Human somatic cells in regenerative medicine

In vitro characterization of mesenchymal stem cells and chondrocytes

Doctoral thesis

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To:

My spirited and forgiving wife,

Dear Effat

For her love and her unconditional support

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Oslo, the 15th of October 2007

Aboulghassem Shahdadfar

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2. LIST OF PAPERS

This thesis is based on the following papers:

Shahdadfar A, Boquest AC, Frønsdal K, Sigurjonsson O, Tunheim SH, Collas P, and Brinchmann JE.

Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression after in vitro cell culture.
Mol Biol Cell. 2005 Mar;16(3):1131-41.

Shahdadfar A, Frønsdal K, Haug T, Reinholt FP, and Brinchmann JE.

In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability.
Stem Cells. 2005 Oct;23(9):1357-66

Shahdadfar A, Løken S, Tunheim SH, Dahl JA, Collas P, Reinholt FP, Engebretsen L, and Brinchmann JE.

Persistence of collagen type II synthesis and secretion in rapidly proliferating human articular chondrocytes in vitro.
Submitted manuscript

3. ABBREVIATIONS

ACI	autologous chondrocyte implantation
ACL	anterior cruciate ligament
ADASC	adipose-derived adult stromal cell
ALK	activin receptor-like kinase
alloHS	allogeneic human serum
AP	alkaline phosphatase
APC	allophycocyanin
ApoE	apolioprotein E
Arg	arginine
AS	autologous serum
b-FGF	basic fibroblastic growth factor
BM	bone marrow
BMI	body mass index
BMMNC	bone marrow mononuclear cell
BMMSC	bone marrow mesenchymal stem cell
BMP	bone morphogenic protein
cDNA	complementary deoxyribonucleic acid
CD-RAP	cartilage-derived retinoic acid-sensitive protein
CFU-F	colony forming units fibroblastic
ChIP	chromatin immunoprecipitation
CK	casein kinase
CNS	central nervous system
COMP	cartilage oligomeric protein
CpG	cystosine-guanine dinucleotides
CRD	cysteine-rich domain
cRNA	complementary ribonucleic acid
CTL	cytotoxic T lymphocyte
CY	cyanine dye
D	dimensional
DC	dendritic acid
Dkk-1	dickkopf-1
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
Dnmt	DNA methytransferase
dpc	days postcoitum
Dvl	dishevelled
EB	embryoid body
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetra acetic acid
EGC	embryonic germ cell
ESC	embryonic stem cell
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Fz	frizzled
GAG	glycosaminoglycan
GSK	glycogen synthase kinase
GVDH	graft versus host disease
H	histone
H3K9	histone H3, K9
H3K9ac	acethylation of H3K9
H3K9m	methylation of H3K9
HA	hyaluronic acid
HA/TCP	hydroxyapatite tricalcium phosphate
hAS	human articular chondrocyte
HDAC	histone deacetylase
hESC	human embryonic stem cell
Hg	hedgehog
HLA	human leukocyte antigen
hLP	human platelet
hMSC	human mesenchymal stem cell
HRPO	horseradish peroxidase
HSC	hematopoietic stem cell
ICM	inner cell mass
IDO	indoleamine 2, 3-deoxygenase

IFN	interferon
IGF-I	insulin-like growth factor-I
Ihh	Indian hedgehog
IL	interleukin
ISCT	international society for cellular therapy
ITS	insulin transferring sodium selenite
JAK	janus kinase
K	lysine
LIF	leukaemia inhibitory factor
LRP	lipoprotein receptor-related protein
Lys	lysine
MAb	monoclonal antibody
MAPC	multipotent adult progenitor cell
MAPK	mitogen-activated protein kinase
MAS	affymetrix microarray suite
MECP	methyl-CpG-binding protein
mESC	mouse embryonic stem cell
MHC	major histocompatibility
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
N-cadherin	neural cadherins
N-CAM	neural cell adhesion molecule
NK	natural killer
NOD	non-obese diabetic
OA	osteoarthritis
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PCM	pericellular matrix
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phycoerythrin
PECAM	platelet endothelial cell adhesion molecule
PerCP	peridinin chlorophyll

PGC	primordial germ cell
PL	platelet lysate
Pthlh	parathyroid hormone-like peptide
RNA	ribonucleic acid
RT	reverse transcriptase
RTK	receptor tyrosine kinase
RT-PCR	real-time PCR
SDF-1	stromal derived factor-1
SDS	sodium dodecylsulfate
Ser	serine
SREBF2	sterol regulatory element binding transcription factor 2
SSC	spermatogonial stem cell
STAT	signal transducer and activators of transcription
SVF	stromal vascular fraction
TAZ	transcriptional coactivator with PDZ-binding motif
TBI	total body irradiation
TGF-β	transforming growth factor β
Thr	threonine
TIPs	tension-induced/-inhibited proteins
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
Wnt	wingless

4. INTRODUCTION

The Greek Titan, Prometheus, is a fitting symbol for regenerative medicine. As punishment for giving fire to humankind, Zeus ordered Prometheus chained to a rock and sent an eagle to eat his liver each day. However, Prometheus´ liver was able to regenerate itself daily, enabling him to survive.

4.1 Stem cells

Stem cells have the remarkable potential to develop into many different cell types and are defined by both their ability to make more cells, a property known as self-renewal, and their ability to produce cells that differentiate.

There are three kinds of stem cells: embryonic, germinal, and somatic or adult stem cells. Embryonic stem cells are derived from the first five or six divisions of the fertilized egg. The progeny of embryonic stem cells are the precursors for all of the cells of the adult organs. Germinal stem cells in the adult produce eggs and sperm and are responsible for reproduction. Somatic stem cells are considered more limited in their potential, and they produce cells that differentiate into mature functioning cells that are responsible for normal tissue renewal (Sell, 2004).

4.2 Embryonic stem cells

The first entity of life, the fertilized egg, has the ability to generate an entire organism. This capacity, defined as totipotency, is retained by early progeny of the zygote up to the eight-cell stage of the morula. Subsequently, cell differentiation results in the formation of a blastocyst composed of outer trophoblast cells and undifferentiated inner cells, commonly referred to as the inner cell mass, ICM. Cells of the ICM are no longer totipotent but retain the ability to develop into all cell types of the embryo, defined as pluripotent embryonic stem cells (ESC) (Wobus and Boheler, 2005) (Figure 1).

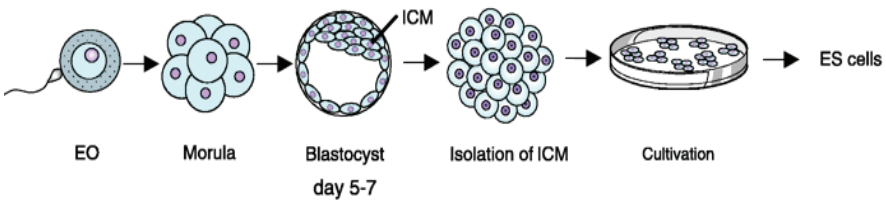


Figure1. Generation of pluripotent embryonic stem cells.

Human pluripotent embryonic stem cells have been derived from in vitro cultured ICM cells of blastocyst after in vitro fertilization (from Wobus AM.and et.al. 2005, with permission of The American Physiological Society).

Embryonic stem cell (ESC) lines from mouse (Evans and Kaufman, 1981; Martin, 1981), human (Thomson et al., 1998) blastocysts and, as recently shown, derived from single blastomeres, any one of the cells formed by the first divisions of a fertilized egg (Chung et al.,2006; Klimanskaya et al., 2006) have been established.

Stem cells from various adult organs that self-renew and differentiate into multiple organ specific cell types are termed multipotent stem cells. Committed cells that have limited or no self-renewal ability and differentiate into only one defined cell type are termed progenitor or precursor cells (Lakshmipathy and Verfaillie, 2005).

4.2.1 Human and mouse embryonic stem cells

ESC forms tight colonies with sharp borders when grown *in vitro* in culture dishes. From the morphological point of view, mouse embryonic stem cells (mESCs) grow as aggregates, but human embryonic stem cells (hESCs) grow as flat colonies. The population doubling time of hESCs is significantly longer than that of mESCs, possibly reflecting the longer gestational period in humans than in mice. Furthermore, hESCs

differentiate more readily and show much lower cloning efficiency compared to mESCs (Scholz et al., 1999; Thomson et al., 1998). Although many signaling pathways are conserved in animal development (transforming growth factor β (TGF-β) superfamily, receptor tyrosine kinase (RTK), Wingless (Wnt), Hedgehog (Hg), JAK/STAT and notch signaling (Pires-daSilva and Sommer, 2003), several key differences have already emerged, particularly the mechanisms underlying self-renewal, cell surface marker expression, signaling pathways and differentiation ability.

4.2.2 Self-renewal and pluripotency of ESCs

In vivo, during mouse embryogenesis, the primitive ectoderm of the epiblast forms three primary germ layers: the ectoderm, the mesoderm, and the definitive endoderm. These germ layers interact to form all tissues and organs of the developing embryo. The differentiation potential of mESCs *in vitro* has facilitated the examination of these processes. Once differentiation has begun, ESCs will aggregate into three-dimensional clusters of cells in an early stage of differentiation, thereby losing pluripotency. These clusters, named Embryoid Bodies (EBs), form the first step of further differentiation into any type of progeny. Within the EBs, a microenvironment exists in which various signals will promote differentiation into all three germ layers (van der Bogt et al., 2006). Initially, an outer layer of endoderm-like cells forms within the EB, followed over a period of a few days by the development of an ectodermal layer and subsequent specification of mesodermal cells. These EBs in tissue culture plates allow for continued differentiation into a variety of specialized cell types including cardiac, smooth and skeletal muscle as well as hematopoietic, pancreatic, hepatic, lipid, cartilage, or neuronal and glial cells. The temporal expression of tissue-specific genes and proteins in mES-derived cells during *in vitro* differentiation indicates that early processes of *in vivo* development into ectoderm, mesoderm and endoderm lineages are recapitulated *in vitro* (Wobus and Boheler, 2005). In a similar manner, hESCs spontaneously differentiate into EBs composed of three embryonic germ layers *in vitro* (Itskovitz-Eldor et al., 2000).

Derived from the ICM of the blastocyst, ESCs can proliferate indefinitely *in vitro* (self-renewal) and differentiate into cells of all three germ layers (pluripotency). These

unique properties make them exceptionally valuable for cell replacement therapies, drug discovery and regenerative medicine.

An interesting feature of these cells is that self-renewal happens indefinitely without loss of pluripotency. The mechanisms of this unique ability are still not well known, but several extrinsic factors support the self-renewal of ESCs *in vitro*. Leukemia inhibitory factor (LIF) supports the undifferentiated state of mESCs by activating the signal transducer STAT3. Another extrinsic factor known to support mESCs self-renewal is BMP4. In the presence of LIF, BMP4 can enhance the self-renewal and pluripotency of mESCs by activating inhibitor of differentiation (Id) genes (Ying et al., 2003). In addition to LIF and BMP4, the Wnt pathway is sufficient to maintain self-renewal of mESCs and hESCs (Sato et al., 2004). Under conditions that would promote self-renewal of mESCs, LIF is not sufficient to maintain hESCs self-renewal and BMPs cause rapid differentiation. Instead, FGF signaling and the balance between TGFβ/activin and BMP signaling appear central to the self-renewal of hESCs (Xu et al., 2005; James et al., 2005; Vallier et al., 2005).

At the molecular level, pluripotency has been linked to a few transcription factors and their expression appears to define whether a cell should be pluripotent. The best characterized of these is *OCT4*, which functions to maintain pluripotency both *in vivo* and *in vitro*. Oct4 is a transcriptional factor that is specially expressed in all pluripotent cells during mouse embryogenesis and also in undifferentiated ESCs (Pan et al., 2002). However, *OCT4* is not the only master gene that controls ESCs pluripotency. Recently, another master gene in ESCs pluripotency, *Nanog*, was discovered. Nanog is a unique homeobox transcription factor that plays a critical role in regulating the cell fate of the pluripotent ICM during embryonic development, maintaining the pluripotent epiblast and preventing differentiation to primitive endoderm (Chambers et al., 2003).

4.2.3 Signaling pathways in ESCs

The unique property of ESCs has opened new possibilities for studying differentiation in early human embryo development, more importantly to understand the underlying molecular mechanisms that control cell growth and differentiation *in vitro*. Furthermore, hESCs and their derivatives have great potential for developing cell therapies for

treatment of ailments such as diabetes, Parkinson's disease and heart failure. However, fabrication of both undifferentiated and differentiated hESCs requires improved control of regulatory mechanisms of differentiation before transplantation of hESCs becomes clinically applicable.

However, if undifferentiated hESCs were to be transplanted into immunosuppressed patients, it is possible that they would form teratomas or teratocarcinomas (generation spontaneous tumors from ESCs) containing a mixture of tissue types. It is therefore of great importance to understand the signaling pathways that direct hESCs along a given differentiation path. Following is a brief description of some of the more important families of molecules known to be involved in stem cell differentiation.

4.2.3.1 TGF-β superfamily

TGF-β is a multipotent growth factor that is involved in regulating cell growth, differentiation, migration, extracellular matrix (ECM) deposition and apoptosis during embryonic development and adult homeostasis and response to injury and diseases (Valdimarsdottir and Mummery, 2005). TGF-β superfamily signals are conveyed through serine/threonine kinase receptors to specific intracellular mediators known as Smad proteins (Massague, 2000; Roberts et al., 1986). Vertebrates possess at least eight Smad proteins falling into three functional classes: (1) Receptor activated Smads (RSmads): Smad1, Smad2, Smad3, Smad5 and Smad8, (2) Comediator Smads: Smad4 and Smad10, (3) Inhibitory Smads: Smad6 and Smad7. This superfamily has more than 40 members, including TGF-β, activin, nodal and bone morphogenetic proteins (BMPs) (Massague, 1998; Roberts et al., 1986). These ligands have all been associated with ES cells. The TGF-β prototype transduces signals from the membrane to the nucleus by binding to a heteromeric complex of serine/threonine kinase receptors known as TGFβ type I (TβRI) and type II (TβRII) receptors (Figure 2). The type I receptor, also known as activin receptor-like kinase (ALK), acts downstream of the type II receptor and propagates the phosphorylation signal through specific downstream Smad mediators. The Smads enter the nucleus, where they activate transcription of their target genes (Heldin et al., 1997; Piek et al., 1999; Massague, 2000) (Figure 2).

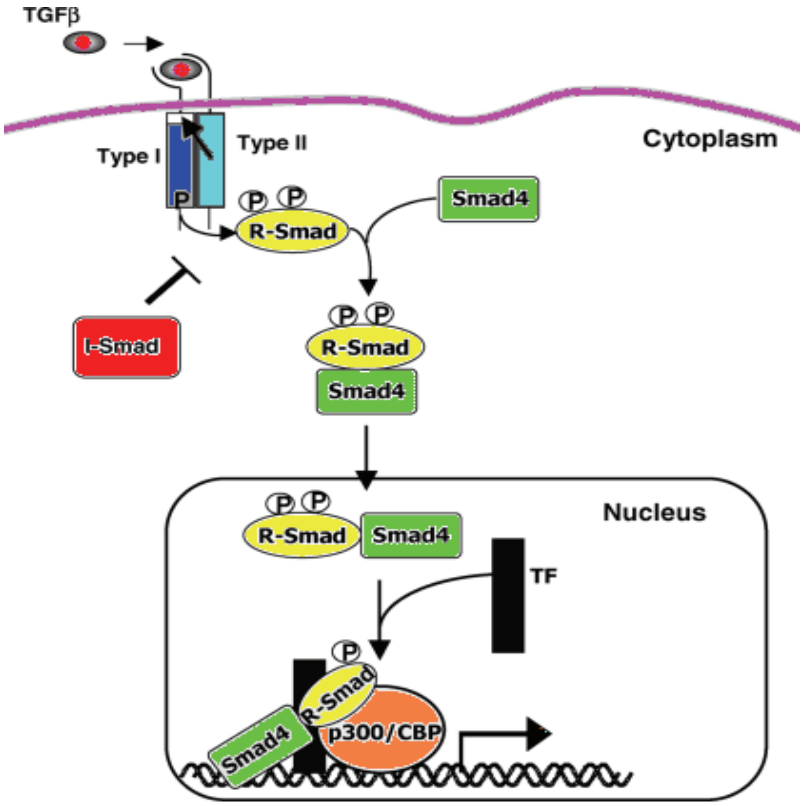


Figure 2. The TGF-β signal transduction pathway.

(from Valdimarsdottir G. and et.al. 2005, with permission of Wiley-Blackwell Publishing Ltd.).

The TGF-β family members have been reported to regulate multiple aspects of ESC fate decisions. Whether ESCs undergo self-renewal or differentiation likely depend on the state of the cells and the levels of cytokines and their interactions with other molecules (Valdimarsdottir and Mummery, 2005).

4.2.3.2 Wnt signaling pathway

WNT (Wingless and *INT-1*) genes encode small secreted proteins that are found in all animal genomes. Wnt signaling is involved in virtually every aspect of embryonic development and also controls homeostatic self-renewal in a number of adult tissues. Germline mutations in the Wnt pathway cause several hereditary diseases, and somatic mutations of *WNT* genes are associated with cancer of the intestine and a variety of other tissues. Genome sequencing has since revealed that mammalian species have roughly 20 secreted Wnt proteins, which can be divided into 12 conserved Wnt subfamilies (Clevers, 2006). Three different pathways are believed to be activated upon Wnt receptor activation: the canonical Wnt/β-catenin cascade, the noncanonical planar cell polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway (Katoh, 2005; Kohn and Moon, 2005). The canonical pathway is the best understood, and is the subject of this section (Figure 3).

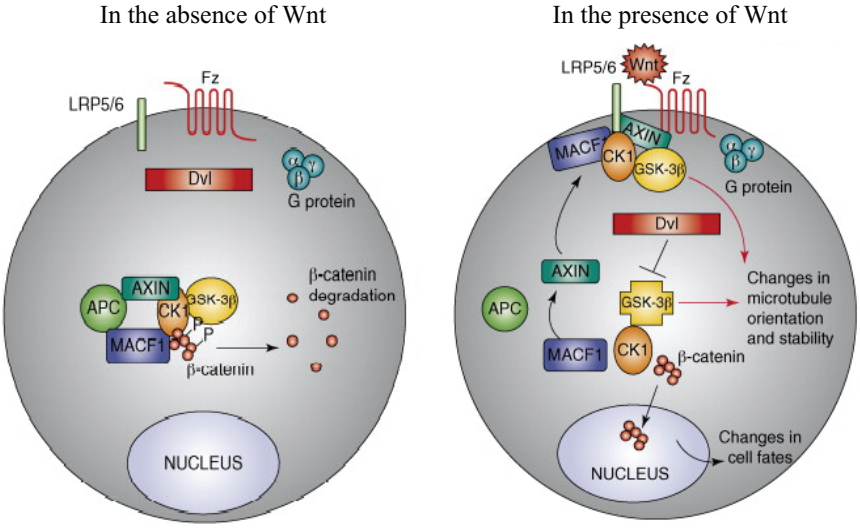


Figure 3. Canonical Wnt signalling pathway.

(from Salinas PC. 2007, with permission of Elsevier Limited).

The central player in the canonical Wnt cascade is β-catenin, a cytoplasmic protein whose stability is regulated by the destruction of a cytoplasmic complex. Axin acts as the scaffold of this complex as it directly interacts with β-catenin, APC, and the two kinase families, CK1 and GSK3. In the absence of Wnt proteins, Wnt receptor complexes are not engaged. CK1 and GSK3α/β sequentially phosphorylate β-catenin at a series of highly conserved Ser/Thr residues near its N terminus. Phosphorylated β-catenin is then recognized by a component of the dedicated E3 ubiquitin ligase complex. As a consequence, β-catenin is ubiquitinated and targeted for rapid destruction by the proteasome (Aberle et al., 1997) (Figure 3).

In the presence of extracellular Wnt ligands, the Wnt family binds Frizzled (Fz) proteins, which are seven-pass transmembrane receptors with an extracellular N-terminal cysteine–rich domain (CRD) (Bhanot et al., 1996). LRP5/6 (lipoprotein receptor related protein 5 or 6) act as a coreceptor for Wnt binding to Fz. The Fz/LRP5/6 complex activates the canonical signaling pathway. Fz can physically interact with Dvl, a cytoplasmic protein that functions upstream of β-catenin and the glycogen synthase kinase 3β (GSK-3) (Clevers, 2006). The coreceptor LRP5/6 interacts with Axin through five phosphorylated PPP(S/T)P repeats in the cytoplasmic tail of LRP (Davidson et al., 2005; Zeng et al., 2005). GSK3 phosphorylates the PPP(S/T)P motif, whereas CK1γ phosphorylates multiple motifs close to GSK3 sites. Thus, the phosphorylated PPP(S/T)P motif mediates the LRP5/6-Axin interaction (Mao et al., 2001; Tamai et al., 2004). The recruitment of Axin away from the destruction complex leads to stabilization of β-catenin. In the nucleus, stabilized β-catenin displaces the corepressor protein Groucho (Lepourcelet and Shivdasani, 2002) and interacts with transcription regulators; including T cell factor /leukocyte enhance factor-1 (Tcf/Lef-1) to promote the transcription of Wnt target genes (Dale, 1998; Gumbiner, 1998; Wodarz and Nusse, 1998) (Figure 3).

4.2.4 hESCs immunogenicity

hESCs are under extensive investigation for their regenerative potential in the treatment of various degenerative and malignant human diseases. The pluripotent character of

undifferentiated hESCs is attractive for cell-based therapy, but their pluripotency can also lead to risk of teratoma formation after transplantation. Another major clinical concern is the possibility of a provoked immune reaction after transplantation of hESCs into a new host, which may severely limit their use in therapeutics. Survival of transplanted cells correlates with the number of differences in major histocompatibility (MHC) antigens between donor and recipient, triggering T-cell responses and rejection of cells with disparate MHC profiles (Janeway, Jr., 1999). Undifferentiated hESCs express low levels of cell surface human leukocyte antigen class I (HLA-I) molecules. The expression of HLA-I was elevated in EBs, with even higher levels observed in teratomas. However, HLA class II (HLA-II) molecules were not expressed (Draper et al., 2002; Drukker et al., 2002). Undifferentiated as well as differentiated hESCs upregulate expression of HLA-I at least 10 fold in response to interferons (IFNs). However, HLA-II expression was not induced, reviewed in (Drukker and Benvenisty, 2004).

HESCs injected into the leg muscle of immunocompetent (CD-1) mice failed to induce an immune response 48 hours after injection. In addition, *in vitro* analyses showed that undifferentiated hESCs and EBs failed to stimulate proliferation of alloreactive primary human T cells (Li et al., 2004). HESCs transplanted into mice were rejected in all recipient strains tested, but when transplanted under the kidney capsule with concomitant hematopoietic reconstitution after whole body irradiation, hESCs were accepted and developed into teratomas (Drukker et al., 2006).

In vitro studies and short term *in vivo* experiments demonstrating that hESCs are immune-privileged or have reduced immunogenicity are in contrast with recent studies in which hESCs were injected into the mouse myocardium demonstrating that hESCs were immunogenic in allogeneic and xenogeneic settings (Grinnemo et al., 2006). Microarray analysis and flow cytometry of hESC lines have shown that both *HLA* classes were expressed at the mRNA level, but there was no surface expression of HLA class II, not even after IFNγ stimulation. It has also been demonstrated that expression of co-stimulatory molecules at the mRNA level was low or absent (Grinnemo et al., 2006). Co-stimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86) were not expressed on undifferentiated or differentiated hESCs (Sharpe and Freeman, 2002). Microarray analysis of molecules known to inhibit immune responses, such as FasL (CD59L) which has been shown to mediate induction of apoptosis in activated T- cells

(Green and Ferguson, 2001; Hamad and Schneck, 2001), IL-10, a cytokine important in reducing inflammation and utilized by regulatory T-cells in the prevention of unwanted immune responses (Roncarolo et al., 2001), revealed that they were not expressed in hESCs (Grinnemo et al., 2006). TGF-β, a key mediator in the prevention of autoimmune disease in various animal models (Roncarolo et al., 2001) was expressed in four of seven hESC lines (Grinnemo et al., 2006). Thus, the lack of expression of high levels of HLA class II and co-stimulatory molecules and the expression of molecules known to inhibit the immune response, theoretically state that ESCs cannot be immunogenic. But surprisingly, in some studies, hESCs induce mouse $CD4^+$ T-cell proliferation equivalent to that of fibroblasts. This is probably due to the fact that all cells which are transplanted into a host with functional dendritic cells (DC) will have their antigen presented by indirect antigen presentation to the immune system (Grinnemo et al., 2006).

Although these are important observations, it is also important to note that such knowledge does not allow for an accurate prediction of immune responses against the cells because multiple factors in addition to direct antigen presentation by hESCs are involved in such responses. Before using hESCs or their derived tissues in therapeutics, a complete understanding of the cellular interactions between hESCs and the immune system should be investigated.

4.3 Germinal stem cells

Following fertilization of the oocyte by a spermatozoon, the zygote begins proliferation and then differentiates to produce many cell types that constitute an individual. At day 5 after fertilization in the mouse, the zygote becomes a blastocyst, and then begins the process of implantation. At this time, the embryo consists of ICM, primitive endoderm and trophoectoderm. The ICM cells are precursors of epiblast cells, which give rise to the three germ layers that produce stem cells for all tissues of the fetus. Epiblast cells gradually commit to specific cell lineages and lose pluripotent developmental potential. Some of these committed cells become cell linage specific primitive somatic stem cells, while others become primitive germline cells (Kubota and Brinster, 2006).

About 7 days postcoitum (dpc) in the mouse, specification of the germ cell lineage

occurs in the proximal epiblast under the influence of surrounding extraembryonic tissues. At this stage, about 100 alkaline phosphatase (AP) positive primitive germline cells, which are also called primordial germ cells (PGCs), are formed. PGCs are the founder cells for the germline. They divide symmetrically and all their descendents are germ cells. In many organisms, PGCs are motile and migrate to the somatic gonads. In the mouse, they migrate to female or male genital ridges and differentiate into oogonia or gonocytes, respectively (Matsui et al., 1992; McLaren, 2003; Seydoux and Braun, 2006). Oogonia in the female gonads quickly enter meiotic prophase as oocytes, which are all arrested in meiosis I before birth. The adult ovary, therefore, does not contain germline stem cells (Kubota and Brinster, 2006). In the male, gonocytes cease mitosis in the seminiferous tubules of the testes and resume mitotic activity after birth. In the fetus, gonocytes are located in the centre of the tubules, but during the first few days after birth in mouse and at later times in other species, they either undergo apoptosis or migrate to the basement membrane in testes and become spermatogonial stem cells (SSCs) (de Rooij and Grootegoed, 1998; Orwig et al., 2002).

4.3.1 Spermatogonial stem cells

PGCs from embryos between 8.5 and 12.5 dpc give rise to pluripotent cells when cultured under appropriate conditions (Matsui et al., 1992; Resnick et al., 1992). These embryonic stem(ES)/embryonic germ (EG) cells have differentiation properties similar to ES cells isolated from the inner cell mass (Evans and Kaufman, 1981; Martin, 1981). These observations suggest that the germline lineage may retain the ability to generate pluripotent cells. Germline stem cells from neonatal mouse testis are also pluripotent and have differentiation potential similar to embryonic stem cells (Kanatsu-Shinohara et al., 2004). Recently, the pluripotency and plasticity of adult spermatogonial stem cells (SSCs), which are responsible for maintaining spermatogenesis throughout life in the male, has been reported. These isolated SSCs respond to culture conditions and acquire embryonic stem cell properties. They express ES cell markers and are able to spontaneously differentiate into derivatives of three embryonic germ layers *in vitro* and generate teratomas in immunodeficient mice. When injected into an early blastocyst, SSCs contribute to chimeras by the development of various organs and show germline transmission (Guan et al., 2006). Thus, the capacity to form pluripotent cells persists in

adult mouse testis. It is extremely fascinating that SSCs can contribute both to spermatogenesis and to chimera formation, indicating that SSCs appear to be able to change their fate quickly according to their microenvironment. No other cells in the body could have such diverse developmental functions at the same time. SSCs may be ideal cells for regenerative medicine if these findings can be reproduced with human cells. These cells can provide a generous supply of healthy pluripotent cells for curing diseases without the ethical concerns associated with ESCs. Finally, SSCs in the adult testis may be more versatile than ESCs (Kanatsu-Shinohara and Shinohara, 2006).

4.4 Adult stem cells

Developmentally, stem cells are categorized either as embryonic stem cells or as postnatal stem cells. These latter cells are also called organ specific, tissue specific or adult stem cells (Pauwelyn and Verfaillie, 2006). Adult stem cells are traditionally thought to be restricted in their differentiation potential to the progeny of the tissue in which they reside. In higher vertebrates, most adult tissues and organs contain stem cells capable of self-renewal, proliferation and differentiation into their own mature and functional progeny. These stem cells are more abundant in tissues with a high renewal rate, such as blood and epithelia, and less abundant in tissues or organs with little renewal capacity such as myocardial muscle or the central nervous system (CNS). In the last few years, a number of reports have claimed a broad multipotency and remarkable plasticity in the differentiation potential of stem cells derived from adult tissues such as bone marrow (BM), the skeletal musculature or the CNS. In all cases, differentiation of stem cells into a non-traditional progeny e.g., muscle or liver from BM stem cells, was a rare phenomenon (Krause et al., 2001; Okamoto et al., 2002). For instance, neurosphere-derived neural stem cells have been differentiated to hematopoetic stem cells (HSC), which contain a completely different genetic program (Bjornson et al., 1999).

However, the origin of stem cells in adults, as well as whether they are distinct populations of cells or represent the remains of embryonic development, is unclear. Another controversy is whether adult stem cells isolated from a particular tissue originated in this tissue or was derived from a pool of stem cells circulating in the blood

that have been temporarily trapped in the tissue from which they were isolated, having thus been subjected to a process called homing (Korbling et al., 2002).

More recent developments have proven that adult stem cells reside in nearly every tissue, including brain, bone marrow, peripheral blood, kidney and epithelia of the digestive system, as well as skin, retina, muscle, pancreas, liver, fat, umbilical cord, scalp and placenta (Keating, 2006; Slack, 2000). The possibility of a broad multipotential capacity of adult stem cells has obvious relevance for repair of damaged tissues other than the tissues in which they reside. In a part of this thesis, adult stem cells from human bone marrow and adipose tissue were isolated and characterized. The following sections highlight recent discoveries in the area of MSCs.

4.4.1 Biology of adult mesenchymal stem cells

Mesenchymal stem cells (MSCs) were first identified in 1966 in studies when bone/ cartilage forming progenitor cells from rat bone marrow cells with fibroblastic-like morphology were isolated (Friedenstein et al., 1966). The first description of the classical tri-lineage differentiation of MSCs was reported in 1999 (Pittenger et al., 1999). Plastic adherent multipotent cells were isolated from many adult tissues and displayed a heterogeneous mixture of cells with varying proliferation and differentiation potentials. Most of the studies focused on MSCs from human and mouse bone marrow. Surprisingly, there is only a small amount of variations between populations, even among cells isolated from different sources. However, a rare cell within the human bone marrow mesenchymal stem cell culture was identified that can be expanded for more than 80 population doublings. This cell differentiates not only into mesenchymal lineage cells but also into endothelium and endoderm (Reyes et al., 2001; Reyes et al., 2002; Schwartz et al., 2002). The same lab has also reported mouse bone marrow-derived multipotent adult progenitor cells (MAPCs) that proliferate extensively without obvious senescence or loss of differentiation from the MSC population as a whole (Jiang et al., 2002a). These MAPCs differentiate, at the single cell level, not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm and endoderm characteristics *in vitro*. MAPCs contribute to most somatic tissues when injected into an early blastocyst and engraft *in vivo*, where they differentiate into tissue specific cell types in response to cues provided by different organs (Jiang et al., 2002a).

Many efforts have been made to develop a cell surface antigen profile for better purification and identification of MSCs. However, even MAPCs do not express specific surface markers and show similarities that may represent a more primitive pluripotent progenitor of MSCs.

4.4.1.1 Adult MSC markers and nomenclature

Many studies have reported a population of stem cells from various tissues known as mesenchymal stem cells, marrow stromal cells and mesenchymal stromal cells, all designated by the acronym MSC. These studies have shown a more or less similar phenotype characteristic of MSCs. Recently, the International Society for Cellular Therapy (ISCT) has clarified the nomenclature by introducing the term multipotent mesenchymal stromal cells, with the same acronym (MSCs). The minimal criteria for defining multipotent mesenchymal stromal cells are as follows: first, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules. Third, MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006).

4.4.1.2 Cell frequency of adult MSCs in different tissues

MSCs are present at low numbers in BM and most other tissues. Thus, most of our present data on MSCs is based on cells expanded in culture. In previous studies, the hMSC precursor frequency has been calculated to be 2 to 5 per 10^6 bone marrow mononuclear cells (BM MNC) (Minguell et al., 2001), 1 in 10^4 BM MNCs based on selection by the D7-FIB monoclonal antibody (MAb) and colony-forming unit fibroblastic (CFU-F) assays, and 3 in 10^4 BM MNCs based on isolation of STRO-1^{bright}/vascular cell adhesion molecule–positive ($VCAM^+$) cells and CFU-F assays in medium supplemented with growth factors(Gronthos et al., 2003b). In paper II of this thesis, the MSC precursor frequency in $CD14^+$-depleted BM MNCs has been calculated

to be approximately 1 in 10^3.

In comparative analyses, colony number per 10^3 nucleated cells derived from synovium, periosteum, adipose tissue and muscle was about 100-fold higher than that derived from BM (Sakaguchi et al., 2005). The number of CFU-F calculated on the basis of 10^6 initially plated cells was highest for adipose tissue, followed by BM, and was lowest for umbilical cord blood (Kern et al., 2006).

Adipose tissue is easy to obtain in large quantities and harbors a large number of cells with CFU-F ability (Zuk et al., 2002). In paper I, we purified the uncultured CD34+CD105+CD31- population from SVF of human adipose tissue using a combination of MAbs to cell surface antigens. About 4% of this selected population, at the single cell level in culture, displayed MSC characteristic.

4.4.1.3 Adult MSC self-renewal and maintenance

Self-renewal refers to the biological pathways and mechanisms that preserve the undifferentiated state of stem cells. LIF, fibroblast growth factors (FGFs) and mammalian homologs of Wnts among other growth factors and cytokines have been shown to be involved in MSC stem cell maintenance (Jiang et al., 2002b; Boland et al., 2004; Tsutsumi et al., 2001). These factors have attracted particular attention because of their demonstrated role in the self-renewal of undifferentiated ESCs, which was discussed in sections 4.2.2 and 4.2.3 of this thesis. LIF cytokine maintains the undifferentiated state of MSCs and may involve paracrine crosstalk with neighboring cells. FGF2 also maintains the self-renewal of MSCs from a variety of species by prolonging their viability in culture. Target genes of FGF involved in MSC stemness are not known, but it is plausible that an autocrine regulatory loop may underlie FGF self-renewal function, as during vertebrate limb development (Marie et al., 2005). Wnt3a treatment increases adult MSC proliferation while inhibiting osteogenic differentiation (Boland et al., 2004). Canonical Wnt functions include the promotion of long term culture expansion of stem cells, increased *in vivo* reconstitution of hematopoietic lineages and Wnt3a specific maintenance of the skin and intestinal stem cell population (Kleber and Sommer, 2004). The mechanism of Wnt signaling has also been described in section 4.2.3.2.

MSCs from a variety of mammalian species also express the embryonic stem cell gene

markers *OCT4*, *SOX2*, and *REX1*, among others (Izadpanah et al., 2006). Recent chromatin immunoprecipitation array studies suggest that some polycomb chromatin-associated proteins are involved in maintaining the repression of differentiation genes. Polycomb protein may indirectly maintain *OCT4*, *SOX2*, and *REX1* activation in MSCs (Ringrose and Paro, 2004).

4.4.1.4 Differentiation of adult MSCs

Regulating the switch between proliferation and differentiation of MSCs is critical for the development of normal tissues, and the prevention of tumors. How MSCs exit from the cell cycle and differentiate into alternative cell fates such as bone, fat, and muscle, is incompletely understood. The ability to modulate biological effectors to manage a desired differentiation program is needed for effective clinical application, as in tissue engineering and regeneration. The differentiation of adult MSCs isolated from a variety of tissues into a number of lineages has been described. In general, extracellular molecular signaling and mechanical inducers of differentiation transduce effects through accepted receptors, channels or other cell surface associated mechanisms. Downstream signaling pathways, including that between distinct mitogen-activated protein kinases (MAPKs) and R-Smads, provides a level of specificity that gives rise to unique lineages, such as chondrocytes and osteoblasts. The specificity of lineage differentiation can also result from the recruitment of master transcriptional switches with binary regulation of cell fate, such as TAZ (transcriptional coactivator with PDZ-binding motif). Depending on the potentially unique multiprotein complexes that it may form in response to specific upstream signaling, TAZ promotes osteogenesis and inhibits adipogenesis. TAZ functions as a transcriptional modulator to stimulate bone development while simultaneously blocking the differentiation of MSCs into fat. These developmental effects occurs through direct interaction between TAZ and the transcriptional factor Runx2 (also called Cbfa1 or Pebp2αA) and peroxisome proliferator-activated receptor γ (PPARγ), resulting in transcriptional enhancement and repression, respectively of selective programs of gene expression (Hong et al., 2005; Hong and Yaffe, 2006).

An attempt to identify common pathways mediating differentiation of MSCs into

osteogenic, adipogenic and chondrogenic lineages has shown that a set of genes that are upregulated during differentiation is needed for differentiation into all three lineages, whereas lineage specific late-differentiation genes are essential for terminal differentiation. One of the genes that appear to have a positive role in early differentiation of these three mesenchymal lineages is FKBP5, a binding protein involved in modulating hormone receptor response and transcription regulation (Liu et al., 2007).

4.4.1.5 Asymmetric and symmetric stem cell division

Stem cells are defined by both their ability to make more stem cells, self-renewal, and their ability to produce differentiated cells. One tactic by which stem cells can achieve these two tasks is asymmetric cell division, whereby each stem cell divides to generate one daughter with a stem-cell fate like the mother, self-renewal, and one daughter (committed cell) that differentiates. The benefit of asymmetric cell division is that it manages both tasks with a single division, and the disadvantage of asymmetric division is that it leaves stem cells unable to expand in number. Thus, asymmetric cell division alone cannot fulfill all stem cell characteristics. However, stem cells are also able to use symmetric cell division to generate more stem cells and differentiated daughters. In principal, stem cells can either depend completely on symmetric division or a combination of symmetric and asymmetric divisions. The balance between these two forms of division is controlled by developmental and environmental signals in order to produce appropriate numbers of stem cells and differentiated daughters (Morrison and Kimble, 2006). The combination of asymmetric and symmetric cell divisions to repair damaged adult tissue has recently been well described in muscle.

In adult tissues such as muscle, satellite cells are the major source of myoblast generation during postnatal growth and after injury. When labeled satellite cells are engrafted, less than ten satellite cells can contribute to over 100 myofibres. In addition, these cells can self-renew after muscle lesion. Satellite cells have the ability to divide asymmetrically, and the frequency of asymmetric cell divisions increases after 24 hours when cells are seeded at higher cell densities and then declines significantly after 72 hours (Shinin et al., 2006).

The descendants of activated satellite cells, called myogenic precursor cells, undergo multiple rounds of division prior to terminal differentiation and fusion to form multinucleated myofibers. Activated satellite cells also generate progeny that restore the pool of quiescent satellite cells (McKinnell et al., 2005). The molecular mechanism regulating self-renewal and differentiation suggests a combination of asymmetric and symmetric cell divisions in muscle satellite cells (Figure 4).

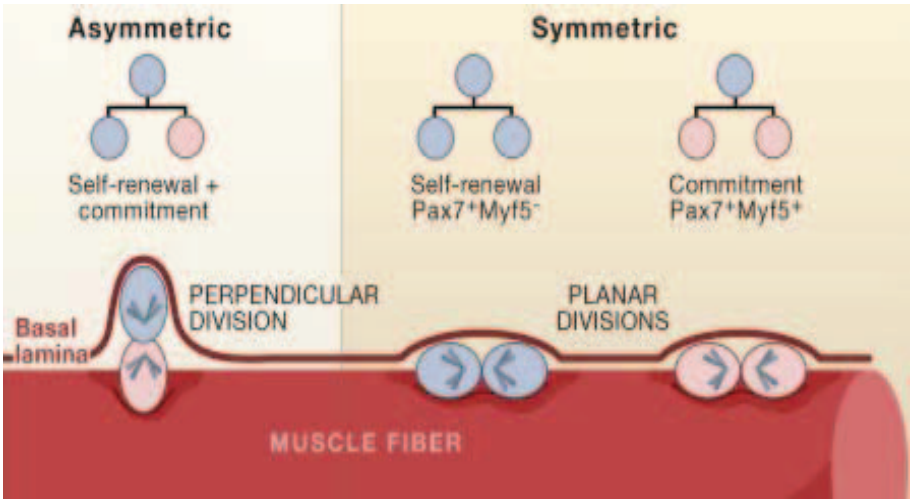


Figure 4. Asymmetric division of stem cells generates self-renewing and committed cells.

Symmetric divisions generate either two self-renewing cells that are $Pax7^{+}/Myf5^{-}$ (blue) or two committed cells that are $Pax7^{+}/Myf5^{+}$ (red). In adult skeletal muscle, symmetric divisions generate two identical (either stem or committed progenitor) daughter cells that both contact the basal lamina and the plasmalemma. Asymmetric division generates one self-renewing cell ($Pax7^{+}/Myf5^{-}$) that remains in contact with the basal lamina and one committed cell ($Pax7^{+}/Myf5^{+}$) that is adjacent to the plasmalemma but has lost contact with the basal lamina (from Cossu G. and et. al. 2007, with permission of Elsevier Limited).

Symmetric cell divisions in the muscle fiber (constituting 90% of all divisions) result in both daughters remaining in contact with the plasmalemma of the myofiber and the basal lamina, which includes the satellite cell. Notably, it is implied from the expression of the marker genes that these symmetric cell divisions can result either in the self-renewal or the myogenic commitment of both daughter cells (Figure 4) (Cossu and Tajbakhsh, 2007). Evidence has also been provided for asymmetric cell divisions (10% of all divisions) that occur perpendicular to the muscle fiber (Kuang et al., 2007). These result in one daughter cell remaining in contact with the basal lamina and the other daughter cell losing this interaction but maintaining contact with the plasmalemma. The cell proximal to the plasmalemma expresses higher levels of Myf5, whereas the cell proximal to the basal lamina preferentially expresses Pax7 (Figure 4). This observation raises the intriguing possibility that the former will fuse with the fiber, whereas the latter will remain in the niche to generate other stem cells or re-enter quiescence (Cossu and Tajbakhsh, 2007).

4.4.1.6 Adult MSC niche

As described earlier, MSCs are now routinely isolated from the bone marrow of many mammalian model organisms, as well as from other tissues of mesodermal and non-mesodermal origins. An interesting study has reported that plastic adherent MSCs derived from the brain, spleen, liver, kidney, thymus, lung, bone marrow, muscle and pancreas of mice all displayed similar morphologies and immunophenotypes in culture after several passages, but MSCs are not detected in peripheral blood. This study hypothesizes that the distribution of MSCs throughout the postnatal organism is related to their existence in a perivascular niche (da Silva et al., 2006). The data from current studies raise questions about the microenvironment of the MSCs, whether there is a common *in vivo* MSC niche in all of these tissues or whether MSCs function independently of their environments.

Recently, the idea of a stem cell niche has gained wide support. Briefly, a niche includes all stem cells and their surrounding elements when they are in their naive state, including non-stem cells that might be in direct contact with them as well as ECM and soluble molecules found in that locale. These elements maintain the stem cells in their

undifferentiated state. It is then assumed that certain signals in the niche regulate the differentiation needed for regeneration or repopulation of a tissue (Li and Xie, 2005; Lin, 2002). However, the perivascular niche hypothesis describes the MSCs location in the basement membrane of blood vessels, throughout the body that gives them easy access to all tissues and provides stem cells to heal many different tissues (da Silva et al., 2006). Cellular, soluble and ECM components that are important in stem cell biology are involved in the MSC niche.

4.4.1.7 MSC homing and wound healing

Another stem cell niche related event is the homing of stem cells to sites of injury and subsequent wound healing. Signaling to progenitors or stem cells to home to the site of injury and differentiate into the required cell type is needed. To understand the niche, it is important to analyze not only what keeps stem cells in their niche but also what signals them to immigrate from it. The homing capacity of MSCs seems to be related in part to the expression of Stro-1. Stro-1 negative cells were better able to aid in the engraftment and survival of HSCs, whereas Stro-1 positive cells were more capable of homing and engrafting to most of the tissues studied (Bensidhoum et al., 2004). In the mouse, irradiation of both the whole animal and specific sites caused injected MSCs to engraft to more organs and in higher numbers than in unconditioned mice (Francois et al., 2006). It is known that injury alters the patterns of migration and differentiation of exogenously added MSCs. However, injected MSCs are capable of specific migration to the site of injury, influencing the idea of using these cells for therapeutic use (Barry and Murphy, 2004).

4.4.1.8 Immunobiological characteristics of adult MSCs

Recent studies, mostly from *in vitro* experiments, show that MSCs contain attractive immunobiological properties. MSCs can evade immune recognition and inhibit the immune response.

Adult MSCs express HLA class I, but not HLA class II on the cell surface. After

differentiation into bone, cartilage, or adipose tissue, adult MSCs continued to express HLA class I, but not class II (Le et al., 2003). Cell surface expression of HLA class II can be induced in culture by treatment with IFN-γ. After induction by IFN-γ to express HLA class II, MSCs still escape recognition by alloreactive T cells (Gotherstrom et al., 2004; Le et al., 2003). FAS ligand or costimulatory molecules, such as B7-1, B7-, CD40 or CD40L, which are necessary for the T cell response, are not expressed by MSCs (Tse et al., 2003). MSCs failed to elicit a proliferative response when cocultured with allogeneic peripheral blood mononuclear cells (PBMC), despite provision of a costimulatory signal delivered by an anti-CD28 antibody and pretreatment of MSCs with IFN-γ (Tse et al., 2003).

MSCs also escape recognition by CTLs and alloreactive NK cells and inhibit the formation of cytotoxic T cells by secreting a soluble factor, but they do not interfere with CTL and NK cell lysis (Rasmusson et al., 2003). A recent study has revealed that MSCs remain resistant to CTL lysis, even after pulsing with specific synthetic peptide at high concentrations. MSCs induced CD25 up-regulation, although at relatively low levels, but were unable to induce CD3 or CD8 down-regulation at the surface of CTLs and also failed to induce IFN-γ and TNF-α production by CTLs (Rasmusson et al., 2007). MSCs neither exhibited the humoral immune response in patients with prior hematopoietic stem cell transplantation nor did express any blood antigens (Sundin et al., 2007).

The observations of these studies support the idea that MSCs may escape immune response and are capable to suppress immune reactions. This suggests that MHC-mismatched MSCs may be utilized as universal donor cells for therapeutic purposes. However, implantation of murine MHC class I and class II- mismatched engineered MSCs for secretion of erythropoietin (Epo) led to robust and specific cellular immune responses in nonimmunosuppressed allogeneic mice (Eliopoulos et al., 2005), showing that in this model system, allogeneic immune responses could recognize and kill MSC.

4.4.1.9 Immunomodulation of MSCs

MSCs cocultured with purified CD19 positive B cells in the presence of a cocktail of

stimuli significantly inhibited B cell proliferation (Corcione et al., 2006). MSCs also inhibited professional antigen-presenting cells. MSCs cocultured with blood monocytes significantly inhibited the generation of differentiated dendritic cells, DCs, and MSCs cocultured with matured DCs caused a significant decrease in the expression of MHC class II molecules, CD80 and CD86 on DCs (Jiang et al., 2005).

Ex vivo expanded MSCs have been shown to suppress the function of a broad range of immune cells, including T cells, B cells, NK cells and antigen-presenting cells. The mechanisms by which *ex vivo* expanded MSCs mediate immunosuppression have recently been studied.

Soluble factors secreted by MSCs or immune cells in response to MSCs play a major role in MSC-mediated immune suppression. Soluble factors such as hepatocyte growth factor, prostaglandin E2, TGF-β1, indoleamine 2,3-dioxygenase (IDO), nitric oxide and IL-10 have been demonstrated to be involved, while other factors remain unknown (Tse et al., 2003; Djouad et al., 2003; Aggarwal and Pittenger, 2005; Beyth et al., 2005; Sato et al., 2007). Some studies reported that contact-dependent mechanisms might also be implicated, including the expression of B7H1 receptor on MSCs (Augello et al., 2005; Krampera et al., 2003). Recent studies have shown that multiple immunosuppressive factors can be released by MSCs, and the nature of these factors varies depending on the nature of the stimuli received (e.g. allogeneic determinants, membrane-bound proteins, mitogens, and cytokines). In support of this, it has been observed that MSCs use different mechanisms to inhibit lymphocyte proliferation induced either by mitogens or alloantigens: the former, but not the latter, relying on the release of prostaglandins (Rasmusson et al., 2005).

Cytokines play a crucial role in regulating MSC-mediated immunosuppression. Tumor necrosis factor (TNF)-α can enhance the production of immunosuppressive prostaglandins by MSCs by as much as 100-fold (Aggarwal and Pittenger, 2005). IFN-γ is another important cytokine that regulates MSCs' immune functions. Studies have shown that IFN-γ plays an active role in the immunosuppression mediated by MSCs (Chan et al., 2006; Krampera et al., 2006). IFN-γ can induce MSCs to release prostaglandins and IDO (Aggarwal and Pittenger, 2005). IDO causes depletion of tryptophan, which is an essential factor for lymphocyte proliferation. However, it has

also been reported that IFN-γ stimulates upregulation of MHC class I and II molecules on MSCs and induces MSCs to present antigenic peptides to CD4+ T cells (Stagg et al., 2006).

4.4.1.10 Therapeutic application of MSCs

MSCs have high expansion potential, genetic stability and can be easily collected and used. In addition, MSCs have two other extraordinary characteristics; they are able to migrate to sites of tissue injury and have strong immunosuppressive properties that can be exploited for successful autologous as well as heterologous transplantations (Le and Pittenger, 2005). GVDH is a form of rejection, in which transplanted cells begin to attack host tissues and organs. MSCs have been shown to have immunosuppressive properties and to delay skin graft rejection (Bartholomew et al., 2002; Di et al., 2002; Le and Pittenger, 2005). Moreover, MSCs produce cytokines that can support hematopoiesis and potentially enhance marrow recovery following chemotherapy or radiotherapy (Koc and Lazarus, 2001).

Several studies have demonstrated that intracoronary injection of mixed populations of bone marrow stem cells or MSCs could represent a simple and successful approach to the treatment of heart diseases (Chen et al., 2004; Strauer et al., 2002). It has also been suggested that MSCs may be used to treat organ allograft rejection. Indeed, cardiac allograft studies have shown that MSCs home to the site of allograft rejection and participate in allograft tissue repair in the heart by giving rise to scar forming myofibroblasts and cardiomyocytes (Wu et al., 2003; Wu et al., 2005).

MSCs have been shown to possess great somatic plasticity, since they are capable of differentiating into non-mesenchymal lineages. In fact, it has been demonstrated that MSCs are capable of differentiating into neurons and astrocytes *in vitro* and *in vivo* (Jori et al., 2005; Pittenger et al., 1999). Marrow stem cells have been shown to improve neurological performance in rats with brain ischemia. Moreover, MSC transplants delay the onset of neurological abnormalities and extend their lifespan (Zhao et al., 2002).

These studies suggest that MSCs preferentially home to damaged tissue and have

therapeutic potential. Possible clinical implications include therapy-resistant severe acute GVHD, treatment of organ allograft rejection and autoimmune disorders (Le, 2006). MSC transplantation is also a form of cellular therapy with the potential for facilitating tissue repair both in inherited and acquired diseases. MSCs can be manipulated and differentiated into many different mesenchymal and non-mesenchymal lineages to produce functional cells. For example, tissue engineering for tendon, bone and cartilage repair is an exciting and realistic goal. MSC seems to be the superior candidates for cell therapy to regenerate injured skeletal tissues.

In this thesis, we have also shown that MSCs are able to undergo chondrogenic commitment that is of great interest in articular cartilage engineering. In contrast to the ethical, immune response and the risk of teratoma formation of ESCs, MSCs lacks these limitations and are the suitable cells for therapeutic applications.

4.4.2 Bone marrow MSCs

Studies have demonstrated that bone marrow mesenchymal stem cells (BMMSCs) are true postnatal stem cells capable of differentiating into variety of cell types (Bianco et al., 2001). These cells were initially identified by their capacity to form clonogenic adherent cell clusters with fibroblastic morphology (Friedenstein et al., 1966). The maintenance and regulation of normally quiescent stem cell populations is tightly controlled by the local microenvironment according to the requirements of the host tissue. It is therefore critical to identify the locality of stem cells residing in different organs in order to further characterize the properties of stem cells with respect to their capacity for self-renewal and developmental potential (Shen et al., 2004). The recent identification of a putative perivascular niche for BMMSCs is a highlight in mesenchymal stem cell research (Bianco et al., 2001; Shen et al., 2004). BMMSCs can now be efficiently retrieved from bone marrow aspirates using various immunoselection protocols based on their high expression of the STRO-1 antigen (Dennis et al., 2002). STRO-1 appears to be an early marker of different mesenchymal stem cell populations that is also expressed by perivascular cells *in situ*. Currently, it is possible to isolate highly purified BMMSCs from the bone marrow using STRO-1 in combination with an antibody directed toward vascular cell adhesion molecule-1 (VCAM-1/CD106) (Gronthos et al., 2003b) or MUC-18 (CD146) (Shi et al., 2002). It is anticipated that

more sophisticated immunoselection methods, using additional markers, may help to accurately identify and purify multipotential human BMMSCs following *ex vivo* expansion. Isolation of BMMSCs was described in section 7.1.3 of this thesis.

Although BMMSCs are capable of multidifferentiation, it appears that they are most readily encouraged to develop towards an osteogenic pathway. When cultured in the presence of mineral-forming inductive medium, BMSSCs show the capacity to form structurally distinctive mineralized deposits in comparison with other mineral-matrix-forming postnatal stem cells, such as dental pulp stem cells (Gronthos et al., 2003a; Shi et al., 2002). The processes by which stem cells settle, differentiate and form tissue in adults do not accurately recapitulate what happens during development, where the environment into which they are introduced may not be as collaborative as that in normal development. One of the most striking characteristics of the xenogeneic transplantation system is that BMMSCs can support and organize hematopoietic marrow during the osteogenic process. This may represent a dynamic process whereby multipotential BMMSCs attempt to reconstruct the microenvironment from which they were derived (Kuznetsov et al., 1997b). Although the detailed mechanisms involved in the initiation and maintenance of the bone marrow organ have yet to be determined, recent studies provide evidence suggesting that basic fibroblast growth factor (bFGF) and matrix metalloproteinase 9 (MMP9) may contribute to BMMSC-mediated osteogenesis and BMMSC-organized hematopoietic marrow formation *in vivo* (Batouli et al., 2003).

4.4.2.1 The role of MSCs in developmental processes in bone marrow

MSCs present in BM are thought to give rise to cells that constitute the hematopoietic microenvironment (Prockop, 1997). MSCs have been isolated from BM and various tissues from humans and many other species, expanded in culture, and shown to differentiate into many lineages under defined conditions *in vitro*. In culture, MSCs produce a number of cytokines and ECM proteins and express cell adhesion molecules, all of which are involved in the regulation of hematopoiesis (Conget and Minguell, 1999; Majumdar et al., 1998). They also support the development of hematopoietic colonies *in vitro* (Majumdar et al., 1998).

To assess the engraftment, spatial distribution and lineage commitment of MSCs as well as their roles in hematopoiesis *in vivo*, it has been shown that transplanted MSCs can integrate into the functional components of the hematopoietic microenvironment and that these MSC-derived cells appear to be actively involved in the maintenance of human hematopoiesis in mice (Muguruma et al., 2006).

Inside the BM niche environment, interactions between BMMSCs and HSCs may determine the homeostasis of the BM environment. A recent study has demonstrated that subcutaneously transplanted human BMMSCs, using hydroxyapatite tricalcium phosphate (HA/TCP) as a carrier vehicle, are capable of organizing hematopoetic progenitors of recipient origin. This study has demonstrated that multiple lineages of hematopoetic cells, including myeloid, lymphoid, erythroid and hematopoetic progenitor cells in BMMSCs transplants were generated. Interestingly, mice receiving lethal total body irradiation (TBI) could be rescued by systemic transplantation of BM cells isolated from the BMMSCs transplants. BM cells isolated from BMMSCs transplants contain functional hematopoetic progenitor cells to engraft and support hematopoiesis in recipients (Miura et al., 2006). These results indicate that the BMMSC-organized BM niche system represents a unique hematopoetic progenitor resource.

4.4.3 Adipose tissue–derived adult stromal stem cells

MSCs can usually be isolated from several organs, such as fetal liver, umbilical cord blood and bone marrow (Wagner et al., 2005; Kern et al., 2006); and paper II). Adipose tissue is a highly complex tissue and consists of mature adipocytes, preadipocytes, fibroblasts, vascular smooth muscle, endothelial cells, resident monocytes/macrophages and lymphocytes (Weisberg et al., 2003; Caspar-Bauguil et al., 2005; Xu et al., 2003). The stromal-vascular fraction (SVF) of the adipose tissue that is obtained immediately after collagenase digestion has become a great focus of stem cell research because it provides a rich source of multipotent adipose tissue-derived adult stromal/stem cells (ADASCs) (Katz et al., 2005; Zuk et al., 2002; and paper I). Comparative analysis of MSCs obtained from bone marrow, adipose tissue, and umbilical cord clearly show that ADASCs are not different with regard to morphology, immune phenotype, success of isolating MSC, colony forming and differentiation capacity (Izadpanah et al., 2006; Kern et al., 2006).

4.4.3.1 Isolation and characterization of ADASCs

Liposuction is one of the most popular cosmetic surgical procedures, yielding a large volume (up to 2 L) of adipose tissue. Adipocytes represent roughly two-thirds of the total cell number and more than 90% of the tissue volume (van, V et al., 2005) and the ratio of adipocytes to ADASCs is constant in humans, independent of body mass index (BMI) and age (van, V et al., 2003). ADASCs yield varies among patients and is affected by many factors including donor site and storage duration. It also depends on isolation methods such as duration of collagenase digestion (Aust et al., 2004; Bakker et al., 2004; von et al., 2004). Fibroblast-like ADASCs are morphologically similar to MSCs obtained from other tissues. Moreover, ADASCs have the capacity to differentiate into cells of mesenchymal origin, such as adipocytes, myocytes, chondrocytes, and osteoblasts (Kern et al., 2006; Zuk et al., 2002; Yamada et al., 2007) and paper I. Factors such as donor age, type (white or brown adipose tissue) and localization, type of surgical procedure, culture conditions, plating density and media formulation can influence both the proliferation rate and differentiation capacity of ADASCs. A comparative study has shown that ADASCs have the same cell surface marker expression and differential potential as described for BM-MSCs. Similarity between ADASC and BM-MSC were further supported by gene expression. Gene array analysis revealed that less than 1% of genes were differentially expressed. However, some characteristics such as colony frequency and maintenance of proliferating ability in culture seem to be superior in ADASC compared with BM-MSC (Lee et al., 2004). Selected populations with a CD45-CD34+CD105+CD31- phenotype from human SVF have been kept in continuous culture without loss in proliferation capacity for more than eight months (paper I), which is much longer than BM-MSC can be maintained in culture (Paper II). Human ADASCs express a functional autocrine fibroblast growth factor (FGF) loop that allows for maintenance of their self-renewal ability *in vitro* (Zaragosi et al., 2006).

Phenotypic analysis of human MSCs derived from adipose tissue, bone marrow and umbilical cord has revealed no phenotypic differences among the three stem cell groups when using a panel of 22 surface antigens. When cultured in the appropriate induction media, MSCs were confirmed to exhibit osteogenic and adipogenic differentiation capability unlike control fibroblasts (Wagner et al., 2005). Global gene expression analysis of these MSCs has shown 25 genes including fibronectin, *ECM2, glypican-4,*

IDI, NFIB, HOXA5 and *HOXB6* that were overlapping and upregulated in the MSC preparations. In contrast, several hundred expressed sequence tags were identified to be differentially expressed when comparing adipose-derived MSCs with bone marrow and umbilical cord MSCs (Wagner et al., 2005). In another comparative study, 24 genes were upregulated in adipose tissue-derived MSCs compared with BM-MSCs, and they describe the differential expression profile of eight surface marker proteins in these cells. According to data from this study, less than 1% of genes are estimated to be differentially expressed between ADASC and BM-MSC (Lee et al., 2004). The published data in the literature support the hypothesis that ADASC and BM-MSC originated from identical precursor cells (Lee et al., 2004).

In the end, ADASCs possess a similar phenotype and gene expression profile to bone marrow stem cells (Zuk et al., 2002; Lee et al., 2004; Wagner et al., 2005). In addition to having the capacity for self-renewal and long-term growth, ADASCs are capable of differentiating into diverse cell types including adipocytes (Rodriguez et al., 2004; Zuk et al., 2002), osteoblasts (Zuk et al., 2002), chondrocytes (Huang et al., 2004; Zuk et al., 2002; Betre et al., 2006) hepatocytes (Seo et al., 2005; Talens-Visconti et al., 2006; Talens-Visconti et al., 2007), myocytes (Rodriguez et al., 2005), cardiomyocytes (Yamada et al., 2007), neurons (Guilak et al., 2006; Zuk et al., 2002), endothelial cells (Cao et al., 2005) and epithelial cells (Brzoska et al., 2005). Thus, ADASC are not only increasingly accepted as real adult stem cells but are also considered to be superior to other types of stem cells for future clinical applications (Strem et al., 2005). Whereas bone marrow can only be obtained in limited quantity because of donor site morbidity, adipose tissue is easily obtained in abundance.

4.5 Epigenetic modifications of gene expression

Just as cells inherit genes, they also inherit a set of instructions that tells the genes when to become active, in which tissue and to what extent. Without this “epigenetic” instruction manual, existence of multicellular organisms would be impossible (Jenuwein and Allis, 2001). Epigenetic is defined as any heritable influence in the progeny of cells or individuals, on chromosome or gene function that is not accompanied by a change in DNA sequence.

The cell nucleus is the most prominent compartment in the eukaryotic cell. In this compartment, many essential cellular activities take place, such as genome replication, control of gene expression and transcription, processing of transcripts and DNA repair. The cell nucleus is also an extremely dynamic structure where many components tend to rapidly and transiently interact with each other, giving rise to a highly ordered compartment (Misteli, 2005). The double strand of DNA, a major component of chromatin, and therefore of the nuclear compartment, can undergo chemical modification in the form of cytosine methylation of cytosine-guanine dinucleotides (CpG). This biochemical modification does not alter the subjacent genetic information of the DNA molecule, and is considered to be an epigenetic mark in the genome (Fuks, 2005).

Histones, which are small and highly conserved basic proteins, are found in the chromatin of all eukaryotic cells. Different types of core histones H2A, H2B, H3 and H4 are associated with DNA to form a nucleosome. Nucleosomes are the basic components of a chromosome, in which the DNA helix is wrapped around core histones. A nucleosome contains two copies of each of the core histones wrapped with a 146-bp DNA to form the simple 'beads on a string' structure that is then folded into higher-order chromatin. Chromatin also contains various proteins that are required for its assembly and packaging as well as for DNA replication, DNA modification, histone modification, transcription, DNA repair and DNA recombination (Narlikar et al., 2002; Zhang and Reinberg, 2001).

Chromatin is not uniform with respect to gene distribution and transcriptional activity. It is organized into domains, such as euchromatin and heterochromatin. Euchromatins are the lightly staining regions of the nucleus that generally contain decondensed, transcriptionally active regions of the genome. Heterochromatins are cytologically defined genomic components that contain repetitive DNA and some protein-coding genes that have different chromosomal architecture, transcriptional activity and replication timing.
Histone modification seems to be a universal regulatory mechanism among eukaryotic organisms from yeast to human. DNA methylation, however, is less conserved, but is a common and rapidly evolving mechanism among higher eukaryotic organisms with more complex genomes. The principal of epigenetic mechanisms by which tissue-

specific gene-expression patterns and global gene silencing are established and maintained are chromatin modification and chromatin remodeling. Chromatin modification includes processes such as DNA methylation and histone modification, acetylation, phosphorylation, methylation and ubiquitylation. Chromatin remodeling includes processes with transient changes in chromatin accessibility. (Bird, 2002; Jenuwein and Allis, 2001; Narlikar et al., 2002; Zhang and Reinberg, 2001).

4.5.1 DNA methylation and histone modification

DNA methylation, chromatin structure and gene silencing are interconnected in mammals. High levels of CpG methylation coincide with heterochromatic regions and, upon integration into the genome, *in vitro* methylated DNA was shown to associate with a repressed chromatin structure. Unmethylated CpG-island chromatin is enriched in hyperacetylated histones (Bird and Wolffe, 1999). The mechanisms underlying these observation and discoveries have suggested the `histone code` hypothesis. According to this hypothesis, histone modifications, acting alone or in specific combinations, provide binding platforms for chromatin-associated proteins that initiate or block gene transcription (Jenuwein and Allis, 2001).

In mammalian cells, DNA methylation occurs predominantly at CpG and is catalyzed by important DNA methyltransferases (Dnmt1, Dnmt3a and Dnmt3b). DNA methylation regulates gene expression through several distinct mechanisms and can directly block transcriptional regulatory factors from binding to their target sequences, although regulation by such a mechanism *in vivo* is relatively rare. DNA methylation can also repress gene expression through several methyl-CpG-binding proteins (MECPs) that 'read' DNA-methylation patterns (Jenuwein and Allis, 2001; Bird, 2002; Bird and Wolffe, 1999).

The modification of core histones at the lysine (K), arginine and serine residues that lie in their amino-terminal tails is far more complex, involving many histone-modification enzymes. For example, Lys9 (K9), 14, 18 and 23 of H3 and K5, 8, 12 and 16 of H4, together with lysines on H2A and H2B, can be acetylated; whereas K4, 9 and 27, Arg2, 17 and 26 of H3, and K20 and Arg3 of H4 can be methylated. Histone acetylation and deacetylation have been shown to determine the transcriptional activity of chromatin

(Strahl and Allis, 2000).

MECP2 is one member of a family of methyl-binding domain proteins with affinity for methylated CpG dinucleotides. Recent studies have suggested diverse roles for MECP2 in chromatin dynamics. MECP2 associates with both histone deacetylase (HDAC) activity and histone methyltransferase activity that directs the silencing mark for histone H3, K9 (H3K9). Acetylation of H3K9 (H3K9ac) is a mark of active chromatin, while a reciprocal pattern of trimethylation of the same residue (H3K9m3) often correlates with repression (Jenuwein and Allis, 2001; Thatcher and Lasalle, 2006).

A variety of modifications on the amino-tails of histones have been characterized, of which methylation of lysine residues has been thought to be particularly stable (Jenuwein and Allis, 2001). The consequence of lysine methylation can differ both according to which lysine residue is modified and also as to how many methyl groups are added where lysine residues can be mono-, di- or trimethylated. For example, methylation of histone H3 at K9 (H3K9) or K27 (H3K27) is generally correlated with transcriptional repression, whereas methylation at K4 (H3K4) is predominantly associated with transcriptional activity. In addition, the level of methylation is important, for example H3K9m3 shows a different distribution from H3K9m1 and H3K9m2 in mammals (Schubert et al., 2006).

As a summary of the epigenetic terms used in this thesis, the transcription status of a gene is affected by modifications of histones H3 and H4. Trimethylation of K9 of H3 (H3K9m3), H3K27m1, and H4K20m3 has been shown to be part of constitutive heterochromatin and associated with long-term gene repression. Facultative heterochromatin, associated with loci that are temporarily inactive, is enriched in H3K9m2, H3K27m3 and H4K20m1. Both forms of transcriptional inactivation are also associated with H3K9 deacetylation. In contrast, di- or trimethylated H3K4, H3K36m3 and H3K79m3, together with acetylated H3K9 (H3K9ac), mark a gene for transcription (Lachner and Jenuwein, 2002). Chromatin immunoprecipitation (ChIP) is a key technique used in paper III of this thesis for studying protein-DNA interactions and mapping epigenetic histone modifications on DNA (Dahl and Collas, 2007).

4.6 Articular cartilage

The fields of tissue engineering and regenerative medicine have made enormous strides over the last decade. The final goal of tissue engineering should be functional recovery of damaged tissue *in vivo*. The development of cell-based treatment for cartilage repair requires a cell source that can efficiently produce and assemble a cartilage matrix. Chondral defects lack the capacity to regenerate new cartilage surfaces due to lack of vessels, nerve supply and isolation from systemic regulation. With full thickness articular injury, a healing response is initiated. This response usually produces collagen type I and generation of fibrous cartilage rather than the preferred hyaline. This, consequently, leads to a tendency for the development of osteoarthritis (Mandelbaum et al., 1998).

Autologous chondrocyte implantation (ACI) using chondrocytes from patients was first described in 1994 (Brittberg et al., 1994) with encouraging results. A part of this thesis has focused on improvement of human articular chondrocyte (AC) expansion *in vitro* for cartilage tissue repair. Some characteristics of cartilage and chondrocyte biology that are essential for understanding repair of damaged tissue are discussed in this section.

4.6.1 Joint

Joints are structures in the body that provide movement and mechanical support. There are several types of joints. The focus of this part of my thesis is on synovial joints, such as those in the knees and shoulders. These joints, found at the ends of bones, have a space that allows for a wide range of motion. Joints are strengthened by a dense fibrous capsule reinforced with ligaments and muscles. The joint space is filled with synovial fluid, a lubricant that also provides nutrients to the joint tissues. The bony surfaces within synovial joints are covered with articular cartilage (Clark, 2007).

4.6.2 Structure and functional properties of articular cartilage

Articular cartilage consists of four layers of tissue. First, a thin superficial layer

provides a smooth surface for two bones to slide against each other. The second layer is very resistant to shear stresses. An intermediate layer is mechanically designed to absorb shock and distribute load or weight efficiently. The fourth or deepest layer is highly calcified and anchors the articular cartilage to the bone (Clark, 2007).

The most important function of articular cartilage is its ability to support large loads during motion. This functionality has been attributed to its highly organized ECM. Chondrocytes, the cell type found in articular cartilage, are responsible for the production, organization, and maintenance of the articular cartilage ECM, and are, therefore, ultimately responsible for the integrity of the cartilage (Chen et al., 2006).

Articular cartilage ECM contains a fluid phase of water (68-85% of total weight) and a solid, organic phase of ECM proteins. Interstitial fluid support can account for more than 90% of the load-bearing capacity of the joint. The articular cartilage ECM comprises three classes of proteins: collagen type II (60-86% of dry weight), proteoglycans (15-40% of dry weight) and quantitatively minor components, such as collagen type IX, XI, V and other noncollagenous proteins including link protein, fibronectin and cartilage oligomeric matrix protein. In addition it also contains smaller proteoglycans, biglycan, decorin and fibromodulin, which also have important roles in controlling matrix structure and organization (Figure 5).

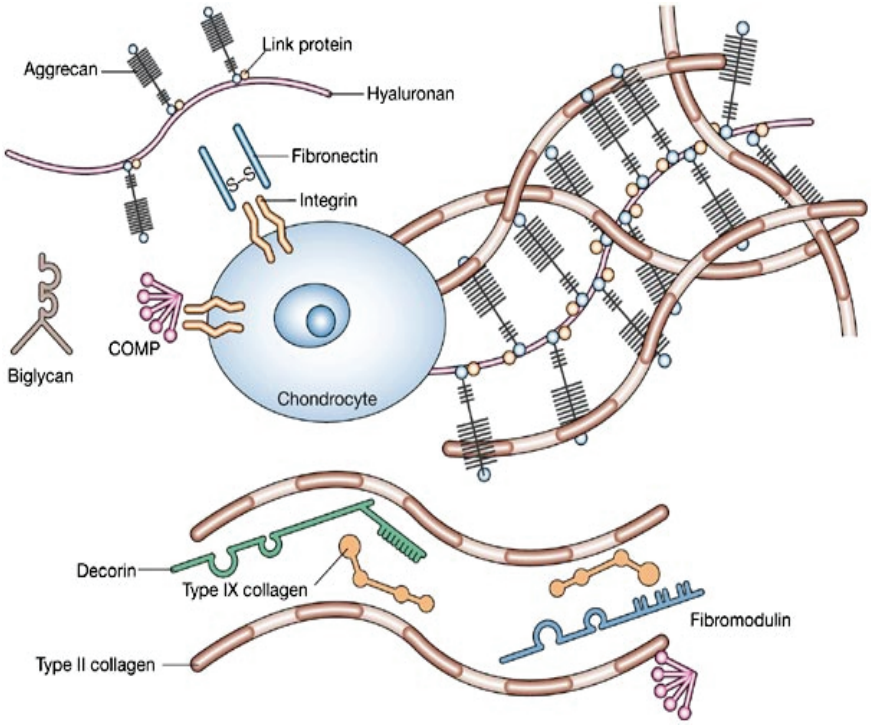


Figure 5. Extracellular matrix of cartilage.

Three classes of proteins exist in articular cartilage: collagens (mostly type II collagen); proteoglycans (primarily aggrecan); and other noncollagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein) and the smaller proteoglycans (biglycan, decorin and fibromodulin). The interaction between highly negatively charged cartilage proteoglycans and type II collagen fibrils is responsible for the compressive and tensile strength of the tissue, which resists load in vivo. Abbreviation: COMP, cartilage oligomeric matrix protein (from Chen FH. and et. al. 2006, with permission of Nature Publishing Group).

The ability of articular cartilage to resist compression is primarily a result of the presence of large proteoglycan aggregates, termed aggracan. Aggrecan monomers consist of a core of protein and keratin sulfate glycosaminoglycan (GAG) chains, filling

the interstices of the collagen meshwork by forming large aggregated complexes interacting with hyaluronic acid (HA) and link proteins (Nagase and Kashiwagi, 2003). HA is a GAG with repeated disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine connected by β-linkages (Tanimoto et al., 2004), playing an essential role in the structural organization and function of cartilage, cell adhesion, migration and differentiation mediated by HA-binding proteins and cell surface receptors such as CD44 (Knudson and Knudson, 1993; Laurent and Fraser, 1992). The high density of fixed negative charges of the sulfated GAG chains of proteoglycans draws water into the cartilage, resulting in high osmotic pressure, which is restrained by the collagen network, thus giving rise to the compressive behavior of cartilage. The tensile resilience and strength of cartilage, on the other hand, is imparted primarily by the network of type II collagen fibers. Overall, the interaction between collagen type II, aggrecan, link protein and HA provides cartilage with its ability to resist compressive loads. The biomechanical properties of articular cartilage thus depend largely on the maintenance of high proteoglycan and collagen contents within the matrix, which represent the current standard for measuring the functionality of engineered cartilage constructs (Chen et al., 2006).

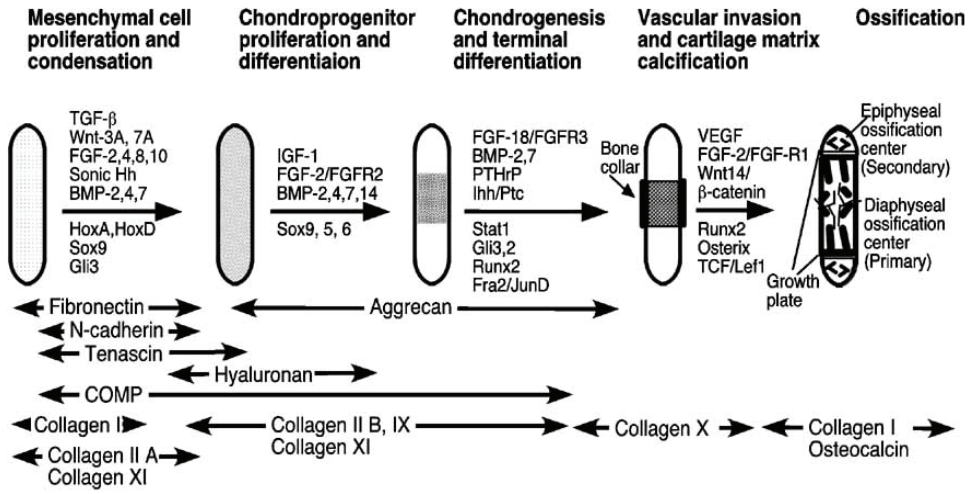
4.6.3 Chondrogenesis

Chondrogenesis is a process that results in the formation of the cartilage intermediate (anlagen) and leads to endochondral ossification during skeletal development, which is essential for proper formation of endochondral bones. The vertebrate skeleton, composed of cartilage and bone, is the product of three distinct embryonic lineages. The craniofacial skeleton is formed by cranial neural crest cells, the axial skeleton is derived from paraxial mesoderm (somites) and the limb skeleton is the product of lateral plate mesodermal cells. Cells in these lineages migrate to the locations in the embryo where skeletal elements will develop, form characteristic mesenchymal condensation of high cell density and differentiate to osteoblasts or chondrocytes (Olsen et al., 2000).

In the human embryo, the appendicular skeleton develops from limb buds that are first visible at around 4 weeks of gestation. Subsequent stages of human limb development are divided into formation of the cartilaginous anlagen that shapes skeletal elements and

subsequently results in joint formation. The joint develops from the primitive avascular, densely packed cellular mesenchyme termed the skeletal blastema. The skeletal elements are prefigured in mesenchymal condensations, and common precursor mesenchymal cells divide into both chondrogenic and myogenic lineages that determine the central differentiation of cartilage and peripheral differentiation of muscle. The surrounding tissues, particularly the epithelium, influence the differentiation of mesenchymal progenitor cells to chondrocytes. Cartilaginous nodules appear in the middle of the blastema and, simultaneously, cells at the periphery become flattened and elongated to form the perichondrium. The differentiated chondrocytes can then proliferate and the complex process of hypertrophic maturation occurs (Goldring et al., 2006; Pacifici et al., 2006). The different stages of chondrogenesis are briefly described below.

(A)



(B)

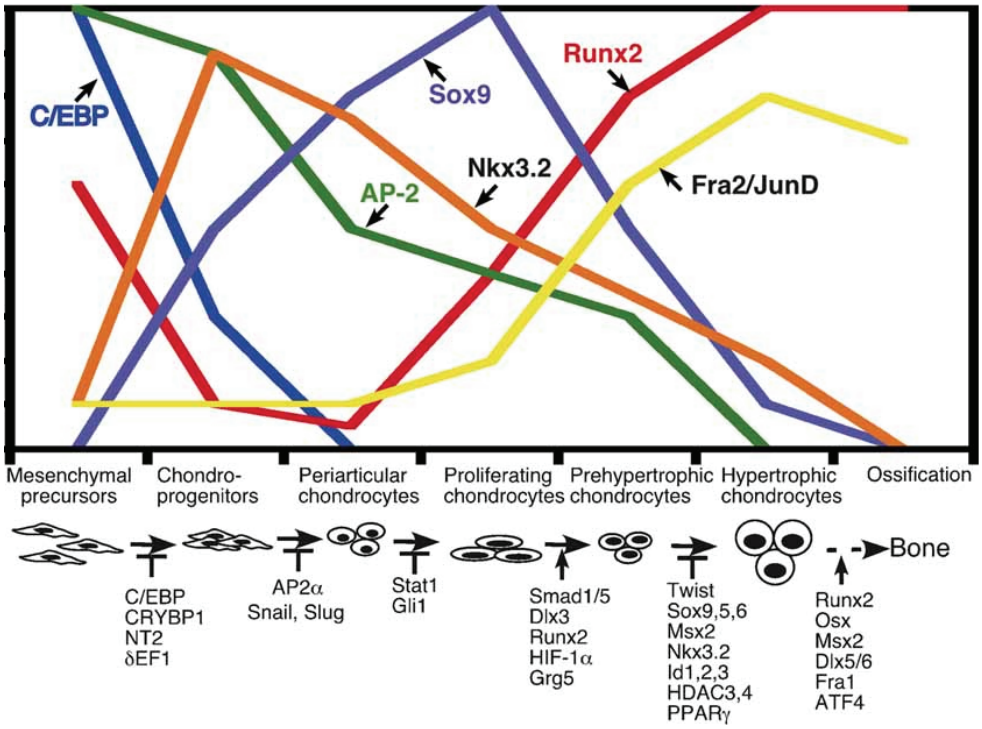


Figure 6. Sequence of events of chondrogenesis until the development of long bones. *__A__: The different stages are represented schematically, showing the temporal patterns of growth and differentiation factors (above the arrows) and the transcription factors involved below the arrows. The extracellular matrix proteins that distinguish the different stages are indicated below. __B__: Schematic representation of the expression of transcriptional regulators at different stages of chondrogenesis and endochondral ossification. Additional transcription factors that are inhibitors or activators at different stages are indicated below the scheme of cellular transitions (from Goldring MB. and et. al. 2006, with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.).*

4.6.3.1 Mesenchymal cell condensation

Chondrogenesis is the earliest step of skeletal development. It is initiated by the migration of mesenchymal cells to the site of future skeletogenesis, followed by

proliferation. The next step is the tissue (epithelial-mesenchymal) interaction that results in condensation and differentiation to chondroblasts or osteoblasts. Each phase involves different cellular processes and separate gene expression patterns (Figure 4A) (Hall and Miyake, 2000). Prior to condensation, the prechondrocytic mesenchymal cells produce ECM that is rich in hyaluronan and collagen type I, as well as collagen type IIA containing the exon 2 encoded amino acid propeptide found in neocartilage collagens (McAlinden et al., 2005). The initiation of condensation is associated with increased hyaluronidase activity and the appearance of the cell adhesion molecules neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM). TGF-β, which is among the earliest signals in chondrogenic condensation, stimulates the synthesis of fibronectin, which in turn regulates N-CAM. Syndecan binds to fibronectin and downregulates N-CAM, thereby setting the condensation boundaries. The ECM molecules, which also include tenascins and thrombospondins, as well as cartilage oligomeric protein (COMP), interact with the cell adhesion molecules to activate intracellular signaling pathways involving focal adhesion kinase and paxillin, to initiate the transition from chondroprogenitor cells to a fully committed chondrocyte (DeLise et al., 2000). N-cadherin and N-CAM disappear in differentiating chondrocytes and are later detectable only in perichondrial cells (Goldring et al., 2006).

4.6.3.2 Chondrocyte differentiation

Investigations of cartilage development explain the differentiation of chondroprogenitors for subsequent stages of skeletogenesis. The nuclear transcription factor Sox9, one of the earliest markers expressed in cells undergoing condensation (Figure 5A and 5B), is required for the expression of the type II collagen gene (*COL2A1*) and certain other cartilage-specific matrix proteins, including collagen XIA2 (Col11a2) and cartilage-derived retinoic acid-sensitive protein (CD-RAP) genes, prior to matrix deposition in the cartilage anlagen, reviewed in (Goldring et al., 2006). Two additional Sox family members, L-Sox5, a long form of Sox 5, and Sox6 are co-expressed with Sox9 during chondrocyte differentiation (Lefebvre et al., 2001). L-Sox5 and Sox6 are required for the expression of Col9a1, aggrecan and link protein, as well as Col2a1, during chondrocyte differentiation (Smits et al., 2001) (Figure 5A). The effect of BMP signaling on chondrogenic differentiation is mediated through Sox9, L-

Sox5 and Sox6 regulation via their receptors, BMPR1A (ALK-3) and 1B (ALK-6) (Yoon et al., 2005). The runt-domain transcription factor, Runx2 (also known as core binding factor, Cbfa1 and Osf2), is expressed during the condensation stage of chondrogenesis, chondrocyte maturation and osteoblast differentiation. Sox9 and Runx2 are coexpressed during mesenchymal condensation and dominance of Sox9 function over Runx2 during this early first step in progenitor cell fate determines between osteoblastic and chondrogenic lineages (Zhou et al., 2006) (Figure 4A and 4B).

4.6.3.3 Chondrocyte proliferation

Chondrocytes mature towards two different fates after commitment to the chondrogenic lineage; they remain as chondrocytes (persistent cartilage) or differentiate to hypertrophic chondrocytes (transient cartilage). The chondrocytes that stop differentiating form the persistent cartilage located on the articular surfaces of joints. These chondrocytes do not differentiate further or mineralize their ECM. Chondrocytes that continue to differentiate to the hypertrophic state proliferate and contribute to the formation of the growth plate, reviewed in (Woods et al., 2007).

Hypertrophic differentiation starts from the center of the cartilage element where chondrocytes undergo a multistep program, differentiating into prehypertrophic, hypertrophic and, finally, terminal hypertrophic chondrocytes. The region of terminally differentiated chondrocytes is subsequently invaded by blood vessels followed by osteoclasts and osteoblasts, which start to replace cartilage with bone and bone marrow. The interactions of fibroblast growth factor (FGF), Indian hedgehog/parathyroid hormone-like peptide (Ihh/Pthlh) and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation (Minina et al., 2002) (Firures 5A and 5B). Throughout chondrogenesis, the balance of signaling by BMPs and FGFs determines the rate of proliferation, thereby adjusting the rate of differentiation. Whereas FGF signaling accelerates the rate of terminal hypertrophic differentiation, BMPs have been shown to hinder this process. The proliferation of chondrocytes in the lower proliferative and prehypertrophic zones is also under the control of a local negative feedback loop involving signaling by Pthlh and Ihh. By simultaneously regulating proliferation, Ihh expression and the rate of terminal hypertrophic differentiation, the

balance of FGF and BMP signals seems to adjust the process of hypertrophic differentiation to the proliferation rate (Minina et al., 2002).

4.6.3.4 Growth plate and chondrocyte hypertrophy

Longitudinal bone growth occurs at the growth plate, a thin cartilage structure situated at the end of tubular bones, by a process called endochondral ossification, in which cartilage is formed and then remodeled into bone tissue. The growth plate consists of three principal layers: the resting zone close to the epiphysis, the proliferative zone in the middle and the hypertrophic zone close to the metaphysis. The newly formed cartilage is invaded by blood vessels and bone cell precursors, which remodel the hypertrophic zone cartilage into bone.

The growth plate contains one cell type, the chondrocyte, at different stages of differentiation. Resting zone chondrocytes replicate at a slow rate (Kember, 1971) and act as stem-like cells that refill the pool of proliferative chondrocytes (Abad et al., 2002). Proliferative zone chondrocytes replicate at a high rate (Kember, 1971), and the resulting daughter cells line up along the long axis of the bone. As a result, chondrocytes are arranged in columns parallel to this axis, a process critical for the formation of bones with an elongated shape (Abad et al., 2002). At a certain point, the cells stop dividing and terminally differentiate into hypertrophic chondrocytes. Hypertrophic chondrocytes undergo apoptosis shortly before vessels invade the chondrocyte lacuna (Gerber et al., 1999; Goldring et al., 2006). Chondrogenesis resulting in cartilage formation is regulated by numerous complex interactions. Understanding these regulative processes is essential for cartilage tissue engineering.

4.6.4 Chondrocyte response to mechanical loading and degeneration of cartilage

The unique biomechanical properties of healthy cartilage ensure that articular cartilage is able to transmit force between the joints while maintaining almost friction-free limb movement. Human articular cartilage experiences a wide range of stress during normal joint loading. Stresses in the range of 300-800% of body weight are normal in the hip,

but above this physiological range compression of cartilage causes deformation of cells and matrix, gradients in hydrostatic pressure, intratissue fluid flow and associated electrokinetic effects (e.g., flow-induced streaming potential). The deformation of the charged ECM will change the ionic concentration, osmolarity and pH of the cellular environment of the cartilage. Tissue fluid flow during loading can also dramatically enhance transport of nutrients and macromolecules such as growth factors and cytokines. Therefore, mechanical and chemical changes during loading can alter chondrocyte behavior and, as a result, matrix synthesis and turnover (Kerin et al., 2002).

4.6.5 Repair of articular cartilage

Recent and more advanced understanding of how the articular cartilage was formed during development and how the adult articular chondrocytes function within their unique environment will enable us to control further development of cartilage diseases at early stages or plan repair strategies for damaged cartilage. Lesions are generated during the course of many joint diseases, especially during osteoarthritis (OA), a number of genetic or metabolic conditions and/or as a result of trauma. Traumatic lesions may occur directly or indirectly as a result of the strength of mechanical loading or following ligament injuries (Newman, 1998). The capacity of adult articular chondrocytes to repair lesions and regenerate the normal cartilage matrix is limited and the damage becomes irreversible unless the destructive process is interrupted (Goldring, 2006).

Numerous experimental and clinical attempts have been made to repair articular cartilage lesions or at least to bring symptomatic relief. In fact, relief of symptoms and improvement in functionality are the main reasons for performing these procedures. These techniques can be divided into four categories: (1) symptomatic treatment: lavage, shaving and debridement; (2) stimulation of bone marrow derived cells: drilling, microfracture, abrasion and deep abrasion (spongiolization); (3) transplantation of osteochondral plugs: allografting and autografting (mosaicplasty), and (4) chondrogenesis within transplanted cells/tissue: periosteal grafting, autologous chondrocyte implantation (ACI). Of these techniques, only the strategy involving transplantation of cells will be further described in this thesis.

4.6.5.1 Autologous chondrocyte implantation (ACI)

ACI falls within the group of therapies that aim to induce chondrogenesis within transplanted cells or tissues. The ability to harvest a sufficient number of autologous primary chondrocytes is the first step. Material is derived from the patient's own cartilage, from non-load bearing areas of healthy regions of hyaline cartilage. The cartilage biopsies are around a few hundred milligrams in weight. Cell isolation procedures rely on enzymatic digestion of the cartilage tissue, followed by *in vitro* expansion of the chondrocytes in adherent, monolayer cell cultures. In the second step, the chondral lesion is surgically prepared and covered with a periosteal flap, which is sutured to the surrounding cartilage tissue. The cultured chondrocytes, now in single cell suspension, are then injected beneath the flap, which is peripherally sealed with biological fibrin glue (Brittberg et al., 1994).

The first study describing repair of human osteochondral defects by transplantation of cultured chondrocytes was reported in 1994 (Brittberg et al., 1994). Since then, several studies using autologous chondrocyte implantation (ACI) have described good or excellent results for the majority of patients (Peterson et al., 2003; Recht et al., 2001). Although ACI is a safe technique, other reports are not as positive and their available data are not supportive of its being more effective than other therapeutic strategies in the treatment of chondral lesions of the knee (Jakobsen et al., 2005; Hunziker, 2002; Giannoni and Cancedda, 2006; Hambly et al., 2006; Ruano-Ravina and Jato, 2006).

However, ACI is a procedure which may be further improved using various tissue engineering techniques. Obviously, transfer of a chondrocyte from its 3D native environment to a 2D monolayer proliferative condition results in a generally diminished chondrogenic potential of the expanded cells. Adhesion to a two-dimensional surface seems to be a prerequisite for chondrocyte proliferation. In the course of adhesion and proliferation, the cells alter their morphology and metabolism in a process known as dedifferentiation (Schnabel et al., 2002). The gene expression profile of the cells is profoundly altered, and the metabolic activity of the dedifferentiated chondrocytes is directed mainly towards proliferation. Endogenous production of collagen type II and aggrecan is significantly reduced and predominately replaced by collagen type I and versican (az-Romero et al., 2005; Fuss et al., 2000; Stewart et al., 2000). The tissue repair resulting from transplantation of dedifferentiated chondrocytes is often

fibrocartilaginous (Darling and Athanasiou, 2005a; Tins et al., 2005). These topics will be discussed further in paper III of this thesis.

5. AIM OF THE STUDY

The overall purpose of this study was to establish optimal expansion protocols for cells of potential use in regenerative medicine, and to use different strategies to carefully characterize these cells.

The specific aims of the present study were:

1) To isolate sufficient human stromal stem cells from adipose tissue (ADASC) and purify and characterize uncultured and cultured expanded cells to investigate their phenotype and global gene expression.

2) To investigate if autologous serum (AS) or allogeneic human serum (alloHS) could replace FBS for the expansion of hMSCs *in vitro*.

3) To determine if human articular chondrocytes (AC) may be expanded *in vitro* to therapeutically useful numbers without losing collagen type II synthesis and sercretion.

6. SUMMARY OF THE PAPERS

Paper I:

Isolation and transcription profiling of purified uncultured human stromal stem cells: Alteration of gene expression after *in vitro* cell culture.

Freshly isolated stromal stem cells have been too few or insufficiently pure to be thoroughly characterized as uncultured progenitors. Most of our current knowledge about these cells is based on their expansion *in vitro*. In this study, we showed that freshly isolated cells from human adipose tissue with $CD45^{-}CD34^{+}CD105^{+}CD31^{-}$ phenotype are the precursors from which multilineage cells are derived. These precursors, compared with another isolated population having a $CD31^{+}$ phenotype, overexpressed transcripts associated with cell cycle quiescence and stemness, and transcripts involved in the biology of cartilage, bone, fat, muscle and neural tissues. In contrast, $CD31^{+}$ cells overexpressed transcripts associated with endothelium and major histocompatibility complex class II.

$CD31^{+}$ cells did not proliferate *in vitro*, but $CD31^{-}$ cells proliferated and could be differentiated to cells with characteristics of bone, fat, cartilage and neural-like tissue. Upon culture, transcripts associated with cell cycle quiescence, stemness, certain cytokines and organ specific genes were downregulated, whereas transcripts associated with signal transduction, cell adhesion, and cytoskeletal components were upregulated. Thus, the $CD45^{-}CD34^{+}CD105^{+}CD31^{-}$ population from adipose tissue contains cells with great potential for therapeutic tissue regeneration.

Paper II:

***In Vitro* expansion of human mesenchymal stem cells: Choice of serum is a determinant of cell proliferation, differentiation, gene expression and transcriptome stability.**

In this study, we have investigated the possibility of using autologous serum (AS) or allogeneic human serum (alloHS) rather than FBS for *in vitro* expansion of (hMSCs) for cell therapy and tissue engineering purposes. We found that the choice of serum affected hMSCs at several different levels. hMSCs expanded in AS proliferated faster but differentiated more slowly toward mesenchymal lineages than hMSCs expanded in

FBS. Changes in gene expression reflected these differences. The use of alloHS resulted in hMSC growth arrest and death. Genome-wide microarray analysis identified transcripts involved in the cell cycle and differentiation that was differentially regulated between hMSCs in FBS and AS. Finally, several transcripts, including some involved in cell cycle inhibition, were upregulated in hMSCs in FBS at late passages, while the hMSC transcriptome in AS was remarkably stable. It seems that FBS induces a more differentiated and less stable transcriptional profile, whereas hMSCs may be expanded rapidly with stable gene expression in AS in the absence of growth factors. This insight may be useful for protocols where hMSC are to be used for cellular therapy.

Paper III:

Persistent collagen type II synthesis and secretion in rapidly proliferating human articular chondrocytes *in vitro*.

In this study, we investigated whether human articular chondrocytes (hAC) may be expanded *in vitro* to therapeutically useful numbers without losing collagen type II synthesis and secretion. hAC were expanded *in vitro* adherent to plastic or supported by their own ECM in loose structures called chondrocytes in autologous ECM (CA-ECM). Real-time PCR, flow cytometry and electron microscopic immunogold techniques were used to determine the presence of key molecules of hyaline cartilage ECM. Promoter methylation and histone modification studies were performed to assess epigenetic mechanisms underlying chondrocyte dedifferentiation. Plastic adherence quickly led to loss of collagen type II production. Culturing hAC as CA-ECM allowed the chondrocytes to remain attached to their own ECM. Here, hAC maintained a rounded shape, proliferated rapidly and continued to synthesize and secrete collagen type II. This was associated with high levels of Sox9 mRNA expression. Collagen type I was found at the mRNA and intracellular protein level, but not in the ECM. Epigenetic changes could not account for the variations in collagen type II production observed in this study.

Supported by their own ECM in CA-ECM, hAC proliferate to clinically useful numbers while maintaining collagen type II synthesis and secretion. *In vitro* expansion of high numbers of collagen type II secreting chondrocytes may improve tissue engineering of joint surfaces.

7. METHODOLOGICAL ASPECTS

7.1 Isolation and culture of cells

7.1.1 Isolation of the SVF from human adipose tissue and isolation of ADASC from SVF

The SVF was separated from adipose tissue using a modified procedure (Zuk et al., 2001). Washed adipose tissue was digested with collagenase. Red blood cells were removed from SVF pellets by lysis buffer and suspended cells were passed through 100-µm and then 40-µm cell sieves. Cell suspensions were applied to Histopaque gradients and interfaces were collected, washed in buffer solution and passed through a 30-µm mesh. Cell counts and viability assessment were performed. SVF analysis by flow cytometry revealed a haematopoietic $CD45^+$ population. SuperMACS magnetic beads coupled to anti-human MAb were used to remove $CD45^+$ cells from SVF according to the instructions provided by the manufacturer. Remaining $CD45^-$ cells contained $CD31^+$ and $CD31^-$ populations which were separated by superMACS. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD31 MAb. After washing, antibody-coated cells were incubated with anti-FITC microbeads. Microbead-coated $CD31^+$ cells were retained in LS columns, allowing for initial isolation of $CD31^-$ cells. Later, $CD31^+$ cells were separately flushed out of the columns. Flow cytometry was used to confirm separation, and purity was less than 8% contaminating cells in both populations.

7.1.2 Culture of isolated human ADASC

After isolation, $CD31^+$ and $CD31^-$ cell subsets and clonal cell lines were cultured in DMEM/F12 containing 20% FBS, antibiotics and amphotericin B (culture medium) at 37°C in an atmosphere of 5% CO_2 in humid air. After one week, attached cells were passaged by trypsin-EDTA and cultured in DMEM/F12 containing 10% FBS without amphotericin B. Clonal cell lines from $CD31^-$ cells were achieved by culturing single fresh cells in separate wells. Using a micropipette, a single cell was placed into each well of 48-well plates containing culture medium. Colony-forming ability was assessed after 3 weeks. At that time, colonies were passaged and cultured further in DMEM/F12

containing 10% FBS without amphotericin B.

7.1.3 Isolation and culture of human bone marrow MSCs

Bone marrow was obtained from the human iliac crest and the aspirate was diluted in DMEM/F12 medium. Mononuclear cells were separated from the interface after performing density-gradient centrifugation. Since mononuclear cells cultured in AS but not in FBS were always contaminated with a plastic attached $CD14^+$ monocyte population, $CD14^+$ cells were removed using magnetic beads coupled to mouse anti-human CD14 monoclonal antibody MAb, superMACS magnet and LS columns. $CD14^-$ cells were washed and allowed to adhere overnight at 37°C with 5% humidified CO_2 in five parallel flasks. Each flask contained DMEM/F12 medium supplemented with antibiotics, amphotericin B and 20% serum from one of the following sources: FBS from 3 different sources, a human commercially available off the clot pooled allogeneic serum and autologous serum. After 24 hours, nonadherent cells were discarded and adherent cells were washed with PBS and then cultured in DMEM/F12 medium with antibiotics and 20% of the same serum. At approximately 50% confluence, the cells were suspended using trypsin-EDTA and replated at approximately 5,000 cells per cm^2. The preferred cell seeding density and cell culture confluency gave rise to optimal cell growth for non-differentiated cell conditions. After the first passage, amphotericin B was removed and 10% instead of 20% serum was used for further cell cultures. Viable cells were counted at each passage.

7.1.4 Isolation and culture of human articular chondrocytes

Human articular cartilage was obtained from a low-load bearing area on the proximal part of the lateral femoral condyle of the injured knee for autologous chondrocyte implantation (ACI) cultures or from the lateral femoral notch of patients undergoing anterior cruciate ligament (ACL) surgery for *in vitro* studies. In all cases informed consent was obtained, and the study was approved by the Regional Committee for Ethics in Medical Research. The biopsies were digested in two different ways: in the single cell procedure, biopsies were minced and digested by collagenase type XI and deoxyribonuclase I at 37ºC in room air with 5% CO2 for 3-5 hours. The digested

biopsies were then filtered through 70 μm cell strainers. The resulting single cells were resuspended in culture medium supplemented with antibiotics, amphotericin B, ascorbic acid and 20% AS.

In the CA-ECM procedure, biopsies were minced to very tiny pieces and then digested in collagenase type XI at 37ºC in room air with 5% CO2 for 90 minutes. The structures formed by this procedure, called CA-ECM, were washed and resuspended in culture medium supplemented with 20% AS. In some experiments, single AC from each of the three donors was established in monolayer cultures (see paper III). In other experiments, CA-ECM from other donors was established in 3D cultures at 37º C in room air with 5% CO2, incubator atmosphere. At the first passage, or the medium change at 8-9 days of culture, amphotericin B solution was removed and 10% AS was used instead of 20% in the culture medium. For each passage, cells were detached from the plastic surface or released from CA-ECM with trypsin-EDTA, and viable cells were counted.

7.2 Preparation of autologous serum

Whole blood was quickly transferred to 10-ml vacutainer tubes without anticoagulants and allowed to clot for 4 hours at 4°C to 8°C. Subsequently, the blood was centrifuged at 1800*g* at 4°C for 15 minutes. Serum was collected and filtered through a 0.2-μm membrane. Aliquots of the sterile autologous serum (AS) were stored at –20°C.

7.3 Flow cytometry

Freshly isolated and cultured cells were examined for surface and intracellular molecule expression using flow cytometry. MAbs conjugated to fluorecein isothiocyanate (FITC), phycoerythrin (PE), Peridinin chlorophyll protein (PerCP), cyanine dye (CY) and allophycocyanin (APC) fluorochromes were used. Irrelevant control MAbs were included for all fluorochromes. Cells were coated with directly conjugated MAbs, washed, and fixed in 1% paraformaldehyde. Cells were also stained with unconjugated MAbs directed against the cell surface antigens. Cells were incubated with primary MAbs, washed and incubated with PE-conjugated goat anti-mouse secondary

antibodies. Cells were then washed, fixed in 1% paraformaldehyde and analyzed. For detection of intracellular proteins, cells were fixed in 1% paraformaldehyde for 4h and incubated overnight with 1% Tween-20. For immunolabeling, cells were then incubated with primary MAbs, washed, and incubated with PE-conjugated goat anti-mouse secondary antibodies. Cells were washed, fixed in 1% paraformaldehyde and analyzed using a FACSCalibur flow cytometer. Gates were set based on staining with combinations of relevant and irrelevant MAbs so that no more than 1% of cells stained with irrelevant antibodies were positive.

7.4 Real-Time quantitative RT-PCR

Total RNA was extracted from cell pellets using Trizol. Following treatment with DNase I, reverse transcription (RT) was performed according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) with 100 ng total RNA per RT reaction. All assays were designed to overlay a junction between two exons to avoid hybridization to genomic DNA. 18S was included as an endogenous normalization control. Quantification of cDNA was performed using the 7300 Real Time PCR system (Applied Biosystems). Gene expression was calculated using the relative standard curve method (User Bulletin 2, Applied Biosystems).

7.5 Microarray analysis

RNA sample preparation and microarray assays were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, freshly isolated or cultured cells were snap-frozen in liquid nitrogen. Total RNA was extracted using Trizole according to instructions provided by the manufacturer. Because of small amounts of RNA in freshly isolated cells, cRNA was obtained according to the GeneChip Eukaryotic Small Sample Target Labeling Assay Version II (Affymetrix). For all samples, 10 μg of cRNA was hybridized to the HG-U133A array (Affymetrix) representing 22,284 probes and ~14,500 genes. Arrays were scanned at 3 μm using the Agilent Gene Array Scanner (Affymetrix). Gene expression data were analyzed using the Affymetrix Microarray Suite (MAS) 5.0, Affymetrix

MicroDB 3.0 and Affymetrix Data Mining Tool (DMT) 3.0 programs. Briefly, a target value of 100 was set for scaling signal intensities of all probe sets. For each comparison, differentially expressed genes were obtained as follows: genes with a present or marginal call in one or both populations were selected. Only genes that showed increased or decreased calls were kept for further analysis. Within these genes, only those with a $\log_2$ ratio >1.6 or < -1.6 in paper I and ratio >1 or < -1 in paper II were selected and published using MicroDB 3.0 into DMT 3.0 to obtain gene names and descriptions. Raw data for the microarray analyses are available at: http://www.ebi.ac.uk/arrayexpress/, under accession no. E-MEXP-167 and E-MEXP-168 for paper I and accession no. E-MEXP-214 and E-MEXP-215 for paper II.

7.6 Mesodermal linage differentiation

Studies on the capability of hMSCs and ADASCs to differentiate along adipogenic, osteogenic, and chondrogenic lineages were performed at passage 4. For adipogenic differentiation, confluent cultures were incubated with DMEM/F12 containing 10% FBS or AS, 0.5 µM 1-methyl-3 isobutylxanthine, 1 µM dexamethasone, 10 µg/ml insulin and 100 µM indomethacin for 3 weeks. After differentiation, cells were fixed with 4% formalin, washed in 50% isopropanol and subsequently incubated for 10 minutes with Oil-Red O to visualize lipid droplets. Cells were then washed in isopropanol and subjected to nuclear staining with hematoxylin. For osteogenic differentiation, cells incubated at 3,000 cells per cm^2 were incubated in DMEM/F12 containing 10% FBS or AS, 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate for 3 weeks. After differentiation, cells were fixed for 1 hour in 4% formalin and rinsed with PBS without Ca^{2+} and Mg^{2+}. Mineralization of the ECM was visualized by staining with 40 mM Alizarin Red S, pH 4.2, for 5 minutes. For chondrogenic differentiation, 1.5-2.0 x 10^5 cells were pelleted in conical tubes in 500 µl chondrogenic induction media containing high-glucose DMEM supplemented with 500 ng/ml bone morphogenic protein-6, 10 ng/ml recombinant human transforming growth factor-β1, 1 mM sodium pyruvate, 0.1 mM ascorbic acid-2-phosphate, 100 nM dexamethasone, 1% ITS (25 mg insulin, 25 mg transferrin, and 25 µg sodium selenite), and 1.25 mg/ml bovine serum albumin. Tissue spheres were collected after 4 weeks and fixed overnight in a 0.1-M cacodylate buffered mixture of

2% glutaraldehyde and 0.5% paraformaldehyde. The samples were embedded in an epoxy resin, and 2-µm-thick sections were cut on a microtome. Sections were then stained with a drop of 0.4% acidic toluidine blue solution for 1 minute, rinsed in distillated water, mounted and immediately micrographed.

7.7 Neurogenic differentiation and immunocytochemistry of ADASC

Cultured cells from polyclonal and clonal cell lines at passage 4 were added to wells in 48-well plates at a density of 1000 cells per cm^2 in DMEM with 4.5 g/l glucose containing 10% FBS and antibiotics. To initiate differentiation, the medium was replaced the following day to also contain B27, 10 ng/ml epidermal growth factor and 20 ng/ml bFGF. After 5 d, cells were washed and incubated with induction media containing DMEM with 5 µg/ml insulin, 200 µM indomethacin and 0.5 mM 1-methyl-3-isobutylxanthine in the absence of FBS for 5 h. Cells were fixed in methanol at 20°C for 10 min and washed. Immunocytochemistry was performed using standard methods. In brief, cells were blocked with 10% FBS and 5% milk and incubated with a polyclonal antibody against Neurofilament 200 or a MAb against glial acidic fibrillary protein overnight at 4°C. After washing, cells were incubated with goat anti-rabbit antibody conjugated to Cy2 for 2 h. An inverted phase contrast microscope with camera and controller software was used to capture images.

7.8 Electron microscopy

CA-ECM were fixed in a phosphate-buffered mixture of paraformaldehyde and glutaraldehyde and embedded at low temperature in Lowicryl HM23 (Chemische Werke, Waldkraiburg, Germany). Ultrathin sections were subjected to immunogold analysis against Col1, Col2 antibodies using protein A coated with 10-nm colloidal gold for detection. Electron micrographs were sampled randomly with respect to distribution of gold particles, and marker density for each tissue compartment was measured by semiautomatic interactive image analysis.

7.9 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed in buffers containing 20 mM sodium butyrate (Dahl and Collas, 2007) with a few modifications. In short, cells were crosslinked with formaldehyde, lysed in lysis buffer/2% SDS, sedimented, and the pellet was sonicated in lysis buffer/1% SDS to produce chromatin fragments of ~300 bp. ChIPs were performed using 100 µl sonicated chromatin (corresponding to ~5000 cells) and antibodies to acetylated H3K9 (H3K9ac), which is known as a marker of active genes, and trimethylated H3K9 (H3K9m3) and H3K27m3, which are epigenetic markers for silent genes. Immunoprecipitated DNA was used as template for quantitative PCR. Primers used were *COL1A1*, *COL2A1* promoter regions and *COL2A1* enhancer region relative to their transcription start sites to investigate mechanisms of chondrocyte dedifferentiation.

8. DISCUSSION

Isolated living and healthy cells from the body in the form of freshly isolated cells, *ex vivo* expanded cells or cells combined with biocompatible carrier materials as explants have been used *in vivo* in a variety of tissues to maintain healthy tissue (homeostasis) and repair damaged tissue. Much progress has been made recently in tissue engineering, particularly with adult stem cells in preclinical research, on the cell source for tissue substitute, ex vivo amplification and differentiation, and 3D tissue organization, but there are still many engineering and biological challenges ahead. Using human adult somatic cells in the clinic requires cells with well characterized profiles, controlled culture conditions during expansion with good quality (Benya and Shaffer, 1982; Miura et al., 2006; Rodriguez et al., 2005; Talens-Visconti et al., 2007; Zhao et al., 2002).

In this thesis, I have tried to characterize some essential biological principles for human adult stem cells and chondrocytes which may be of use in regenerative medicine. Freshly isolated MSC from bone marrow have been too few or insufficiently pure to be thoroughly characterized. We took advantage of the availability of large numbers of MSC from adipose tissue to perform characterization of freshly isolated human ADASC. These results were then compared with similar results obtained from cultured ADASC in order to understand the changes induced in these cells by in vitro culture. Furthermore, optimal culture conditions for expansion of hMSCs and alternative autologous nutrition for FBS has also been explored. To produce therapeutically useful hAC numbers without losing collagen type II synthesis and secretion has also been studied.

Paper I:

Recent studies have shown that human SVF of adipose tissue contains a potent supply of a subset of hMSC. Although cultured stem cells isolated from SVF have been extensively characterized, little was known about the phenotypical and gene expression characteristic of freshly isolated cell populations in the SVF of human adipose tissue.

In paper I, after removing the $CD45^+$ population, we isolated two populations of $CD45^-$ $CD34^+CD105^+$ from human SVF of adipose tissue, which were separated based on CD31 expression. CD31 (PECAM-1) is commonly used for labeling endothelial cells

(EC), but is also expressed by monocytes, platelets, lymphocytes, and granulocytes (Wosnitza et al., 2007). Freshly isolated $CD31^+$ and $CD31^-$ and cultured $CD31^-$ cells were extensively examined for cell surface and intracellular molecule expression. We demonstrated that all $CD31^+$ cells expressed both CD34 and CD105 with high intensity, while most, but not all $CD31^-$ cells expressed these markers (Figure 1A, and 1B, paper I).

The most remarkable differences between $CD31^-$ and $CD31^+$ cells was expression of HLA-DR. $CD31^-$ cells were consistently negative whereas all $CD31^+$ cells expressed cell surface HLA-DR (Figure 1C, paper I). Expression of integrins and endothelium-related molecules was also found more frequently and with higher intensity in $CD31^+$ cells than $CD31^-$ cells (Table 1, paper I).

Global gene expression analysis has shown that only a few hundred probes out of 22,000 were differentially expressed more than three fold between $CD31^-$ and $CD31^+$ subsets. Briefly, a number of transcripts involved in cell cycle arrest, stem cell biology, development and biology of adipose tissue, bone, cartilage, muscle and neuronal tissue were upregulated in $CD31^-$ cells. Transcripts associated with endothelium, transcripts related to the MHC class II complex, antigen presentation, cytokines and cytokine receptors and proteins involved in signal transduction and transcription were upregulated in $CD31^+$compaired with $CD31^-$.

In contrast to $CD31^+$cells, the $CD31^-$ population in culture displayed proliferative and multilineage differentiation capacity *in vitro*. Culture of $CD31^-$ and $CD31^+$ cells separately revealed that the $CD31^+$ cells did not plate and remained as suspended aggregates that did not proliferate under standard culture conditions used for adherent cells. In contrast, about half of the freshly isolated $CD31^-$ cells attached to the plastic surface displayed fibroblastic-like morphology after 1-2 days. Of the remaining $CD31^-$ cells, some of them attached later, while the rest remained in suspension and finally died. Surprisingly, the $CD31^-$ population, which contained predominantly $CD34^+$ cells, lost its expression of CD34 in culture at passage 4. Results of other studies characterizing human SVF cells are in line with our observations showing that $CD34^+$ expression was not found in cells cultured for more than 2 weeks under conventional culture conditions (Zuk et al., 2002; Katz et al., 2005; Lee et al., 2004). This

observation was also confirmed at the transcriptional level, where CD34 antigen expression was downregulated 6 fold in cultured cells at passage 4 compared with uncultured $CD31^-$ cells (Table 5, paper I).

We generated several monoclonal colonies from the $CD31^-$ populations. Of note, only 16% (63/384) of seeded single cells adhered to the plastic. Many of these cells failed to produce CFU-F after 3 weeks, and only 15 clones (15/384, or 4% of entire $CD31^-$ cells) could be expanded to more than 10^6 cells (20 population doublings). However, when polyclonal $CD31^-$ cells were seeded, approximately half of the cells attached to plastic after two days, and most of these spread and formed colonies. The difference in attachment and colony forming efficiency between the seeding of single and polyclonal cells may be explained by the observation that MSCs, upon attachment to plastic, secrete the Wnt inhibitor dickkopf-1 (Dkk-1). The addition of Dkk-1 to MSC cultures has been shown to induce entry into the cell cycle (Gregory et al., 2005). In our polyclonal cultures, some cells may be early attachers and secrete Dkk-1 upon attachment. The presence of Dkk-1 in the culture may induce slow attachers to attach and proliferate. These cells could potentially be lost in clonal cultures, where Dkk-1 from neighboring cells would not be present. It should be noted that the culture conditions used here are extremely stringent, with no growth factors added and no coating on the plastic surface, so the cloning and colony forming efficiency described must be considered as low estimates. The observation that approximately half of the $CD31^-$ cells in polyclonal cultures attached and formed colonies strongly suggest that some colonies are formed from $CD34^+$ cells, as the proportion of $CD34^-$ cells within the $CD31^-$ population never approached 50% (data not shown).

After *in vitro* expansion, $CD31^-$ cells were studied by flow cytometry and microarray analyses and compared with their uncultured counterparts. Surface marker expression did not differ greatly between uncultured and cultured $CD31^-$ cells. The most apparent changes in cultured cells were found within the group of integrins and adhesion molecules, where expression of several molecules was upregulated in culture. Other obvious changes were upregulation of MSC-related CD105 and loss of CD34 expression. Cultured $CD31^-$ cells were capable of undergoing multilineage differentiation.

The phenotype and gene expression profile of $CD31^-$ and $CD31^+$ cells are sufficiently similar to suggest ontogenetic linkage. The data from our study and others may also suggest interactions between $CD31^-$ and $CD31^+$ cells in adipose tissue. Very recently, the functional properties of $CD31^- CD34^+$ as progenitor cells that may be induced to differentiate towards an endothelial phenotype in the presence of conditioned medium from cultures of $CD31^+ CD34^+$ cells has been reported (Sengenes et al., 2007). This study demonstrated that the G-protein-coupled seven-span transmembrane receptor CXCR-4 is expressed on $CD31^- CD34^+$ progenitor cells, and stromal derived factor-1 (SDF-1), the sole ligand of CXCR-4, is produced by $CD31^+ CD34^+$ cells. $CD31^+ CD34^+$ cells induce chemotaxis and promote the differentiation and organization of $CD31^- CD34^+$ progenitor cells into capillary-like structures via a SDF-1/CXCR-4-dependent pathway *in vitro* (Sengenes et al., 2007).

Our data also demonstrates that $CD31^-$ and $CD31^+$ cells are two large and distinct populations in SVF of human adipose tissue. Live cell imaging from this tissue has revealed that adipogenesis takes place within adipogenic/angiogenic clusters that also contain various stromal cells and blood vessels where angiogenesis is an essential part of adipogenesis (Nishimura et al., 2007). Recent research findings has also postulated that adipocytes and endothelial cells may share a common progenitor (Planat-Benard et al., 2004). SVF cells have been shown to be able to induce change from mature fat cells into endothelial cells (Planat-Benard et al., 2004). During the reverse differentiation pathway, $CD31^+$ mature endothelial cells from the SVF have been shown to be converted to adipocytes (Wosnitza et al., 2007). Our data in paper I also demonstrated that $CD31^+$ cells overexpressed endothelial-related genes such as *VEGFR1* and *VEGFR2*. Administration of anti VEGF antibodies have been shown to inhibit not only angiogenesis but also formation of adipogenic/angiogenic cell clusters, indicating that coupling of adipogenesis and angiogenesis is essential for differentiation of adipocytes, and that VEGF is a key mediator of that process (Nishimura et al., 2007).

In comparison, freshly isolated, uncultured $CD31^-$ cells overexpressed transcript associated with different mesodermal organs. We also observed overexpression of transcripts related to fat metabolism and several complement components, which is likely to be related to the function of those cells as precursors of adipocytes (Kershaw and Flier, 2004). Recently, it has also been shown that freshly isolated $CD31^-$ cells are

100% positive for S100 as an early marker of adipogenesis (Wosnitza et al., 2007). Thus, adipose tissue contains a $CD31^-$ population with preadipocyte characteristics and the capacity to differentiate into adipocytes as well as endothelial precursor and mature endothelial cells. In addition, $CD31^+$ cells with an endothelial phenotype can be converted into adipocytes under adequate differentiation conditions. Consequently, $CD31^+$ and $CD31^-$ populations in human adipose tissue are well connected to each other and control their own regulation and differentiation. Since several cell types reside in adipose tissue, the thorough interactions, functions and ontogenetic linkage of these cells need to be investigated in greater detail.

Paper II:

While clinical studies using hMSC have already been performed, the challenge of generating the best possible culture conditions for clinical scale production of hMSCs is still being discussed (Sotiropoulou et al., 2006b); (Dimarakis and Levicar, 2006); (Berger et al., 2006; Sotiropoulou et al., 2006a). Although hMSCs themselves are not highly immunogenic (Le et al., 2003; Le, 2003), when expanded in FBS they are likely to generate xenogeneic immune responses. Recently, it has been shown that uptake of FBS protein by MSCs is an active process that leads to an intracellular accumulation of bovine antigen even when concentrations as low as 2% of FBS are used in the expansion medium (Gregory et al., 2006). A previous study by the same group has also shown that a single preparation of 10^8 hMSCs grown under standard conditions in FBS could carry 7-30 mg of FBS proteins (Spees et al., 2004).

In a clinical trial using MSC for the treatment of osteogenesis imperfecta, a patient was identified with antibodies against bovine serum proteins after treatment with hMSCs expanded in FBS. This patient did not exhibit successful cellular engraftment (Horwitz et al., 2002). Anaphylactic reactions have been reported in several patients who have received repeated administrations of dendritic cells or lymphocytes cultured in FBS (Mackensen et al., 2000); (Selvaggi et al., 1997; Tuschong et al., 2002). Most recently, an interesting study has shown that FBS-primed dendritic cells induce a strong anti-FBS immune response in the non-obese diabetic (NOD) mouse (Kadri et al., 2007). Also, FBS proteins have been implicated in prion transmission (Kuznetsov et al., 2000). Thus, the use of FBS as a cell culture supplement is not recommended (Doucet et al., 2005).

Obviously, these data indicate that both clinical and regulatory issues necessitate a search for serum supplements other than FBS. Allogeneic human serum might represent an acceptable alternative, but expansion of hMSCs in allogeneic serum has not been successful. We used a commercially available pool of human sera and observed growth arrest in hMSC cultures (paper II). Other investigators were also unable to grow hMSCs in commercially available human serum (Spees et al., 2004). Serum free and serum substituted media have been investigated, but no protein or peptide free culture medium has been published to support the proliferation of hMSCs in the absence of growth factors. Most studies have concluded that serum free media cannot promote MSC growth without the addition of cytokines (Gronthos and Simmons, 1995; Kuznetsov et al., 1997a). This is probably due to the fact that serum contains nutritional components essential to MSC proliferation and differentiation.

Recently, human platelet lysate (hPL) has been identified as a promising supplement to culture media (Doucet et al., 2005; Kocaoemer et al., 2007). Human platelets contain factors such as platelet-derived growth factors (PDGFs), basic fibroblastic growth factor (b-FGF), vascular endothelial growth factor (VEGF), insulin-like growth factors-I (IGF-I) and TGF-β (Doucet et al., 2005; Eppley et al., 2004). PL is a fluid containing high concentration of these growth factors. It is obtained by lysing the platelet bodies, and then mixing the lysate in a small volume of blood plasma (Bernardo et al., 2007). Several studies have successfully expanded hMSCs in hPL better than in FBS-containing media, but the differentiation capacity and immunosuppressive function of expanded MSCs in hPL-containing media have been contradictory (Gruber et al., 2004; Doucet et al., 2005; Bernardo et al., 2007; Kocaoemer et al., 2007; Lange et al., 2007; Muller et al., 2006).

In fact, the main aim of *in vitro* MSC expansion for therapy is, first and foremost, maximal increase in cell numbers while retaining an undifferentiated profile. Growth factors present in hPL promote MSC proliferation. However, MSCs express receptors for PDGF-A, -B, bFGF, TGF-β and IGF-I growth factors (Doucet et al., 2005), which may induce differentiation signals in the proliferating MSCs. Also, since hPL is derived from platelets obtained from a pool of allogeneic donors, concerns about immunological reactions must again be raised. Human platelet surface glycoprotein molecules are suitable targets of alloimmune recognition and can result in activation of T cells and

formation of alloantibodies in recipients (Rozman, 2002). Investigators intending to use human allogeneic serum or platelet derived media instead of autologous serum, desire to obtain an "off-the-shelf" product that will be available in large quantities for the expansion of large numbers of MSCs required for repetitive transplantations (Baksh et al., 2004; Kocaoemer et al., 2007).

In paper II of this thesis, I have shown that expansion of hMSCs in AS in the absence of FBS and without addition of any cytokines is as effective as supplementing the culture medium with FBS. However, in hMSC cultures supplemented with commercially pooled alloHS, fewer cells attached and formed colonies, and cultured cells never reached 60% subconfluence in the first passage, which is in line with other studies (Spees et al., 2004).

Using staining assays, we showed that hMSCs expanded in AS differentiated more slowly towards adipogenic and chondrogenic lineages than did hMSCs expanded in FBS. Changes in gene expression confirmed these differences. Genes associated with differentiation in osteoblasts, adipocytes, and chondrocytes were overexpressed in hMSC cultures supplemented with FBS rather than in AS. Interestingly, differentiation of hMSCs cultured in media supplemented with hPL was also inhibited (Bernardo et al., 2007; Lange et al., 2007). A time-delay of fat droplets and a decreased number of cells converting to adipocytes in line with downregulation of genes participating in fatty acid metabolism, e.g. apolioprotein E (ApoE) and sterol regulatory element binding transcription factor 2 (SREBF2) has been reported in hMSCs expanded in human platelet-derived medium (Lange et al., 2007). Together, this pattern of genes overexpressed in hMSCs grown in FBS suggest that FBS may contain differentiation factors that are not found in human supplements such as AS and PL. Thus, compared with cells expanded in FBS, hMSCs expanded in AS seemed less changed and remained transcriptionally more stable after four passages. This tendency was confirmed by long term cell expansion where the lists of genes differentially expressed in FBS and AS supplemented hMSCs between passage 4 and 10 were analyzed. Many genes were up- and downregulated in hMSCs expanded in FBS between passages 4 and 10. In contrast, gene expression in hMSCs supplemented with AS was extremely stable over this same period of time. Surprisingly, no genes were upregulated in any of the donors examined, and only a few genes were downregulated. Thus, hMSCs could be expanded in culture

medium supplemented with AS for therapeutic purposes without risk of transcriptome instability.

The immunosuppressive effect exerted by hMSCs has been linked to production of prostaglandins (Aggarwal and Pittenger, 2005). Several prostaglandin synthesis genes were highly upregulated in FBS-supplemented hMSCs, suggesting that hMSCs expanded in AS may have a lesser immunosuppressive effect than cells cultured in FBS. In harmony with our results, hMSCs cultured in human platelet lysate displayed an apparently weaker immunosuppressive effect than cells expanded in FBS (Bernardo et al., 2007). Whether these weaknesses in differentiation potential and immunosuppressive effect of hMSCs cultured in human supplements such as AS and PL interfere with their *in vivo* functions, or just represent the consequence of *in vitro* culture, remains to be investigated.

Genes associated with the cell cycle were overexpressed in hMSCs cultured in FBS and were all associated with prolongation of the cell cycle, which is consistent with different rates in cell proliferation. At passage 10, hMSCs in FBS were closer to replicative senescence than hMSCs in AS. In addition, cumulative cell counts were always much higher for hMSCs cultured in AS as compared to FBS. Thus, this study has shown that hMSCs could be expanded rapidly to very high cell counts in the presence of AS without growth factors.

Undoubtedly, in the clinical setting where patient blood collection is not possible, platelet-derived culture medium for cell expansion may be a proper alternative to FBS.

Paper III

Trauma, inherited abnormalities and osteorthritis may lead to damage of articular cartilage. With the protective cartilagenous layer damaged or gone, joint movement may lead to the grinding of one bony surface on the exposed, osseous surface of the other. This may be very painful, and lead to a decrease in quality of life. Poor healing capacity of cartilage has resulted in the emergence of numerous strategies to repair or replace damaged cartilage surfaces.

Chondrocytes residing within cartilage are the most applied cell source to regenerate cartilage tissue, but they are limited in number, and monolayer expansion leads to

dedifferentiation or loss of phenotype. The dedifferentiation of hAC cultured monolayers is fast, occurring as early as first passages (az-Romero et al., 2007; Darling and Athanasiou, 2005b). During this process, chondrocytes lose their rounded shape and produce less of hyaline cartilage-specific matrix proteins, such as collagen type II, and behave more like a fibroblastic cell type, producing increased amounts of collagen type I (Schnabel et al., 2002). ACI studies, in which the patient's own cells have been isolated from cartilage biopsy, expanded in monolayers and then sealed in a defect with a periosteal patch, have demonstrated the occurrence of fibrous tissue formation that is indicative of dedifferentiated chondrocytes (Grigolo et al., 2005; Knutsen et al., 2004). To obtain regenerated cartilage with adequate mechanical properties, these cells need to synthesize appropriate amounts of hyaline cartilage-specific proteins. It is desirable either to prevent dedifferentiation or to induce redifferentiation capacity during chondrocyte expansion. Therefore, for cartilage tissue engineering using autologous chondrocyte transplantation to be more successful, the cells expanded in vitro need to expresses a hyaline chondrocyte phenotype.

In paper III, we tried to improve the ACI cell proliferation protocol in order to expand AC *in vitro* to clinically useful cell numbers without losing its hyaline cartilage characteristics. ACs were cultured attached to their own pericellular matrix (PCM)/ECM in loose structures termed chondrocytes in autologous ECM (CA-ECM). Cultures with chondrocytes adherent to plastic as monolayer or supported by their own ECMs, CA-ECM, were established.

In monolayers, *COL2A1* expression was very low or absent. For CA-ECM cultures, biopsies were briefly digested with collagenase, and ACs were allowed to proliferate in their own PCM/ECM. Here, chondrocytes were found to be small and rounded. ACs in CA-ECM proliferated rapidly, expressed *COL2A1* and secreted translated protein into the ECM in an organized manner at P0 and P1. EM images of immunogold staining demonstrated long and ordered fibrils containing dense COL2 epitopes within newly formed ECM.

At the end of P1, *COL2A1* mRNA expression was quite well conserved in CA-ECM after 2 weeks of culture. At this stage, chondrocytes in CA-ECM migrated increasingly onto the plastic surface to form a monolayer culture and started to develop a

fibroblastic-like appearance. *COL2A1* mRNA expression was gradually reduced with each passage, but only reached very low levels from P2 onward, when CA-ECM were completely dissolved and the cells were expanded as monolayer cultures. However, after 2 weeks in culture, the mRNA level of *COL2A1* in CA-ECM was at least 100-fold higher than in those initially expanded as filtrated single cells in monolayer. At this stage, an average cell number of 40 x 10^6 or more were obtained.

The *SOX9* mRNA levels followed the pattern of *COL2A1* mRNA expression when chondrocytes were cultured in CA-ECM, and its expression declined when CA-ECM dissolved and cells were expanded as a monolayer. *SOX9*, which is expressed during embryonic development, is closely related to cartilage matrix synthesis (Bell et al., 1997) and exerts its properties by activating the gene for collagen type II and other cartilage-specific genes (Lefebvre et al., 1997; Ng et al., 1997; Xie et al., 1999). Recently, direct application of a recombinant adeno-associated virus (rAAV) *SOX9* vector *in vitro* and *in situ* has indicated that sustained expression of a *SOX9* gene cassette significantly increased the proteoglycan and type II collagen content in 3D cultures of human normal and OA chondrocytes (Cucchiarini et al., 2007), consistent with the effects of *SOX9* leading to expression of cartilage matrix components (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Tew et al., 2005). This is consistent with the possibility that, also in CA-ECM cultures, COL2 synthesis is predominantly regulated at the level of the transcription factor *SOX9*. Moreover, our results indicate that the synthesis of proteoglycan aggrecan and COL2, two main key elements of cartilage ECM, are persistent in CA-ECM without any exogenous induction.

The concentration of *COL1A1* mRNA was high at the end of P0 after about 9 days, and could be found to remain at this level through several passages as monolayer cultures (data not shown). This trend was associated with high levels of intracellular COL1 protein as shown by flow cytometry analysis. However, COL1 was not detected in newly generated ECM in CA-ECM nor in the original ECM, as shown by EM immunogold staining. A possible explanation for the absence of COL1 in the new ECM of CA-ECM could be that release of COL1 into the ECM was blocked.

To identify epigenetic mechanisms involved in the dedifferentiation process of AC, we performed ChIP assays (Dahl and Collas, 2007) to map histone H3 modification

associated with the *COL2A1*, *COL1A1* promoter and *COL2A1* enhancer regions at P1 and P3 in two different incubator gas compositions. We found that *COL1A1* promoter was associated with a high level of H3K9ac, which is a marker of active genes, while trimethylated H3K9 and H3K27, which are epigenetic markers of silent genes, were absent. The H3 modifications on *COL2A1* showed low levels of H3K9ac. At the same time, H3K9m3, which is considered to be associated with constitutive transcriptional repression, was also low for both loci at both time points. These H3 modifications on *COL2A1* suggest that the gene is accessible to transcription factors, and that this may be the predominant mechanism involved in the regulation of *COL2A1* expression. However, a high level of H3K27m3 in the promoter region is consistent with an epigenetic mechanism of transient transcriptional repression.

In conclusion, we have shown that ACs expanded in CA-ECM *in vitro* proliferate rapidly, while the dedifferentiation process is slowed sufficiently to yield clinically useful numbers of chondrocytes producing COL2 at the end of two weeks in culture. These results may improve clinical protocols for ACI.

9. CONCLUSION

The data presented in this thesis demonstrate that;

- Human adipose tissue contains a $CD45^{-}$ $CD34^{+}CD105^{\pm}$ $CD31^{-}$ population with stem cell properties.

- Cultured $CD45^{-}CD34^{+}CD105^{+}CD31^{-}$ cells differ from their uncultured counterparts.

- hMSCs are expanded rapidly to high cell counts in the presence of AS without growth factors.

- Compared with cells expanded in FBS, hMSCs in AS are less differentiated and remained transcriptionally more stable over time in culture.

- hACs expanded *in vitro* in CA-ECM are rapidly proliferated to clinically useful numbers while maintaining COL2 expression.

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11. APPENDIX

Supplementary information;

For more detailed supplementary information connected to paper I and II, respectively, refer to online supplementary information at:

http://www.molbiolcell.org/cgi/data/E04-10-0949/DC1/1

http://stemcells.alphamedpress.org/cgi/content/full/23/9/1357/DC2