoxylin.

For the visualization of the mineralized matrix, cells were fixed in 70% ice-cold ethanol for 1 h at -20°C, and stained with 40 mM Alizarin red S (pH 4.2; AR-S; Sigma-Aldrich) for 10 min at room temperature.

Bone nodules were detected with von Kossa staining. Cells were fixed in 4% PFA for 10 min at room temperature and incubated with 2% $AgNO_3$ solution for 1 hour and, subsequently, with 2.5 % sodium thiosulfate for 5 min at room temperature.

Adipocytes were visualized by Oil red O staining for lipid droplets. Cells were fixed in 4% PFA for 10 min at room temperature, rinsed with a 3% isopropanol solution, and stained with Oil Red O (Sigma-Aldrich) solution for 1 h at room temperature. Cells were counterstained with Mayers-Haematoxylin.

4.4.3 Immunohistochemistry (I, II, III, IV, V)

The paraffin sections were rehydrated and digested with bovine testicular hyaluronidase (1000- 2000 U/ml) in PBS (pH 5.5) for 60 min at 37°C or by heating in 10 mM Tris-Na-citrate (pH 6.0) at 95°C to facilitate antibody access. Non-specific peroxidase activity was blocked by 0.3% H_20_2 in methanol and nonspecific antibody binding by 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS or 2% rabbit normal serum and 2% BSA in PBS, or a specific blocking reagent when supplied with the secondary detection kit. The antibodies and secondary detection kits used in the immunology-based assays are listed in Table 4.

For the detection of type II collagen, the paraffin sections were incubated with a monoclonal antibody raised against chicken type II collagen (6B3 Chemicon Millipore), overnight at 4°C. Binding of the primary antibody was detected by Mouse Link and AP Label (BioGenex) with Fast Red (Sigma-Aldrich) as a substrate for the colour development.

For the other antibodies (Table 4), anti-goat IgG and ABC detection reagents (Vector Laboratories) or a Histostain Broad Spectrum kit (Zymed) were used for the detection of the primary antibody, and diaminobenzidine (DAB) chromogen (Zymed) for the visualization. After counterstaining with haematoxylin, the sections were mounted in Aquamount Improved (BDH, Poole). In control sections, the primary antibody was replaced with normal serum.

Table 4. Antibodies

Antibody	Characteristics	Supplier/ reference	Secondary antibody	Application	Used in
beta III-Tubulin	Mouse MAb	AH-diagnostic	Powervision+	ICC	III
BMP-2	Goat PAb	SantaCruz Biotechnology	Vectastain ABC	IHC	I
BMP-2/4	Goat PAb	R&D Systems	Vectastain ABC	IHC	I
BMP-5	Goat PAb	SantaCruz Biotechnology	Vectastain ABC	IHC	I
BMP-6	Goat PAb	SantaCruz Biotechnology	Vectastain ABC	IHC	I
BMP-7	Goat PAb	SantaCruz Biotechnology	Vectastain ABC	IHC	I
CD31	Mouse MAb	Dako	Powervision+	ICC	III
CD34	Mouse MAb	Vision Biosystems	Powervision+	ICC	I, V
CD45	Mouse MAb	Dako	Powervision+	ICC	I, V
CD56	Mouse MAb	Novocastra	Powervision+	ICC	III
FA1	Rabbit PAb	(Jensen *et al.*, 1994)	Histostain Plus	IHC, ICC FACS	III, IV
Oct 3/4	Goat PAb	SantaCruz	Envision+	ICC	III
Sca-1	Rat MAb	BD Biosciences	Powervision+	ICC	I
sFRP-1	Rabbit PAb	SantaCruz Biotechnology	Powervision+	ICC, WB	II
Sox9	Goat PAb	R&D	Vectastain ABC	IHC	III, IV
Type I Collagen	Mouse MAb	Chemicon	Powervision+	ICC	III
Type II Collagen	Mouse MAb 6B3	Chemicon Millipore	Mouse Link and label	IHC	I, II, III, IV, V
Type IIA procollagen	Rabbit PAb	(Salminen *et al.*, 2001)	Histostain Plus	IHC	I, III
Type X collagen	Mouse MAb X53	Quartett	Histostain Plus	IHC	I, II, III, IV

* Abbreviations: MAb = monoclonal antibody PAb = polyclonal antibody, IHC = immunohistochemistry, ICC = immunocytochemistry, WB=Western blot

4.4.4 Evaluation of type II collagen production (I)

Progression of the chondrogenic differentiation was evaluated in LTS cell pellets by determining the relative matrix area that was stained with the type II collagen antibody. Tissue sections at 120 μm intervals from each pellet were analysed for the global threshold values using the LabView 7.1 program (National Instruments). The relative area of the type II collagen matrix was quantified by comparing the number of pixels exceeding the global threshold value to the total number of pixels in the pellet.

4.4.5 Immunocytochemistry (I, II, III, V)

Cells were cultured in chamber slides at a density of 10 000 cells/cm^2 for 48 hours and fixed with 4% PFA for 15 min, washed with PBS and fixed with 0.3% H_2O_2 in methanol for 15 min. For surface epitope characterization, primary antibodies (Table 4) were diluted in the ChemMate antibody diluent (Dako) and incubated for 1 hour. Immunostaining was performed using the DAKO EnVision$^+$ kit (Dako) or the DPVO system (Immunovision Technologies Co.). For detection with EnVision$^+$, the cells were incubated with an anti-rabbit Ig/HRP (Horseradish peroxide)-conjugated polymer. In DVPO, after post-blocking (DPVO+500Post), the cells were incubated with a poly-HRP anti-Mouse/Rabbit IgG polymer (DPVO+500HRP). The secondary antibodies were visualized with DAB chromogen and the nuclei were counterstained with haematoxylin.

4.4.6 Immunodepletion (II)

The conditioned medium containing 400 µg of proteins were incubated with 2 µg of sFRP-1 or normal rabbit IgG antibody overnight at +4°C. Protein A/G-sepharose beads (Santa Cruz Biotechnology) were added to the medium and incubated in a rotator for 3 hours at 4°C. After centrifugion for 5 min at 4°C, the supernatant, representing immunodepleted CM, was collected.

4.4.7 Western blotting (II)

The conditioned media were concentrated with a concentrator (VivaSpin, GE Healthcare Life Sciences) by centrifuging at 3000 rpm for 2 hours. Their total protein concentrations were determined with the Pierce Coomassie Plus Bradford assay kit (Thermo Fisher Scientific Inc.). Proteins were separated by SDS-PAGE and transferred into nitrocellulose filters. After blocking the membranes with 5% non-fat milk for 1h at room temperature, they were incubated with a primary antibody overnight at +4°C. The membranes were incubated with an HRP-conjucated secondary antibody for 45 min at room temperature, and the protein bands were visualized with the Amersham ECL chemiluminescence detection system (GE Healthcare Bio-Sciences Corp.). To confirm an equal loading of the samples, the gel was stained with Coomassie Brilliant Blue stain for 20 minutes and photographed with the FUJIFILM LAS-4000 luminescent image analyzer (FUJIFILM Corp.).

4.4.8 Flow cytometry (FACS) (III)

For the flow cytometry (FACS) analysis, the cells were centrifuged and resuspended into blocking buffer (PBS with 0.5 % BSA, 0.05 % normal human serum, 5% FBS) at a concentration of 10^6 cells/ml. The cells were stained with a non-conjucated primary antibody (Table 4) for 30 minutes on ice, followed by incubation with an immunofluorescent secondary antibody for 30 minutes on ice. After washing, the cells were resuspended in 0.5 % BSA in PBS, and analysed by FACScan linked with the cell-Quest 3.1 software or sorted with the FACSDiVa cell sorter (Becton Dickinson).

4.5 Gene expression analysis

4.5.1 RNA extraction and RT-PCR analysis (I, II, III, IV, V)

The total RNA was isolated with a mirVana RNA isolation kit (Ambion) or a single-step method of TRIzol (Invitrogen A/S). Genomic DNA was digested with DNase I (NEB) and cDNA was synthesized from 1-2 µg of total RNA using a RevertAid H minus first strand cDNA synthesis kit (Promega Corporation; Fermentas).
For the RT-PCR analysis, cDNAs were amplified using DyNAzyme II DNA Polymerase (Finnzymes) for 20-35 cycles and analysed on a 1.5% agarose gel. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with BioRad or Applied Biosystems 7500 Real-Time system using the Fast SYBR Green Master Mix (BioRad; Applied Biosystems). The data were normalised for housekeeping gene *β-actin*, *Gapdh* or *L19* mRNA, and the relative expression level and fold induction of each target gene were calculated using a comparative C_T method [(1/ ($2^{\Delta C}{}_T$) formula, where ΔC_T is the difference between C_T target and C_T reference] with Microsoft Excel 2007®. The primer sequences used are listed in Table 5.

Table 5. The primer sequences

Gene	Forward primer	Reverse primer	Used in
Actb	TTC CTT CTT GGG TAT GGA AT	GAG CAA TGA TCT TGA TCT TC	I, II, V, IV
Bglap	GAA ACT CTT CCA AGC AAT TC	GGA CTA GCT TGT CCT TGT GG	IV
Alpl	TCC TGA CCA AAA ACC TCA AAGG	TGC TTC ATG CAG AGC CTG C	II
Bsp	CAG AAG TGG ATG AAA ACG AG	CGG TGG CGA GGT GGT CCC AT	II
Col1a1	GGT GAA CAG GGT GTT CCT GG	TTC GCA CCA GGT TGG CCA TC	I, II, V
Osx/Sp7	GCA GCA GCG GCT GGG CTG AGGA	GGA GCT GGA GAC CTT CCT CTA C	I, II, V
Runx2	AGC AAC AGC AAC AAC AGC AG	GTA ATC TGA CTC TGT CCT TG	I, II, V
Acan	CCC GGT ACC CTA CAG AGA CA	ACA GTG ACC CTG GAA CTT GG	I, II, III, IV,V
Col2a1	ACA TGT CAG CCT TTG CTG GC	CAT GGT CTC TCC AAA CCA GA	I, II, III,IV,V
Col10a1	CCT GCA GCA AAG GAA AAC TC	TGG CTT AGG AGT GGG AGC TA	I, II, III, IV,V
Comp	GTT CAG CGG ACC CAC CCA CG	GCA TCT CCG ATG CCG TCC CG	IV
Ihh	TGG CCA TGG GGG AGG ATG GG	GCC ACG TGG GTG GAG ACA GC	IV
PTHrP	CCACGACCCAGAGTGCTGCC	GGCCACTCGCAGCTGTGCTT	IV
S100	AGACCCTTGGAGGAGGCCCT	TCCGGGGCTCCTTATCTGGGC	IV
Sox9	CGA CTA CGC TGA CCA TCA GA	AGA CTG GTT GTT CCC AGT GC	I, II, III, IV,V
Dcn	CTG GGC GGC AAC CCA CTG AA	CCC AGC AGG CAC CCT GAG GA	II
Col11a1	CCG TGC AGG CCC AAC TGG AG	GTT GGA CCA CGC TGT CCC CG	II
Adipoq	GAC GTT ACT ACA ACT GAA GAG C	CAT TCT TTT CCT GAT ACT GGT C	II
Agt	GGC TGG CCG TGG GAT CTA	GGC CGC CGA GAA GCT AGA	II
Agt	GGC TGG CCG TGG GAT CTA	GGC CGC CGA GAA GCT AGA	II
C/ebpa	AAG CCA AGA AGT CGG TGG A	CAG TCC ACG GCT CAG CTG TTC	II
Lpl	CAG AGT TTG ACC GCC TTC C	AAT TTG CTT TCG ATG TCT GAG AA	II
Pparg	GGG TCA GCT CTT GTG AAT GG	CTG ATG CAC TGC CTA TGA GC	II
Cntn1	TTG TCT AGG AGA CTT TAC CTG GC	AAA TGG TAT TGA TTG GCT GCT CT	II
Dpep1	GCA CAA CGA CTT GCC TTG G	ATG CGG TGT ATC ACA TCC ATC	II
Emb	TGA GGG CGA TCC CAC AGA T	CCG TCA CTG AGA TAT TAC AGC TC	II
Kitl	CCC TGA AGA CTC GGG CCT A	CAA TTA CAA GCG AAA TGA GAG CC	II
Kng1	ACA GGT GGT GGC TGG CCT GA	TGG GAA GGG CCA CGC AGT CT	II
Mef2c	ATC TGC CCT CAG TCA GTT GG	CAG CTG CTC AAG CTG TCA AC	II
Mmp13	GAT GAC CTG TCT GAG GAA G	ATC AGA CCA GAC CTT GAA G	II
Lox	ACT CCC TGC GAC CCA TCC CC	TCC CGG CTC GTC CCT TCT CG	II
Pdpn	ACC GTG CCA GTG TTG TTC TG	AGC ACC TGT GGT TGT TAT TTT GT	II
Lef1	AGT GCA GCT ATC AAC CAG AT	TTC ATA GTA TTT GGC CTG CT	II
Tcf7	ACA GCA CTT CCC TGC CCC CA	AGT GCA TGC CAC CTG CGA CC	II
Sfrp1	GCC ACA ACG TGG GCT ACA A	ACC TCT GCC ATG GTC TCG TG	II

4.5.2 Microarray analysis (I, II)

For analysis of the gene expression profile during the chondrogenesis of developing mouse limbs, epiphyseal samples were microdissected from the entire limb buds (embryonic day p.c. E9.5 - E11.5), and the developing knee epiphyseal cartilage (E12.5 - E20.5). The samples were ground into a fine powder and the total RNA was extracted by sedimentation through 5.7M cesium chloride density gradient by ultracentrifugation (Saamanen *et al.,* 2007). $mMSCs^{Adipo}$ and $mMSCs^{Bone}$ were cultured in triplicate under standard culture conditions. At 90% confluence, the total RNA was isolated with an RNeasy Kit (QIAGEN Nordic).

cDNA was synthesized from 8 µg RNA using the SuperScript Choice System (Invitrogen) according to the manufacturer's instructions and hybridized to the Affymetrix GeneChip® Mouse Genome 430A 2.0 Array (Affymetrix) and the arrays were scanned using the GeneArray (Affymetrix).

4.5.3 MicroRNA expression analysis (V)

The expression profiles of the miRNAs were detected by qRT-PCR. Amplifications were performed using the Taq DNA Polymerase (ABgene) and the mirVana qRT-PCR miRNA Detection Kit (Ambion) according to the manufacturer's instructions. The reactions were performed with 50 ng of DNAse-treated total RNA in triplicate with mirVana qRT-PCR Primer sets. The RT-reactions and real-time PCR reactions were performed in an MJ Research PTC-200 DNA Engine Cycler. The microRNA expression data were normalised to U6 snRNA and 5S rRNA, and the relative quantification of miRNA expression was calculated with the 2−delta-deltaCt method in which undifferentiated cells were set as a calibrator sample.

4.5.4 Wnt signalling superarray (II)

For a detailed analysis of the genes related to Wnt signalling, $mMSCs^{Adipo}$ and $mMSCs^{Bone}$ were cultured in a basal medium until 70 % confluent. The medium was supplemented with Wnt3a (50 ng/ml) (R&D Systems) for 24 hours and Wnt SuperArray (GIAGEN Ab) was used to quantify the expression profiles of 84 genes related to the Wnt-mediated signal transduction.

4.5.5 Luciferase assay (II)

Cells were cultured in 24-well plates for 24 hours to reach 80% confluence and transfected with 2 µg of the Topflash plasmid and 10 ng pRL-SV40 using FuGENE6 reagent (Roche) according to the manufacturer's protocol. To induce Wnt signalling, the cells were cultured with 25% Wnt-3 conditioned medium (Qiu *et al.*, 2007). The luciferase activity was measured after 24 hours with a dual luciferase reporter assay (Promega) in a luminometer (BMG Labtechnology) according to the manufacturer's instructions.

4.6 Signalling pathway analysis (IV)

To study the signalling pathways regulating *Dlk1* expression, MEF and limb bud cultures were supplemented with activators or inhibitors of specific pathways (Table6).

Table 6. Recombinant proteins

Recombinant protein	Concentration	Supplier
TGF-β1	1, 10, 100 ng/ml	R&D
TGF-β3	1, 10, 100 ng/ml	R&D
Activin A	1, 10, 100 ng/ml	R&D
Activin B	1, 10, 100 ng/ml	R&D
BMP-2	10, 100, 200 ng/ml	R&D
BMP-4	10, 100 ng/ml	R&D
SB431542	10nM, 100 nM, 1 μM,	Sigma-Aldrich
SB505124	10nM, 100 nM, 1 μM,	Sigma-Aldrich
SD-208	100nM, 1 μM, 10 μM	Tocris Bioscience
LY 364947	1 μM, 10 μM, 100 μM	Tocris Bioscience
Follistatin	5, 50, 500 ng/ml	R&D
DAPT	100nM	Calbiochem
PD169316	2μM	Calbiochem

4.7 Bioinformatics (I, II, V)

Table 7. The programs used for bioinformatics

Program	Reference	Application	Used in
Kensington	www.inforsense.com	Expression profile analysis	I
GeneSpring		Expression profile analysis	I
Affymetrix GCOS	www.affymetrix.com	Preprocessing and normalisation of microarray data	I
dChip	(Li and Wong, 2001)	Analysis of microarray data	II
DAVID 2.0	(Dennis *et al.*, 2003)	Functional analysis	II
Ingenuity Pathways Analysis v. 5.0	www.Ingenuity.com	Signalling pathway analysis	II, V
TargetScan	www.targetscan.org	miRNA target predictions	V
PicTar	www.pictar.bio.nyu.edu	miRNA target predictions	V
miRanda	www.microrna.org	miRNA target predictions	V
Conreal	Berezikov, E. *et al*., 2004	Transcription factor binding site predictions	V

4.8 Statistical analysis (I, II, III, IV, V)

Statistical testing was performed using ANOVA with Tukey's post hoc test. Pair wise comparisons were performed using the two-sample independent Student's *t*-test.

5. RESULTS

5.1 The influence of the cell population composition on the characteristics and chondrogenic differentiation potential of mMSCs (I, II)

5.1.1 Distinct cell morphology and surface epitope expression in mMSC populations (I)

With the aim of optimizing the *in vitro* pellet culture model and studying the effect of cell populations on chondrogenesis, plastic adherent mMSCs were isolated from the mouse bone marrow and characterized by their morphology, surface epitope profile and differentiation capacity after culturing and selection for plastic adherence for different periods of time. After 48 hours of culture, a majority of the plastic-adherent cells were small and spindle-shaped, and expressed surface markers CD34 and CD45 (I: Figure 2). After 1 week of culture, the STS cells had lost their expression for haematopoietic markers and flatter, stem cell antigen-1 (Sca-1)-positive cells were observed compared to the primary cultures (Table 8). Long-term selection removed haematopoietic cells from the culture and the remaining LTS cells formed a homogenous population of Sca1-positive, large, flat cells.

5.1.2 Cell population dependent differentiation of mMSCs (I, II)

To study the effect of cellular composition on mesenchymal differentiation, primary (STS), more selected (LTS) and committed ($mMSC^{Adipo}$ and $mMSC^{Bone}$) bone marrow derived mMSCs were induced to undergo adipogenic, osteogenic and chondrogenic differentiation (Table 8).

Table 8. The expression of surface markers and differentiation capacity

	STS	LTS	$mMSC^{Adipo}$	$mMSC^{Bone}$
Surface epitope profile				
CD-34	-	-	-	-
CD-45	-	-	-	-
Sca-1	+	++	++	++
Differentiation capacity				
Adipogenic	+	+	++	-
Osteogenic	+	+	-	++

Both the STS and LTS cells exhibited a potential to differentiate into adipogenic and osteogenic lineages, demonstrated by the histochemical staining for Oil Red O, and von Kossa and alkaline phosphatase, respectively, and by gene expression analysis of *Osx, Col1a1* and *Runx2* (I: Figure 3). Despite their similar surface epitope profiles (Table 8), the mouse bone marrow-derived clonal cell strains, $mMSC^{Bone}$ and $mMSC^{Adipo}$, have been previously shown to differ in their adipogenic and osteogenic differentiation capacities (Post *et al.*, 2008). The $mMSC^{Bone}$ cells have the ability to differentiate into

osteogenic lineage but not into adipocytes while the mMSC^{Adipo} cells differentiate into adipocytes but not into osteoblasts, indicating that the bone marrow contains committed cell populations that have a limited differentiation potential.

Under chondrogenic induction in a pellet culture system, the STS cells differentiated in 2 weeks into round, proteoglycan and type II collagen-producing chondrocytes (Figure 9, I: Figure 4A). BMPs enhanced the differentiation process, rhBMP-2 and rhBMP-7 more efficiently than rhBMP-6. After 4 weeks of culture, chondrogenesis in rhBMP-2 and rhBMP-7 -induced pellets progressed to terminal differentiation, with type X collagen producing hypertrophic cells and matrix mineralization (I: Figure 4B).

Compared to the STS cells, chondrogenic differentiation occurred at a slower rate in the LTS cells. After 3 weeks in culture, the cells induced with rhBMP-2, rhBMP-4, rhBMP-6 or rhBMP2/7 heterodimer differentiated into proteoglycan and type II collagen-producing chondrocyte-like cells (Figure 9, I: Figure 5). At the transcriptional level, *Sox9* and *Col2a1* as well as *Col10a1* were expressed in all samples at day 21 (I: Figure 6). No type X collagen protein was observed in any of the cultures. *Acan* was not expressed in control pellets and was only expressed at a very low level in BMP-7-induced pellets, indicating a limited ECM production in these pellets.

Similarly, differences were observed amongst the committed mMSCs in their chondrogenic potential. Following a chondrogenic induction in a pellet culture system, mMSC^{Bone} cells readily differentiated into the chondrogenic lineage. After 3 weeks in culture, the mMSC^{Bone} pellets consisted uniformly of proteoglycan and type II-positive cells that highly expressed chondrogenic marker genes (Figure 9, II: Figure 1). In contrast, although early chondrogenic marker genes *Sox9* and *Col2a1* were expressed at a low level in the mMSC^{Adipo} cells, no proteoglycan or type II collagen production was observed, denoting that the mMSC^{Adipo} cells did not exhibit the capacity to produce cartilage matrix and differentiate into mature chondrocytes.

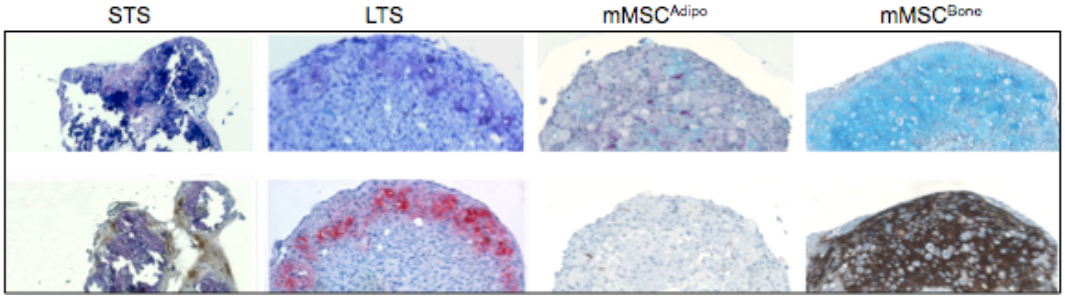


Figure 9. Chondrogenic differentiation of the STS, LTS, mMSC^{Adipo} and mMSC^{Bone} cells. Chondrogenesis was evaluated by Toluidine blue (STS, LTS) or Alcian blue staining (mMSC^{Adipo}, mMSC^{Bone}) of proteoglycans (upper panel) and type II collagen at day 14 (STS) or at day 21 (lower panel).

5.1.3 The molecular signature of committed mMSCs (II)

With the aim of understanding the molecular mechanisms determining the lineage commitment of the MSCs, a microarray analysis was performed to compare the basal gene expression profiles of committed mMSC progenitors $mMSC^{Bone}$ and $mMSC^{Adipo}$. In the $mMSC^{Bone}$ cells, 198 genes were over three-fold up-regulated in comparison to the $mMSC^{Adipo}$ cells, and in the $mMSC^{Adipo}$ cells, 210 genes were up-regulated compared to the $mMSC^{Bone}$ cells.

The gene expression analysis revealed a cell type-specific gene expression profile in the committed undifferentiated mMSCs (Tables 9 and 10; II: Supplemental Table 2). When highly expressed genes were annotated based on their biological function, an over-representation of genes related to extracellular matrix and skeletal development was found in the $mMSC^{Bone}$ cells of 25% vs 1% in the $mMSC^{Adipo}$ cells (II: Supplemental Figure 1). Genes involved in adipogenic differentiation and lipid metabolism were highly expressed in the $mMSC^{Adipo}$ cells (20% vs 5%). Interestingly, an impairment of osteoblast and chondrocyte differentiation in the $mMSC^{Adipo}$ cells was related to an up-regulation of inflammation and immune response genes (35% vs 14%).

Table 9. Selected up-regulated genes in the $mMSC^{Bone}$ cells categorized by their biological function.

Gene Title	Gene Symbol	Fold Change
Skeletal development		
Bone sialoprotein, Integrin binding sialoprotein	*Bsp/Ibsp*	7.6
Bone morphogenetic protein 2	*Bmp2*	4.3
Sry-box containing gene 6	*Sox6*	3.4
Extracellular matrix		
Procollagen, type XI, alpha 1	*Col11a1*	10.7
Fibronectin	*Fndc1*	9.7
Decorin	*Dcn*	7.7
Collagen triple helix repeat containing 1	*Cthrc1*	7.7
Periostin osteoblast specific factor	*Postn*	6.3
Fibromodulin	*Fmod*	5.5
N-cadherin	*N-cad*	4.8
Wnt signalling		
Wingless-related MMTV intergration site 10a	*Wnt10a*	34.6
Transcription factor 7, T-cell specific	*Tcf7*	13.9
Wingless-related MMTV integration site 7b	*Wnt7b*	13.2
Tumor necrosis factor receptor superfamily, 19	*Tnfrsf19*	9.5
Cyclin D1	*Ccnd1*	8.2

Table 10. Selected up-regulated genes in the mMSC^{Adipo} cells categorized by their biological function

Gene Title	Gene Symbol	Fold Change
Immune response and inflammation		
Interleukin 1 receptor antagonist	*Il1rn*	9.9
Interleukin 1 receptor-like 2	*Il1rl2*	7.7
Interferon alpha-inducible protein 27	*Ifi27*	6.6
Ectodysplasin-A	*Eda*	6
Chemokine (c-x-c motif) ligand 7	*Cxcl7*	5.2
Adipogenesis and lipid metabolism		
Adiponectin C1Q and collagen domain	*Adipoq*	7.8
Lipoprotein lipase	*Lpl*	6.7
Fatty acid binding protein 4 adipocyte	*Fabp4*	6.5
CCAAT/Enhancer binding protein (C/EBP), alpha	*Cebpa*	4.4
Hydroxysteroid 11-beta dehydrogenase	*Hsd11b1*	4.2
Peroxisome proliferator activated receptor	*Pparg*	3.5

5.2 Signalling pathways regulating chondrogenesis (I, II)

5.2.1 Wnt signalling and sFRP-1 mediated cell fate determination (II)

Signalling pathway analyses annotated Wnt signalling as the most significantly up-regulated signalling pathway in the mMSC^{Bone} cells compared to the mMSC^{Adipo} cells ($p<0.006$). Wnt superarray analysis revealed a high expression of several Wnt signalling genes in mMSC^{Bone} including receptor tyrosine kinase-like orphan receptor 2 (*Ror2*) and target genes of canonical Wnt signalling: transcription factor 7, T-cell specific (*Tcf7*), Cyclin D1 (*Ccnd1*) and tumor necrosis factor receptor superfamily member 19 (*Tnfsrf19*) (Table 9; II: Table 1). Moreover, Wnt ligands *Wnt10a* and *Wnt7b* were highly expressed by mMSC^{Bone}. The significant difference in Wnt activity was further confirmed by a luciferase assay and gene expression analyses of Wnt target genes (II: Figure 3).

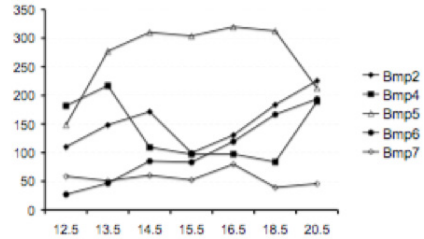
Interestingly, low Wnt signalling activity in the mMSC^{Adipo} cells was associated with high expression of Wnt inhibitor, secreted frizzled-related protein -1 (*Sfrp-1*) (II: Figure 3). SFRP-1 was also detected at the protein level in the mMSC^{Adipo} cells and was secreted in the conditioned medium by the mMSC^{Adipo} cells (mMSC-CM^{Adipo}). Addition of mMSC-CM^{Adipo} suppressed the osteoblastic differentiation and chondrocyte maturation while the removal of sFRP-1 from mMSC-CM^{Adipo} partially restored the differentiation capacity, suggesting a role for sFRP-1 and Wnt signalling in cell fate determination (II: Figure 2, 4). Indeed, when MSCs were induced into adipogenic and ostegenic differentiation in the presence of recombinant sFRP-1, exogenous sFRP-1 exerted a dose-dependent inhibitory effect on osteoblast differentiation while enhancing adipogenesis in a dose-dependent manner (II: Figure 5), demonstrating the important role of sFRP-1 in determining the mesenchymal cell fate decision between adipogenesis and osteoblastogenesis.

5.2.2 In vivo *expression profile and* in vitro *effect of BMPs in chondrogenesis (I)*

A comprehensive expression analysis was conducted to characterize the gene expression profiles of chondrogenic genes during mouse limb development. Expression profiles of all BMPs were extracted from the microarray data covering chondrogenic differentiation from the limb bud outbreak at E9.5 to birth. BMPs displayed distinct expression profiles (Table 11, I: Figure 1). The expression of *Bmp-2* and *Bmp-6* gradually increased until birth. The *Bmp-4* expression was high at E12.5 and E13.5; thereafter, it decreased and remained low until E18.5 and increased again at E20.5. The *Bmp-5* expression increased two-fold from E12.5 to E14.5 and remained high until birth. The *Bmp-7* mRNA was expressed at a relatively low and stable level throughout the measured time period. The highest expression of all BMP proteins was observed in the prehypertrophic and hypertrophic zones. The BMP-7 protein had a distinct distribution in E16.5 day-old hind limbs, where the expression was restricted to the hypertrophic cartilage while other BMPs were more widely expressed throughout the epiphyseal cartilage.

Based on the gene expression profiles during *in vivo* chondrogenesis (Table 11, I: Figure 1) and published data on human MSCs (Sekiya *et al.*, 2005), five bone morphogenetic proteins (BMPs -2, -4, -5, -6, -7 and BMP2/7 heterodimer) were selected for investigation to compare their ability to induce chondrogenic differentiation on mMSCs. Simultaneously, the pellet culture assay was optimized for an *in vitro* chondrogenesis model. BMPs exhibited different abilities to induce chondrogenesis *in vitro*. The effect of BMPs on chondrogenic differentiation of the LTS cells was evaluated by comparing the pellet sizes and analysing the relative area of type II collagen stained matrices. Supplementation with any BMP significantly increased the pellet size in comparison to the control experiments (Table 11, I: Table 2). Induction with rhBMP-2, rhBMP-4, rhBMP-6, or rhBMP-2/ BMP-7 significantly increased the type II collagen-positive area compared to the control pellets (Table 11). The rhBMP-5 and rhBMP-7 induction increased type II collagen production by five to six-fold compared to the control cells. These results suggest that the *in vivo* profiling of regulatory proteins may be used as a tool to predict the *in vitro* differentiation potential.

Table 11. Comparison of the *in vivo* expression and *in vitro* effects of BMPs. The pellet sizes and relative volumes of the type II collagen matrix in the LTS cells are presented as fold increase to control.

***In vivo* expression profile**	**Treatment**	**Diameter (%)**	**Type II collagen matrix (%)**
	rhBMP-2	40	17
	rhBMP-4	36	17
	rhBMP-5	26	5
	rhBMP-6	47	16
	rhBMP-7	24	5
	rhBMP-2/7	39	18

5.3 Expression and regulation of Dlk1 in chondrogenesis (III, IV)

5.3.1 Dlk1 as a marker for embryonic chondrogenesis (III)

The microarray approach was further utilized to identify novel genes that are involved in chondrogenesis in the developing mouse limb cartilage. *Dlk1* was identified in the group of genes up-regulated during chondrogenesis along with several other annotated cartilage matrix genes. The microarray data and subsequent validation by qRT-PCR revealed low expression of *Dlk1* at the time of mesenchymal condensation (Figure 10). At E14.5, the mRNA expression increased, reached the highest level at E16.5 and remained high until birth. Thereafter, the *Dlk1* expression rapidly decreased and very low expression levels, if any, were detected in the adult mouse articular cartilage. As at the time no published data were available on the role of dlk1 in chondrogenesis, it was selected for further investigation.

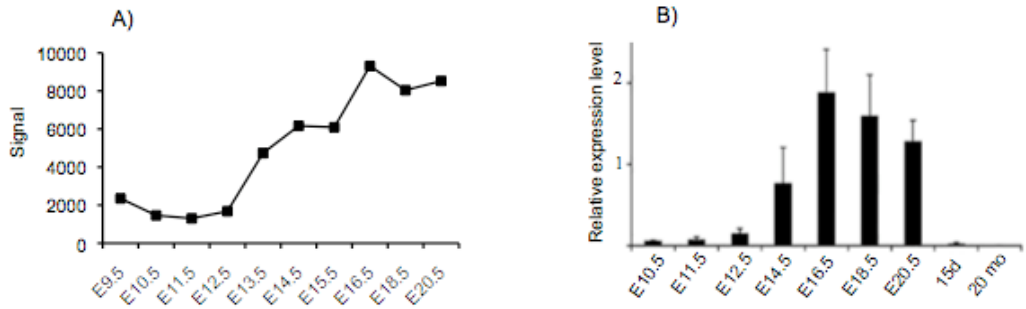


Figure 10. The expression of *Dlk1* in developing mouse limb cartilage. The *Dlk1* expression obtained with an Affymetrix microarray (A) was verified by a quantitative RT-PCR analysis (B).

At the protein level, dlk1/FA1 was weakly expressed at E12.5 in the central regions of the mesenchymal condensations (III: Figure 1). At embryonic days E14.5-18.5 it was detected throughout the immature epiphyseal chondrocytes, including the proliferating and prehypertrophic zones. However, no dlk1/FA1 expression was observed in the hypertrophic zone. During the postnatal development of articular surfaces, positive staining for dlk1/FA1 was first observed throughout the proliferating chondrocytic layer at birth. Thereafter, the tissue distribution gradually narrowed towards the articular surface as the chondrocytes matured and hypertrophied in the deeper zones. By 20 days of age, dlk1/FA1-positive staining was limited to the most superficial 3-6 chondrocyte cell layers, and by 2 months dlk1/FA1 was no longer expressed in the articular cartilage.

Similarly to the *in vivo* findings, *DLK1* was expressed during the *in vitro* differentiation of human embryonic stem cells (hESCs) into embryoid bodies (EBs) upon the down-regulation of totipotent marker Oct3/4 (III: Figure 2). In teratomas derived from hESCs, dlk1/FA1 was expressed by a number of cell types, including cells of extra-embryonic tissues, epithelial cells and chondrocytes (III: Figure 3). Consistently with the *in vivo* observations, dlk1/FA1 was co-expressed with early chondrogenic markers Sox9, type IIA collagen and S100A&B, and no dlk1/FA1 expression was detected in hypertrophic chondrocytes. Additionally, dlk1/FA1 was co-expressed with endothelial and epithelial/chondrocyte markers, while no co-expression was observed with markers of ectodermal or endodermal lineages.

Examination of the effect of several members of the TGF-β/BMP family on the mesoderm induction and *DLK1* expression in hEB model revealed a significant dose-dependent increase in the *DLK1* mRNA expression in response to Activin B (III: Figure 4). When Activin B was used for enriching the mesodermal dlk1-positive population of hESCs, it markedly increased the number of dlk1/FA1$^+$ cells and up-regulated the gene expression of the mesoderm-specific and early chondrogenic markers, while it down-regulated expression of the neuroectoderm-specific markers. Dlk1/FA1$^+$ and dlk1/FA1$^-$ cells were isolated from Activin B-induced EB-outgrowth cells using FACS cell sorting (III: Figure 5). After *in vitro* culturing, the dlk1/FA1$^+$ cells expressed significantly higher levels of mesoderm marker genes and MSC CD markers compared to the dlk1/FA1$^-$ cells. Markers for the ectoderm and endoderm were not detected in the dlk1/FA1$^+$ cell population. Interestingly, the dlk1/FA1$^+$ cells exhibited the ability to differentiate into the chondrogenic lineage *in vitro* in micromass pellets in contrast to the dlk1/FA1$^-$ cells, which were unable to differentiate into chondrocytes, suggesting a role for dlk1 in the cell commitment into chondrogenic lineage (III: Figure 6).

The limb bud derived micromass culture system is a well established model to study embryonic chondrogenesis and endochondral ossification *in vitro* (Mello and Tuan, 1999). Micromass differentiation can be divided into four phases; mesenchymal cell condensation (day 1-3), chondrogenesis (day 4-9), chondrocyte hypertrophy (day 10-13) and matrix mineralization (day 14 onwards) based on stage specific gene expression profile and morphological changes (IV: Figure 1A). *Sox9* and *S100* were expressed during the mesenchymal condensation while matrix genes *Col2a1*, *Acan* and *Comp* were up-regulated during chondrogenesis (IV: Figure 1B). Late chondrogenic genes *PTHrP*, *Ihh* and *Col10a1* were expressed during chondrocyte prehypertrophy and hypertrophy, after which their expression decreased upon the expression of osteoblast genes *Alpl* and *Bglap*, and matrix mineralization. Corroborating our *in vivo* observations, *Dlk1* was expressed in parallel with *Sox9* during the early chondrogenic differentiation (Figure 11 A, IV: Figure 1B). Its expression increased until day 6 and thereafter dramatically decreased until it was abolished completely by the time the late chondrogenic genes, *Ihh, PTHrP* and *Col10a1*, were up-regulated. Similarly, the dlk1 protein was observed in proliferating chondrocytes but was abolished at hypertrophy. Additionally, as demonstrated using ELISA, FA1 was secreted from the limb bud cells into the culture medium with a pattern similar to that of the gene and protein expression

(IV: Figure 1C, D).

Mouse embryonic fibroblasts, MEFs, provide a suitable model to study embryonic differentiation in cell culture. Similarly to other embryonic cells, MEFs expressed *Dlk1* at the undifferentiated stage. The expression markedly increased during early chondrogenesis, reaching the highest level at day 5 (Figure 11 B, IV: Figure 2A). Thereafter, the *Dlk1* expression decreased and was barely detectable at day 20 of differentiation.

In contrast to the embryonic cells, adult MSCs do not endogenously express *Dlk1*. During the chondrogenic differentiation of mMSCs, the relative expression level of *Dlk1* increased in accordance with the early chondrogenic marker genes, but the absolute expression level remained low (Figure 11 C).

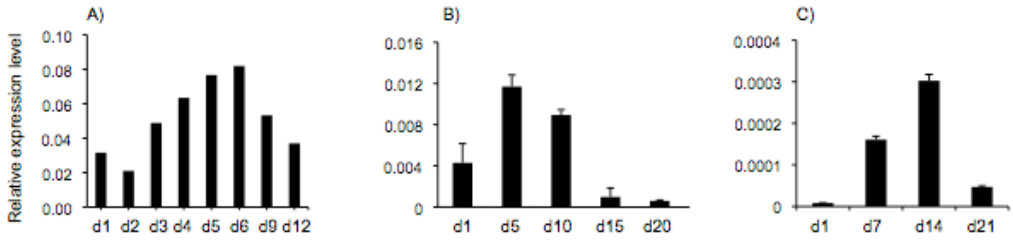


Figure 11. The expression of *Dlk1* mRNA during chondrogenic differentiation. A qRT-PCR analysis of the *Dlk1* gene expression in A) limb bud, B) MEF and C) mMSC culture models.

5.3.2 Enhanced chondrogenic differentiation in the absence of Dlk1 (IV)

The *in vivo* and *in vitro* profiles of *Dlk1* expression suggested that dlk1 plays a role in chondrogenesis. To further characterize the potential role of dlk1 in chondrogenesis, embryonic MEF and limb bud cells and adult mMSCs were isolated from WT and *Dlk1* $^{-/-}$ mice and induced to undergo chondrogenic differentiation. Enhanced chondrogenesis was observed in the absence of *Dlk1* in the cells of embryonic origin, evaluated by gene expression analyses and histological stainings (IV: Supplemental Figure 1A, data not shown). In addition, histological analyses revealed an increased chondrocyte size in the *Dlk1* $^{-/-}$ derived MEF and limb bud cultures compared to WT cells. No marked differences were observed in the chondrogenic differentiation of mMSCs between the WT and *Dlk1* $^{-/-}$ cells (IV: Supplemental Figure 1B), suggesting that dlk1 negatively regulates terminal maturation of chondrocytes during embryonic development and is not necessary for adult chondrogenesis.

5.3.3 The regulation of Dlk1 by TGF-β1 (IV)

To investigate the signalling pathways by which dlk1 is regulated, a comprehensive signalling pathway analysis was performed in the MEF cells and in mouse limb bud cells during chondrogenesis. Signalling pathways known to have a function in chondrogenesis were stimulated or inhibited, and the expression of *Dlk1* was evaluated in association with chondrogenic marker genes.

Supplementation of limb bud culture with BMP-2 or suppression of the Notch signalling by gamma secretase inhibitor DAPT markedly promoted chondrocyte maturation and hypertrophy, as evidenced by increased gene expression of *Col10a1* and *Ihh* without significantly affecting the expression of *Dlk1* (IV: Supplemental Figure 2). Treatment with PD169316, a specific inhibitor for p38 MAPK pathway, inhibited chondrocyte differentiation and hypertrophy by decreasing the expression of *Sox9*, *Col2a1*, and *Ihh*, but did not modulate the expression of *Dlk1*.

Interestingly, TGF-β1 significantly down-regulated the *Dlk1* expression in MEF and limb bud cells in a dose-dependent manner, while the other investigated TGFβ superfamily members, had no significant effect on *Dlk1* expression (IV: Figure 3, 4). In contrast, blocking of the TGF-β1 pathway by its specific inhibitors, SB431542, SB505412, SD-208 and LY364947, significantly increased the expression of *Dlk1*, whereas blocking Activin signalling with follistatin and BMP signalling with Noggin had no effect on the *Dlk1* expression level. These results indicate that TGF-β1 is a specific regulator of the *Dlk1* expression. TGF-β1 has been shown to induce early chondrogenesis (Mello and Tuan, 2006). Consistently with these data, our results revealed increased early chondrogenesis in the MEF and limb bud cells supplemented with TGF-β1, evidenced by the increased gene expression of early chondrogenic markers *Sox9* and *Col2a1* (IV: Figure 3 A). Addition of TGF-β1 suppressed chondrocyte hypertrophy and matrix mineralization, as demonstrated by the decreased expression of *Ihh*, *Col10a1* and osteocalcin gene *Bglap*. In contrast, blocking the TGF-β pathway by supplementation of SB431542 completely inhibited the chondrogenic process, demonstrated by the decreased expression of chondrogenic genes and absence of proteoglycan and type II collagen production (IV: Figure 3 B).

Dlk1 and TGF-β1 have been shown to play a role in chondrogenesis, and our findings suggested that TGF-β1 regulates dlk1. To investigate the putative connection between TGF-β1 and dlk1 in chondrogenesis, WT and *Dlk1*$^{-/-}$ MEFs were induced to chondrogenesis in the presence of TGF-β1. Interestingly, TGF-β1 significantly accelerated the early chondrogenic differentiation and chondrocyte maturation of *Dlk1*$^{-/-}$ MEFs compared to WT MEFs, as demonstrated by the gene expression of *Sox9, Col2a1, Acan* and *Col10a1* and histological analyses for the production of type II collagen and proteoglycans (IV: Figure 5) indicating that the role of TGF-β1 in chondrogenesis is, at least in part, mediated by dlk1.

5.4 Regulation of chondrogenesis by microRNAs (V)

5.4.1 The microRNA expression profile during chondrogenesis of mMSCs (V)

To study the post-transcriptional regulation of chondrogenesis and osteogenesis by microRNAs, 35 miRNAs were selected for expression analysis based on their known relevance for stem cell differentiation, or a computationally predicted importance for chondrogenic or osteogenic differentiation. During the osteoblast differentiation of mMSCs, expression of two of the 35 analysed miRNAs changed over five-fold as compared to undifferentiated mMSCs, while during chondrogenesis, seven miRNAs were differentially expressed (V: Figure 2, Table 1). Additionally, eight miRNAs were differentially expressed over two-fold between osteoblasts and chondrocytes.

5.4.2 Target prediction, promoter and pathway analyses of differentially expressed microRNAs (V)

Differentially expressed miRNAs were subjected to target prediction analyses to further understand their role in mMSC differentiation. Target prediction analyses were performed with the Target Scan, PicTar and miRBase programs, and the target genes annotated by all three programs were selected for further analysis. Signalling pathway analysis of the putative target genes revealed significant biological functions for the target genes of five miRNAs (V: Figure 4). Additionally, upstream regions of the differentially expressed miRNAs were subjected to a transcription factor binding site analysis. When the results of target prediction, signalling pathway and transcription binding site analyses were combined, three transcription factors were annotated as predicted targets for the differentially expressed miRNAs while having transcription factor binding sites in the promoter of the the same miRNAs. *Pbx* (pre-B-cell leukemia homeobox) and *HIF1a* (Hypoxia-inducible factor 1a) are the potential targets of the miRNAs upregulated in chondrocytes, *miR-101* and *miR-199a,* respectively, and *Pparg* is potentially regulated by *miR-130b*, which was up-regulated in osteoblasts. Two of the miRNAs were located in clusters (*miR-199a/miR-214* and *miR-96/miR-182/miR-183*) with a potential functional relevance in strengthening the regulation of Sox5-Sox6 leading to type II collagen responses, or of HIF1-PGF-axis leading to various hypoxia responses. These results suggest a regulatory network where miRNAs in concert with transcription factors modulate the differentiation of MSCs into various lineages (V: Figure4,5).

6. DISCUSSION

Repair of articular cartilage lesions is challenging due to the complex structure and demanding biomechanical function of the tissue. The internal repair capacity of articular cartilage is poor due to the lack of vascularization and of a source of stem cells in the tissue that could contribute to the repair. Autologous chondrocyte transplants and other surgical procedures have been used for the treatment of chondral injuries (Safran and Seiber 2010 review). Recently, a great deal of interest has been addressed towards MSCs as a source of cell-based therapeutic strategies due to their various beneficial properties. Multipotency, secretion of paracrine factors and immune-modulatory properties make MSCs attractive candidates for cell-transfer-based tissue engineering and regenerative approaches. Due to the increased interest in the potential therapeutic applications, it is important to better understand the MSC biology and regulatory mechanisms of their differentiation.

In the present study, several approaches were used to study the regulation of MSC differentiation into chondrocytes. First, the expression of *Bmps* during *in vivo* embryonic chondrogenesis and their influence on adult MSC differentiation was studied. Simultaneously, a pellet culture model for *in vitro* chondrogenesis was optimized for use in the later studies. The contribution of the population composition to chondrogenic potential was studied by evaluating the chondrogenic differentiation potential in primary plastic adherent cells (STS), in long-term selected cells (LTS) that were uniformly stem cell marker Sca-1-positive, and in two progenitor cell lines ($mMSC^{Adipo}$ and $mMSC^{Bone}$) isolated from the mouse bone marrow stroma. Lineage commitment was further investigated by analysing the basal expression profiles of adipogenic $mMSC^{Adipo}$ and osteo-chondrogenic $mMSC^{Bone}$ clonal cells using the microarray approach and characterising the possible cross-talk mechanisms between these two cell lines during the differentiation.

Further, novel genes in chondogenesis were sought using the microarray approach. This lead to an identification of an embryonally expressed imprinted gene, *Dlk1*, that had been previously described as a stem cell fate-determining factor in adipogenic and osteogenic differentiation. Its role in chondrogenesis and regulation by the TGF-beta family members was investigated by using embryonic and adult tissue-derived *in vitro* differentiation models.

Finally, post-transcriptional regulation by miRNA networking during chondrogenic and osteogenic differentiation was characterized by studying the expression profiles of 35 miRNAs during mMSC differentiation. The following chapters discuss the central findings in these studies, which are presented in more detail in the enclosed original publications and manuscripts (I - V).

6.1 The impact of stromal cell composition on mMSC differentiation

MSCs from human and rat bone marrow have been intensively investigated, in part because they are easy to isolate and can be expanded in culture through many generations while retaining their differentiation capacity. The expansion and chondrogenic differentiation of mouse MSCs is more difficult to achieve (Peister *et al.*, 2004). Yet, due to its extensive utilisation in generating genetically modified animal models, and smaller size and shorter life span, both contributing to more economic maintenance expenses, mouse has many advantages over the rat as a model organism in biomedical research. Therefore, the first object in this study was to optimize the chondrogenic differentiation assay for mouse MSCs and to study the contribution of the MSC population to the chondrogenic potential.

Controversial results have been published on the isolation and characterization of mMSCs, where homogenous, haematopoietic cell-free mMSC populations have been obtained by negative or positive cell sorting or by extensive passaging of the cells. However, characteristics of the mMSCs have been shown to vary dramatically between the culture methods and mouse strains (Baddoo *et al.*, 2003; Peister *et al.*, 2004). The approach used here to isolate and culture bone marrow-derived mMCSs resulted in a homogenous, plastic-adherent, colony-forming and multipotent population of MSCs. In a manner consistent with the previous reports, primary cultures were rich in haematopoietic cells, and only a few Sca-1-positive cells were detected. Haematopoietic cells were successfully removed by long-term culture in a nutrient-deficient medium. The remaining multipotent cells were negative for haematopoietic surface markers and positive for Sca-1, thus fulfilling the criteria for MSCs. Further expansion of the selected cells yielded a homogenous population of large, flat MSCs (LTS cells), which uniformly expressed Sca-1. Although the exact role of Sca-1 is unclear, it is considered a marker for precursor/stem cells and has been used for the selection of haematopoietic, mesenchymal or endothelial precursor/stem cells (Holmes and Stanford, 2007). However, our observations indicated that Sca-1 does not serve as an inclusive early marker for a true multipotential mMSC, since the $mMSC^{Adipo}$ and $mMSC^{Bone}$ cells, which are 83% and 96% positive for Sca-1, respectively, (Post *et al.*, 2008) represent uni- and bi-potential cells rather than multipotent MSCs. Despite possibly not being true stem cells, the Sca-1-positive cells may represent a more primitive stage of progenitors than the Sca-1-negative cells.

Interestingly, the short-term selected MSC population which consisted of a relatively heterogeneous combination of cells rapidly differentiated into the chondrogenic lineage. However, chondrogenesis in the STS cells progressed to hypertrophy and terminal differentiation associated with mineralization, which is an unwanted phenomenon in cartilage repair (Pelttari *et al.*, 2006). Compared to the primary cell population, the more selected MSCs differentiated at a slower rate without undergoing chondrocyte hypertrophy and terminal differentiation, and may thus represent a selected population of primitive chondroprogenitors, as suggested by the uniform Sca-1 expression in these cells. It is also possible that the more committed chondroprogenitors among the primary

STS cells were removed along with the haematopoietic cells during the selection by passaging, thereby leading to an enrichment of a more primitive MSC population. Premature chondrocyte mineralization and calcification of the MSCs has been reported to occur in the *in vivo* environment in MSCs that do not undergo chondrocyte hypertrophy *in vitro* (Pelttari *et al.*, 2006). The premature hypertrophy in the STS cells may be explained by the heterogeneous nature of the cell population, which resembles more the endochondral ossification-supportive *in vivo* micro-environment.

As discussed above and in the literature review, the bone marrow stroma consists of a heterogeneous population of cells, including haematopoietic cells, multipotent MSCs and more committed uni- and bi-potential MSCs (Kuznetsov *et al.*, 1997; Muraglia *et al.*, 2000). Clonal analyses of the MSCs have demonstrated their heterogenity with respect to their differentiation potential (Kuznetsov *et al.*, 1997). These findings were supported by the identification of two mouse bone marrow-derived MSC strains, $mMSC^{Adipo}$ and $mMSC^{Bone}$, representing committed pre-adipocytic and pre-osteblastic cells, respectively (Post *et al.*, 2008). The present study demonstrated a difference in their capacity for chondrogenic differentiation. The adipogenic $mMSC^{Adipo}$ cells exhibited no chondrogenic potential, while the $mMSC^{Bone}$ cells readily differentiated into chondrocytes, thus extending $mMSC^{Bone}$ to a bi-potential osteo-chondroprogenitor cell line. These findings are consistent with previous studies in which the MSCs were shown to sequentially lose their potential for trilineage differentiation, giving rise to bi-potential osteochondrogenic cells, and eventually to uni-potential osteogenic precursors (Russell *et al.*, 2010). Our results indicated that the committed mMSCs already have a defined phenotype in *ex vivo* cultures, as standard differentiation assays were not able to trigger the differentiation process of mMSCs into an alternative lineage.

To understand the molecular basis of the mMSC lineage commitment, the basal gene expression levels in the $mMSC^{Adipo}$ and $mMSC^{Bone}$ cells were examined by a microarray approach. The multipotent undifferentiated MSCs are shown to exhibit a unique gene expression profile with high expression levels of genes of multiple cell lineages (Wieczorek *et al.*, 2003). The findings in our study indicated that the commitment of MSCs to a particular lineage entails activation or down-regulation of a large number of genes, including lineage-specific transcription factors, explicit for the particular lineage. A lineage-specific molecular signature may thus offer a potential approach for prospectively identifying different subpopulations of MSCs. The gene expression analysis of the $mMSC^{Adipo}$ and $mMSC^{Bone}$ cells revealed high expression levels of adipogenic genes in the $mMSC^{Adipo}$ cells, whereas both osteogenic and chondrogenic genes were highly expressed in the $mMSC^{Bone}$ cells, thus supporting the bi-potential nature of the $mMSC^{Bone}$ cell line. Together, these findings suggest that the phenotype determination can be explained by significant differences in the molecular signature of mMSCs.

Stimulation of immune response pathways inhibits osteogenic and chondrogenic differentiation and induces bone and cartilage degradation in arthritis and other inflammatory diseases (Mann *et al.*, 1994). A high expression of immune response-related genes is also predictive of a low bone formation capacity in the MSCs (Larsen *et*

al., 2010). Consistently, an impairment of osteoblast and chondrocyte differentiation in the committed $mMSC^{Adipo}$ cells was associated with up-regulation of the expression of cytokines, interferon-activated genes and other inflammatory and immune response genes. MMPs are stimulated by proinflammatory cytokines and MMP-13 has been shown to mediate cartilage degradation by cleavage of type II collagen fibrils in OA and rheumatoid arthritis. MMP-13 was highly expressed in the $mMSC^{Adipo}$ cells, indicating a proinflammatory phenotype (Dahlberg *et al.*, 2000). Secretion of proinflammatory factors may thus be one mechanism by which cells modulate the microenviroment and contribute to the cross-talk between different cell populations in the bone marrow.

Comparison of the short- and long-term selected and committed MSCs illustrated the importance of the composition of the stromal cell population for their differentiation capacity. Cells in the stem cell niche secrete various cytokines and other factors, thereby influencing the fate determination and differentiation of other cells, as well as tissue homeostasis (Caplan and Dennis, 2006). It is thus possible that rather than being the actual cellular reservoir for tissue repair, a more beneficial feature of the MSCs may in fact be their trophic effect; the MSCs secrete immunosuppressive factors and cytokines, thereby contributing to the tissue homeostasis and perhaps triggering the tissue's intrinsic repair capacity (da Silva Meirelles *et al.*, 2008).

6.2 The effect of Wnt signalling on the commitment and differentiation of MSCs

The pivotal role of the Wnt pathway in mesenchymal cell fate decision is well established (Moon *et al.*, 2002a). Canonical Wnt/β-catenin signalling has been shown to inhibit adipogenesis and promote MSC differentiation into osteogenic or chondrogenic lineage through high or low signalling activity, respectively (Ross *et al*., 2000; Day *et al*., 2005b; Ryu *et al.*, 2002). The present study demonstrated the importance of Wnt signalling in the lineage commitment. Corroborating previous findings, active Wnt signalling was observed in the osteo-chondrogenic $mMSC^{Bone}$ cells but not in the adipogenic $mMSC^{Adipo}$ cells indicating that the osteogenic lineage commitment is associated with enhanced Wnt signalling responsiveness and the opposite is true for adipocytic differentiation.

Although high canonical Wnt signalling has been demonstrated to inhibit chondrocyte differentiation (Rudnicki *et al*.,1997), active Wnt signalling in osteo-chondroprogenitors consolidates more recent reports in which mice harbouring inactivated β-catenin in chondrocytes displayed abnormal growth plates, implicating that an appropriate level of β-catenin signalling is required for the cells to commit to the chondrogenic lineage (Day *et al*., 2005b; Ryu *et al.*, 2002). β-catenin is highly expressed in mesenchymal cells committed to the chondrocytic lineage and together with N-cadherin it is required to induce prechondrogenic mesenchymal condensation (Ryu *et al.*, 2002). High expression of N-cadherin (*N-cad*) in the $mMSC^{Bone}$ cells suggests that it is involved in determining the cell fate commitment of the $mMSC^{Bone}$ cells to the osteo-chondrogenic lineage, possibly in concert with β-catenin. The importance of Wnt signalling in chondrogenesis

and osteogenesis was further supported by high expression of a considerable number of genes associated with Wnt signalling in the mMSC^{Bone} cells, including *Wnt10a* and *Wnt7b*, Wnt ligands known to be involved in osteogenesis and chondrogenesis (Zhou *et al*., 2004, Zhou *et al* 2008), tumour necrosis factor receptor superfamily member 19 (*Tnfrsf19*), a novel Wnt inducible factor mediating the determination of hMSCs into osteoblasts or adipocytes (Qiu *et al*., 2010) and receptor tyrosine kinase-like orphan receptor 2 (*Ror2*), a co-receptor enhancing the canonical Wnt signalling pathway. Ror2 is selectively expressed in the cartilage and is suggested to be required for the growth plate development (DeChiara *et al.*, 2000). Interestingly, it interacts physically and functionally with Wnt5a and may thus serve as a receptor for Wnt5a to activate non-canonical Wnt signalling (He *et al.*, 2008). The potential involvement of non-canonical Wnt signalling provides an interesting aspect to the regulation of cell commitment and should be elucidated in further studies.

On the contrary, adipogenic commitment was associated with a low Wnt signalling activity and up-regulation of the PPARγ pathway. Canonical Wnt signalling has been shown to inhibit adipogenesis through an inhibition of adipogenic transcription factors C/EBPα and PPARγ (Ross *et al.*, 2000). Observations with the mMSC^{Adipo} cells suggest that low Wnt signalling activity in the MSCs committed to the adipocytic lineage allowed the expression of *C/ebpα* and *Pparγ* and thus differentiation into the adipogenic lineage but not into osteogenic or chondrogenic lineages. Interestingly, low Wnt signalling activity in the mMSC^{Adipo} cells was associated with high expression of Wnt antagonist *Sfrp-1*. Furthermore, committed preadipocytic cells highly expressed the sFRP-1 protein and secreted it into the conditioned medium (mMSC-CM^{Adipo}), suggesting a biological role in a pericellular environment. In support of this, our results demonstrated impaired osteoblast differentiation and chondrocyte maturation in the presence of mMSC-CM^{Adipo} or an exogenous sFRP-1 recombinant protein. The differentiation capacity was partially restored by removal of sFRP-1 from mMSC-CM^{Adipo}, confirming the involvement of sFRP-1 in mediating the inhibitory effect of preadipocytes on osteoblast differentiation.

Our results describing the suppressive effect of sFRP-1 on osteogenic differentiation, and chondrocyte maturation and hypertrophy are in line with previous findings by other groups in sFRP-1-deficient and over-expressing mouse models, as well as *in vitro* studies (Bodine *et al.*, 2004a; Gaur *et al.*, 2006b; Gaur *et al.*, 2009; Trevant *et al.*, 2008a; Yao *et al.*, 2009). While the inhibitory role of sFRP-1 on osteogenesis is well established, its function in adipogenesis has not been investigated in detail. Recently, sFRP-1 was associated with obesity and was shown to have a physiological role in Graves' ophthalmopathy (GO), an autoimmune inflammatory disorder characterized by increased volume of the orbital adipose tissues (Kumar *et al.*, 2004; Lagathu *et al.*, 2010). Our results provided novel insights into the function of sFRP-1 in MSC biology and lineage commitment by demonstrating its role in determining the fate decision of the MSCs by suppressing osteogenesis and enhancing adipogenesis. sFRP-1 was also shown to display a paracrine effect, and may thus serve as a molecular switch between osteogenesis and adipogenesis in the bone marrow micro-environment. Moreover, sFRP-1 exerted its effect on different cell types suggesting that the observed inverse

relationship between osteoblasts and adipocytes can be mediated through interaction between committed cell populations and not only at the level of multipotent MSCs.

It is possible that not only sFRP-1 but also other secreted Wnt proteins and humoral factors are involved in the cross-talk of different cell populations in the bone marrow. Further characterization of other factors, including the above-discussed proinflammatory cytokines that mediate the complex cross-talk between different cell lineages within the bone micro-enviroment may result in identification of novel therapeutic targets that can be employed to prevent the age-related increase in adipogenesis and to enhance bone formation.

6.3 BMPs in *in vivo* and *in vitro* chondrogenesis

BMP signalling is essential for chondrogenesis and osteogenesis (Pizette and Niswander, 2000; Retting *et al.*, 2009; Wozney *et al.*, 1988). The effect of BMP-2, -4 and -6 on chondrogenesis has been previously demonstrated in human MSCs (Sekiya *et al.*, 2005). Due to the reported variation in BMP responsiveness between different species (Osyczka *et al.*, 2004), we compared the effect of BMPs in mouse MSCs. In the mouse system, rhBMP-2, rhBMP-4, rhBMP- 6 and rhBMP-2/BMP-7 heterodimer markedly increased the pellet size, chondrogenic gene expression and proteoglycan production. Less advanced chondrogenic differentiation was observed in rhBMP-5 and rhBPM-7-induced LTS pellets. Consistent with our result, BMP-5 has been previously reported to enhance cell proliferation rather than chondrogenic differentiation (Mailhot *et al.*, 2008). The BMP-2 and BMP-7 proteins co-localize during the mouse embryonic development and form heterodimers *in vivo* and *in vitro* (Lyons *et al.*, 1995; Israel *et al.*, 1996). These heterodimers have been shown to be more potent in inducing osteoblastic differentiation than the corresponding homodimers (Zhu *et al.*, 2004; Zhu *et al.*, 2006). Similarly, the rhBMP-2/BMP-7 heterodimer efficiently promoted chondrogenic differentiation of mMSCs but not significantly exceeding the effect of rhBMP-2 alone.

MSCs extracted from different tissues respond differently to BMPs (Hennig *et al.*, 2007). The findings with the STS and LTS cells demonstrated that also different MSC populations from the same tissue vary in their BMP responsiveness, possibly due to differences in their expression of BMP receptors. BMP-2 induced chondrogenesis equally in the STS and LTS cells but the efficacy of BMP-6 and BMP-7 was different in these cell populations. BMP-7 strongly induced chondrogenesis and terminal differentiation in the STS cells, but only poorly in the LTS cells, while BMP-6 promoted chondrogenesis in the LTS cells but induced terminal differentiation in the STS cells at a slower rate. Assuming that the LTS cells represent an enriched population of more primitive cells than the STS cells, these data suggest that BMP-6 is a better inducer of primitive stromal cells while BMP-7 induces partially differentiated progenitor cells present in the STS cell population. BMP-7 is extensively used for fracture healing and bone repair purposes (Khosla *et al.*, 2008). It is thus possible that osteogenic precursors had been enriched in the STS population and BMP-7 induced these cells to osteogenesis through endochondral ossification. However, BMP-7 has also

been reported to promote cartilage homeostasis (Chubinskaya *et al.*, 2007) indicating a more complex regulation of the stromal cells by BMPs greatly depending on the experimental conditions.

Although MSCs provide a promising cell source for autologous repair of knee defects, the challenge is to generate cells with the features of stable chondrocytes. Among the problems in using MSCs for articular cartilage repair is the limited formation of type II collagen-producing hyaline cartilage in the repair site. Instead, the transplanted MSCs have been reported to give rise to fibrocartilageous repair tissue with a high content of type I collagen and a tendency to hypertrophy (Buckwalter, 2002). This constraint was also observed in our model. While the STS cells progressed to hypertrophy and thus avoided maintaining stable cartilage phenotype, the LTS differentiation was restricted to the peripheral cell layers of the pellet, both models resulting in a limited formation of hyaline cartilage. The present study demonstrated the synergistic effect of TGF-β and BMPs in promoting chondrogenesis. However, in addition to the optimal combination of growth and differentiation factors, successful chondrogenic differentiation of MSCs has also been obtained by using mechanical loading and the modulation of hydrostatic pressure. Mechanical stress has been demonstrated to influence the maintenance of hyaline cartilage and cyclic mechanical compression to enhance the formation of cartilageous matrix in MSCs (Guilak *et al*., 2004; Schumann *et al*., 2006). Therefore, a concomitant stimulus with growth factors and mechanical loading may provide an approach for improved chondrogenic differentiation of MSCs and the formation of stable and functional hyaline cartilage.

6.4 The role of Dlk1 in chondrogenesis

Dlk1 is an important modulator of various developmental processes and an inhibitor of MSC differentiation into the osteogenic and adipogenic lineages (Abdallah *et al*., 2004b). It is highly expressed in preadipocytes and preosteoblasts but is down-regulated during the differentiation and abolished in mature adipocytes and osteoblasts (Abdallah *et al.*, 2004b; Smas *et al*., 1997). In the present study, dlk1 expression was demonstrated in immature proliferating chondroytes *in vivo* and *in vitro*. In the condensing mesenchyme during limb development, it co-localized with type IIA procollagen, an isoform of type II collagen that is expressed by chondroprogenitors. Dlk1 was abolished in hypertrophic chondrocytes in the developing mouse limb and in the adult articular and growth plate cartilage. This detailed stage-specific expression analysis covering specifically limb chondrogenesis and endochondral ossification corroborates and elaborates on the previous findings describing its expression in the cartilage among other embryonic tissues expressing *Dlk1* (Yevtodiyenko and Schmidt, 2006b). Moreover, expression of *Dlk1* during early chondrogenesis in a hESC-derived *in vivo* teratoma as well as in the hESC, MEF and limb bud cells, indicates that the expression of *Dlk1* by immature and developing chondrocytes is consistent among diverse *in vitro* and *in vivo* models in both mice and men. Yet, *Dlk1* expression was not detected in adult bone marrow-derived MSCs suggesting that the stage-specific expression is limited to embryonic chondrogenesis.

Based on the observation that dlk1 is co-expressed with mesodermal markers but not with markers for self-renewal, ectoderm or endoderm, it was used as a surface marker to identify and sort mesodermal/prechondrogenic cells. Supported by the finding that dlk1 expression was induced by Activin B, and this was associated with an increase in chondrogenic markers, we developed a two-stage culture protocol for hESCs in which mesodermal cells were induced with Activin B to express dlk1, followed by cell sorting using dlk1 as a surface marker (III: Figure 6). This protocol facilitated the isolation of homogenous population of mesenchymal progenitor cells of embryonic origin with chondrogenic potential and may serve as a novel strategy in generating clinical applications to direct hESCs into chondrogenic lineage. Interestingly, the obtained mesodermal dlk1-positive population exhibited an enhanced capacity to differentiate into the chondrogenic lineage, supporting the role of dlk1 in mesenchymal cell commitment.

Despite the requirement of dlk in the cell commitment to the chondrogenic lineage, absence of *Dlk1* enhanced chondrocyte maturation and hypertrophy indicating that it functions as negative regulator of chondrocyte hypertrophy. Together, the restricted spatio-temporal expression of dlk1 in premature chondrocytes, the complete lack of dlk1 in hypertrophic chondrocytes, the requirement of dlk1 for ECS chondrogenesis and enhanced hypertrophy in the absence of *Dlk1* strongly suggest that dlk1 plays a role in controlling lineage commitment and differentiation by maintaining cell proliferation and inhibiting chondrocyte maturation. The dual role of dlk1 in chondrogenesis corroborates recent findings where dlk1 was proposed to promote chondrocyte early differentiation and inhibit terminal maturation through modulation of *Sox9* (Wang and Sul, 2009b).

Although extensively studied, it is not known whether dlk1 functions as a receptor or a ligand and pathways upstream of dlk1 have not been identified (Wang *et al.*, 2010). In the present study, TGF-β1 was identified as a novel negative regulator of dlk1. Interestingly, blocking the TGF-β signalling significantly increased the expression of dlk1 in parallel with the inhibition of chondrogenesis, suggesting that the down-regulation of *Dlk1* by TGF-β1 is required for chondrogenesis. Furthermore, TGF-β1 induction in the absence of *Dlk1* resulted in significantly enhanced chondrogenesis in comparison to the wild type, further supporting that dlk1 mediates the effect of TGF-β1 in chondrogenic differentiation. The down-regulation of the dlk1/FA1 expression by TGF-β signalling suggests a mechanism by which the stimulatory effect of TGF-β1 on chondrogenesis is mediated, at least in part, by dlk1 (IV: Figure 6). The proposed mechanism may also function *in vivo* to regulate the restricted expression pattern and the stage specific down-regulation of *Dlk1* to allow chondrocyte maturation and hypertrophy during endochondral ossification. A significant enhancement in chondrogenesis in the presence of TGF-β1 and absence of *Dlk1* may also indicate an interaction in which dlk1 binds to TGF-β1 to suppress its positive effect on the chondrogenic differentiation. However, the detailed mechanisms by which TGF-β1 and dlk1 interact to regulate chondrogenesis need to be elucidated in further studies.

6.5 Post-transcriptional regulation of chondrogenesis

MicroRNAs have been identified as important post-transcriptional regulators in diverse differentiation processes, including chondrogenesis and osteogenesis (Hu *et al.*, 2010). However, their contribution to these processes is not fully elucidated. The results obtained in this study suggested a central role for miRNAs in the transcription factor network regulating the MSC commitment, proliferation and differentiation. Previous computational analyses suggest that thousands of human genes are regulated by specific miRNA-transcription factor interactions (Shalgi *et al.*, 2007). Our comprehensive computational analysis of miRNAs that were differentially expressed during osteogenic and chondrogenic differentiation revealed complex interaction networks in which miRNAs are regulated by specific transcription factors. In turn, miRNAs target and regulate transcription factors and other lineage-specific genes. MicroRNAs that were down-regulated in chondrogenesis targeted important chondrogenic factors, and, similarly, osteogenic factors appeared as potential target genes for miRNAs that were down-regulated in osteogenesis. In contrast, up-regulated miRNAs were predicted to target important regulatory genes of other lineages, thus suppressing their expression and preventing the differentiation of MSCs into these lineages. In Figure 12, a model for MSC differentiation is proposed, in which the interaction of miRNAs, transcription factors and regulatory genes modulate the differentiation of cells into various lineages.

The majority of miRNAs are intergenic, have their own promoters and are thus regulated by specific transcription factors (Lee *et al.*, 2007). Interestingly, several transcription factors were among the predicted target genes for differentially expressed miRNAs, resulting in complex interaction networks. Putative binding sites for various transcription factors were observed in the upstream regions of differentially expressed miRNAs. In addition, composite loops were observed for three transcription factors: PBX1, PPARγ and HIF1. These transcription factors had binding sites in the promoters of specific miRNA genes, *miR-101*, *miR-130b* and *miR199a*, respectively, while they were potential target genes of the same miRNAs.

This study demonstrated that the physiological response in cells is a result of several miRNA responses and a complex regulatory network. In addition, it illustrated the importance of post-transcriptional regulation in fine-tuning the differentiation of the MSCs.

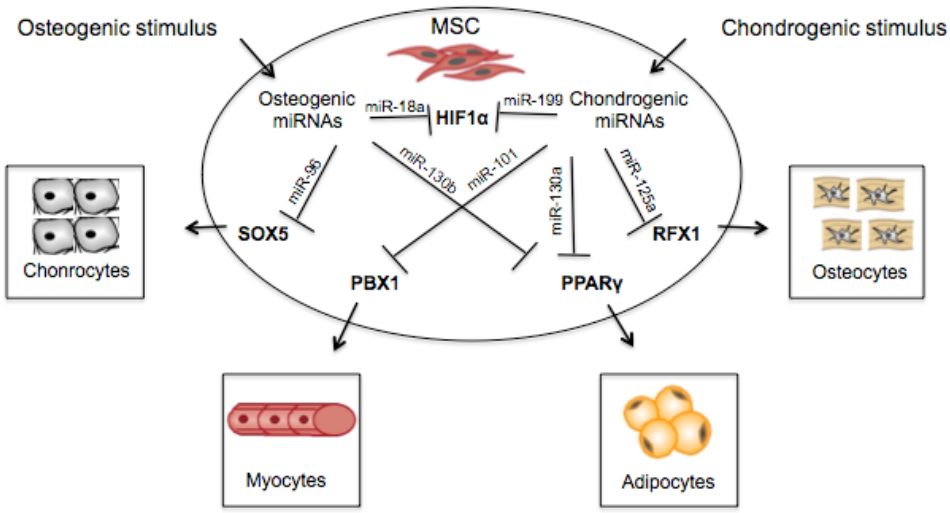


Figure 12. A schematic model for miRNA – transcription factor interactions during the MSC differentiation into mesenchymal lineages. The results of this study suggest that the miRNAs that are down-regulated in chondrogenesis target important chondrogenic genes while those miRNAs that are up-regulated in chondroblasts target genes that are important for osteogenesis. HIF-1α was targeted by the osteogenic and chondrogenic miRNAs, indicating that hypoxia signals play an important role in the regulation of the MSC differentiation into both lineages (Modified from V).

6.6 Future perspectives

MSCs hold great promise as a future cell source in regenerative medicine. Although successful clinical trials have been reported where MSCs have been used in the treatment of degenerative diseases, several hurdles need to be cleared prior to their large scale use in the clinic. Identification of the factors regulating the commitment and differentiation may provide novel tools for the development of therapeutic treatments for various degenerative diseases. The results presented in this study provide novel insights into the commitment and regulation of the differentiation of stem cells. However, further studies are needed to evaluate the potential use of these factors in therapeutic applications.

One of the main difficulties in MSC research appears to be the variation in the culture conditions and cell types used. As also demonstrated in this study, the controlled differentiation of MSCs is dramatically dependent on the MSC population. Therefore, standardization of the expansion method and culture conditions is of importance in the future to minimize this variability and facilitate the proper comparison of studies. Moreover, it has become evident that not only the biology of the MSCs themselves but also the influence of the surrounding niche needs to be fully elucidated in future studies in order to control the fate of the MSCs. Cells in the stem cell niches secrete cytokines and other humoral factors, thereby affecting their surrounding cells. Identification of

these secreted factors may provide novel molecular targets in the treatment of several diseases. Modulation of Wnt signalling by targeting the soluble Wnt inhibitors provides a promising approach for the treatment of bone loss states. Thus further investigation should focus on the development of specific compounds against the negative regulators of Wnt signalling. Small molecule compounds have been generated to antagonize sFRP-1 (Bodine *et al.*, 2009b). More detailed analysis of their function in the bone marrow micro-environment in inhibiting sFRP-1 secreted by preadipocytes, and subsequently promoting osteogenesis may aid in the identification of novel compounds with clinical relevance for bone loss diseases.

Using stem cells, particularly MSCs, as a platform for gene therapy provides an intriguing application in the development of new therapeutic strategies. Dlk1 was identified as a molecule negatively regulating chondrocyte hypertrophy. Therefore, the delivery of *Dlk1* to MSCs may have potential for cartilage regeneration therapy to control the chondrogenic differentiation of transplanted MSCs. As the regulatory role of Dlk1 appeared strictly stage-limited, further studies are required to define the correct timing of Dlk1 delivery to prevent unwanted inhibition of chondrogenesis.

Recent advances in miRNA research have provided new perspectives on the regulation of skeletal development. Moreover, understanding the function of miRNAs and their association with the molecular pathogenesis of various diseases, including OA and osteoporosis, has provided novel insights into the development of therapeutic treatments. Using miRNAs as therapeutic targets by manipulating the miRNA levels to promote osteoblast and chondrocyte differentiation may well develop into a powerful tool in therapeutic approaches. However, numerous questions, including the prevention of off-target effects and efficient delivery *in vivo* need to be solved before miRNAs can be used in therapeutic approaches.

The identification of novel regulatory factors and understanding their mechanisms of function in detail will help us in developing more specific and targeted therapies in the future. In association with the increasing knowledge of the regulation of cellular differentiation, the complexity of the regulatory networks becomes more evident. Transferring the information obtained from basic science to the generation of safe and controlled therapeutic strategies for regenerative diseases in the clinics remains the main future challenge.

7. SUMMARY AND CONCLUSIONS

The MSC lineage commitment and their differentiation into specific cell types is a complex process and tightly regulated by several factors. The observations made in this study suggest that MSC differentiation is modulated at the cellular, transcriptional and translational levels, and is mediated by signalling pathways and their convergence (Figure 13). Based on the results, the following conclusions can be made:

1. MSC characteristics and their differentiation capacity are influenced by stromal cell composition. Culturing MSCs *in vitro* alters their biological properties, such as the cell surface profile, differentiation capacity and response to growth factors. The composition of the MSC population contributed to the chondrogenic differentiation potential. A heterogeneous MSC population had a high chondrogenic potential with a tendency to hypertrophy and mineralization, while more a selected primitive MSC population differentiated at a slower rate but avoided terminal differentiation and mineralization.
2. Committed mMSCs expressed lineage-specific genes, including key transcription and growth factors at a basal stage. Thus, molecular signatures of multipotent or committed mMSCs can be used to prospectively identify different subpopulations of MSCs.
3. Wnt signalling mediates the lineage fate determination of MSCs. Secreted Wnt and, as demonstrated in this study, Wnt inhibitors modulate the commitment of the MSCs. sFRP-1, which is highly expressed in committed pre-adipocytes, inhibited Wnt signalling and osteochondrogenic differentiation. In addition to the direct effect on the sFRP-1-expressing cells, secreted sFRP-1 exerted its effect on adjacent cells, thus modulating the commitment and differentiation of other cell populations in the bone marrow micro-enviroment. Moreover, these results suggest that this interaction is not limited to multipotent MSCs but may also occur between committed cell populations.
4. Dlk1 is a novel surface marker of mesenchymal/chondrogenic progenitor cells that undergo embryonic lineage progression from proliferation to the prehypertrophic stage. Dlk1 exhibited a restricted spatial-temporal expression pattern during embryonic chondrogenesis in which it was expressed in immature chondrocytes but abolished during hypertrophy. Using dlk1 as a marker to enrich cells with chondrogenic potential may provide a new strategy for the development of protocols with clinical use to direct stem cell differentiation into the chondrogenic lineage.
5. Dlk1 inhibits chondrocyte maturation and hypertrophy, possibly by mediating the effect of the TGF-β pathway. TGF-β1 negatively regulated dlk1 expression, and the interaction between the TGF-β1 and dlk1 pathways may function in concert to maintain chondrocyte proliferation and prevent premature hypertrophy.
6. MicroRNAs function as post-transcriptional modulators of MSC differentiation. The results suggest a regulatory network where transcription factors regulate the expression of specific miRNAs, which, in turn, target lineage-specific transcription factors and other crucial genes. Specific miRNAs expressed in a certain MSC prevent its differentiation into other lineages by targeting the key regulators of the alternative lineage.

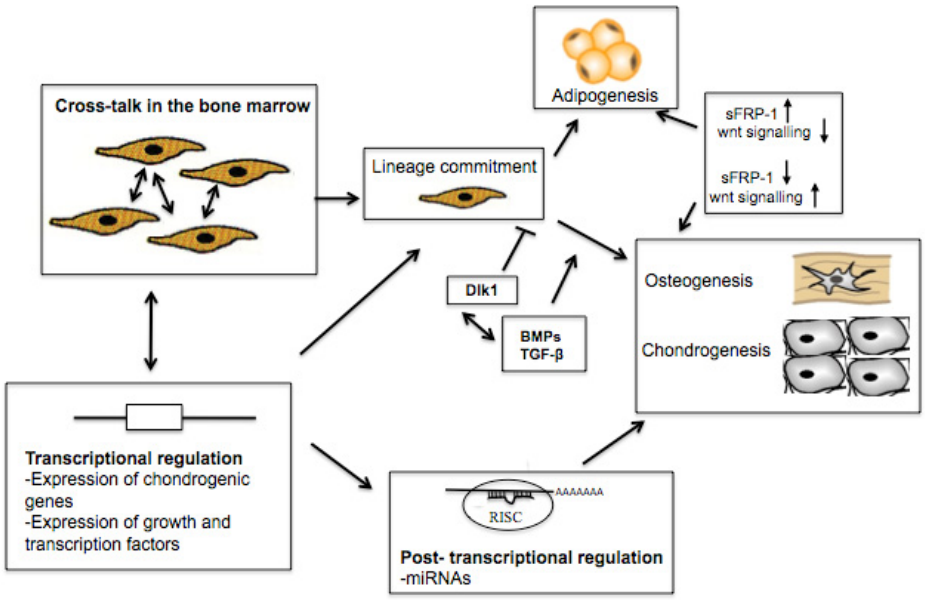


Figure 13. Regulation of MSC differentiation. MSC differentiation is regulated by the cross-talk between multipotent and committed MSCs as well as other cell populations in the bone marrow microenviroment. The transcription and growth factors expressed by certain cell types determine the lineage specificity and induce the expression of cell type-specific genes. MiRNAs exhibit a cell type-specific expression profile. Their expression is controlled by transcription factors. MicroRNAs, in turn, fine-tune the expression of the transcription factors and other genes of the alternative lineages at the post-transcriptional level. Dlk1 is expressed in the beginning of the mesenchymal differentiation processes and negatively regulates the differentiation. Dlk1 down-regulation is necessary for cells to undergo differentiation into the osteogenic and adipogenic lineages, while during chondrogenesis it is downregulated at the onset of chondrocyte maturation and hypertrophy. Growth factors, such as TGF-β1 and BMPs, promote chondrogenesis by activating Wnt and other crucial signalling pathways. The interaction between signalling pathways, for instance TGF-β1 and dlk1, plays a critical role in cell differentiation. Wnt signalling mediates the cross-talk and cell commitment of MSCs. sFRP-1 modulates the Wnt signalling activity and may function as a molecular switch that determines the balance between the adipogenic and osteo-chondrogenenic differentiation of the MSCs.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Medical Biochemistry and Genetics, University of Turku and the Molecular Endocrinology Laboratory (KMEB), Odense University Hospital during the years 2005-2010. Professors Klaus Elenius, Marko Salmi, Risto Penttinen and Jukka Finne at the Department of Medical Biochemistry and Genetics and Professor Moustapha Kassem at the KMEB are acknowledged for providing excellent working facilities.

I owe my warmest gratitude to my supervisor, Adjunct Professor Anna-Marja Säämänen, for introducing me to the fascinating world of stem cells and skeletogenesis. I truly appreciate her enormous support and encouragement as well as the freedom and responsibility I was given during these years. I wish to thank Professor Moustapha Kassem for the opportunity to join the dynamic KMEB group and for motivating scientific discussions. Associate Professor Basem M Abdallah is warmly thanked for supervision and guidance during my stay in Odense.

I am sincerely grateful to Professors Teuvo Hentunen and Mikko Lammi, the members of my supervisory committee, for their interest and valuable advice concerning my studies. I wish to thank Turku Graduate School of Biomedical Sciences and its director Professor Olli Lassila for financial support and for providing a pleasant scientific athmosphere in TuBS.

I wish to thank Professors Anitta Mahonen and Petri Lehenkari for reviewing my thesis manuscript and their constructive criticism and valuable comments. Maaria and Damon Tringham are acknowledged for revising the language of the thesis manuscript.

My co-authors Drs. Tiina Laitala-Leinonen, Teuvo Hentunen, Salla Laine, Abdullah Aldahmash, Linda Harkness, Amer Mahmood, Ulrike Frandsen, Kenneth Hauberg, Anne Seppänen, Tommi Ripatti and Professor Kalervo Väänänen are warmly acknowledged for their contribution to the work.

I am deeply grateful to Professor Eero Vuorio for his help and never-ending support and encouragement. I wish to thank Professor Hannu Aro for inspiring discussions and his optimistic attitude towards my work, and Docent Heli Salminen-Mankonen for help, advice and friendship.

All my former and present colleagues in Turku, especially Salla Laine, Ville Veikkolainen, Jussi Heinonen, Jessica Alm, Tim Wilson, Jutta Jokinen, Annele Sainio and Kari Kurppa are acknowledged for enjoyable company in the lab. Karoliina Vuoriluoto, Jonna Nevo, Saara Tuomi and Raine Toivonen are thanked for unforgettable moments in numerous TuBS arrangements. I wish to warmly thank Drs. Chen Li, Jorge S Burns, Weimin Qiu, Ann-Dorte Porneki, Nicholas Ditzel, Hamid Saeed, Tom E. Andersen, Abbas Jafari, Kenneth Hauberg, Olena Kucheryavenko, Diyako Qanie, Pernille Lund Hansen, Helle Christiansen, Gunnhildur Asta and other present and former members of KMEB for creating an inspiring working enviroment.

My special thanks belong to Linda Harkness for help and friendship and Tilde Eskildsen for fruitful collaboration and for sharing the joys and sorrows of life.

I want to sincerely thank Marja Nykänen, Tuula Oivanen, Merja Lakkisto, Raili Salonen, Liisa Peltonen, Bianca Jorgensen, Tina Kamilla Nielsen, Lone Cristiansen and other personnel at the Department of Biochemistry and Genetics and at KMEB for their excellent technical assistance. Rakel Mattson, Nina Widberg and Tiina Hansen-Barbisan are acknowledged for indispensable secretarial help.

I am truly grateful to Satu, Maria, Jennina, the members of Ihoden Dollsit, my course mates in Oulu and all other friends for reminding me of the beauty of life outside of work. I want to dedicate my special thanks to Eric for his invaluable support and encouragement.

Finally, I want to express my sincere gratitude to my dear family for their love, encouragement and patience. My sisters Laura, Noora, Sonja and Eeva are warmly acknowledged for brightening my days and always being there for me. My deepest gratitude belongs to my parents Marjut and Seppo for their unlimited love and support.

This work has been financially supported by the Academy of Finland, Southewestern regional fund of The Finnish Cultural Foundation, Orion Farmos Research Foundation, Turku University Foundation, Emil and Blida Maunula Foundation, Cirius Foundation, Paulo Foundation and Turku Graduate School of Biomedical Sciences.

Turku, November 2010

Hanna Taipaleenmäki

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