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CHARACTERISATION OF HUMAN FETAL MESENCHYMAL STEM CELLS

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Människan är inte som ett pussel
– de nyfunna bitarna kompletterar inte bilden,
de skapar bara nya frågor.
Sven Delblanc

CONTENTS

1 SUMMARY

Mesenchymal stem cells (MSCs) are present in various tissues of fetal and adult origin. I isolated and expanded MSCs from human fetal 1st trimester livers and observed that they have a shorther cell doubling time compared to adult bone marrow-derived MSCs. Fetal MSCs differentiated into osteogenic, chondrogenic and adipogenic lineages when induced *in vitro*. Furthermore, functional assays of adipocyte-differentiated fetal and adult MSCs demonstrated that the intracellular pathways, expression of proteins involved in lipolysis, lipolytic activity and secretion of leptin and adiponectin were similar to that of mature adipocytes, although some differences were noted. Fetal MSCs did not differentiate into adipocytes as readily as adult MSCs did.

Fetal MSCs exhibit a similar morphology and surface expression pattern as adult MSCs. Analysis of surface proteins by flow cytometry showed presence of CD29, CD44, CD73, CD105 and CD166 but not of the hematopoietic markers CD34, CD45 or CD14. HLA class I expression was lower in fetal than in adult MSCs. HLA class II could not be detected on the surface, and fetal MSCs had no intracellular deposits of HLA class II as did adult MSCs. Seven days of IFNγ exposure was required for full surface expression of HLA class II, compared to two days for adult MSCs. Fetal MSCs, like adult MSCs, did not upregulate HLA class II during differentiation to osteogenic, chondrogenic and adipogenic lineages.

Further analysis of differences between fetal and adult MSCs was conducted by gene array. Transcriptomes of fetal and adult MSCs differed mainly in genes coding for developmental, cell cycle regulatory and immunologic transcripts. Fetal MSCs showed increased expression of transcripts involved in germ plasm and limb patterning and in brain and early muscle development. The expression of transcripts implicated in cell cycle promotion, chromatin regulation and DNA repair were also more abundant in fetal MSCs. Transcripts with reduced expression in comparison to adult MSCs were those involved in smooth muscle and keratinocyte differentiation. Fetal MSCs expressed fewer transcripts for immunologic genes, which could imply that fetal MSCs are less immunologically mature than adult MSCs.

The effect of fetal MSCs on proliferating lymphocytes was investigated by co-culture experiments and mitogen assays. Mitogen stimulation of lymphocytes was inhibited by fetal MSCs. Neither undifferentiated nor differentiated fetal MSC induced proliferation of allogeneic lymphocytes. Unlike adult MSCs, fetal MSCs did not inhibit proliferating lymphocytes, while fetal MSCs treated with IFNγ for seven days did. These results suggest that fetal MSCs are immunologically privileged cells and have potentials for allogeneic transplantation.

MSCs may be used in cellular therapies. A female fetus with multiple intrauterine fractures, diagnosed as severe osteogenesis imperfecta, was transplanted with HLA-mismatched male fetal MSCs in the 32nd week of gestation. At 35 weeks, the baby girl was delivered by cesarean section. At nine months of age a centromeric XY-specific probe revealed 0.3% of XY-positive cells in a bone marrow biopsy. Whole Y genome FISH staining showed a median of 7.4% Y-positive cells. Patient lymphocyte proliferation against donor MSCs was not observed in co-culture experiments performed *in vitro* before and after MSC injection, indicating that the patient was not immunised against the allogeneic cells. During the first two years of life three fractures were noted and growth followed the same curve. Allogeneic mis-matched MSCs can be safely transplanted *in utero* to a patient with severe OI, where the cells engraft in bone.

To conclude, fetal MSCs may be a valuable source for transplantation.

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3 LIST OF ABBREVIATIONS

AR	Adrenoceptor
BSP	Bone Sialoprotein
cAMP	Adenosine 3', 5'-cyclic monophosphate
CPM	Counts per Minute
COL1	Collagen Type 1
ConA	Concanvalin A
ECM	Extra Cellular Matrix
ELISA	Enzyme Linked Immuno-Sorbent Assay
FBS	Fetal Bovine Serum
FLCs	Fetal Liver Cells
FISH	Fluorescence In Situ Hybridization
GM-GSF	Granulocyte Macrophage-Colony Stimulating Factor
GPDH	Glycerol-3-Phosphate Dehydrogenase
GVHD	Graft-Versus-Host-Disease
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplantation
HSL	Hormone-Sensitive Lipase
IFNγ	Interferon Gamma
IL	Interleukin
IUT	*In utero* transplantation
LIF	Leukemia Inhibitory Factor
MAPK	Mitogen Activated Protein Kinases
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompability Complex
MLC	Mixed Lymphocyte Culture
MLD	Metachromatic Leukodystrophy
MSCs	Mesenchymal stem cells
NK-cell	Natural Killer Cell
OI	Osteogenesis Imperfecta
PBLs	Peripheral Blood Lymphocytes
PBSCs	Peripheral Blood Stem Cells
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PHA	Phytohemagglutinin
PWM	Pokeweed Mitogen
SAGE	Serial Analysis of Gene Expression
SCF	Stem Cell Factor
SCID	Severe Combined Immune Deficiency
SI	Stimulation Index
SpA	Protein A from *Staphylococcus Aureus*
TNF-α	Tumor Necrosis Factor alpha
TGF	Transforming Growth Factor
UCB	Umbilical Cord Blood

4 INTRODUCTION

Embryonic and Fetal Hematopoiesis

In adults, hematopoiesis takes place in the bone marrow. However, no bone exists in the embryo and until 12 weeks' gestation the long bones are not of sufficient size to develop a marrow cavity that can support hematopoiesis [1]. Prior to this time, the first hematopoietic cells are developed extra-embryonically in the yolk sac as early as day 16 of development [2]. Hematopoiesis in the yolk sac is primitive and restricted to erythropoiesis, since no other blood cells are needed for the fetus during this stage of gestation [3]. Late in the 4th week of gestation, cells with a characteristic primitive hematopoietic behaviour are found in the ventral endothelium of the dorsal aorta and disappear in the 6th week of gestation [4, 5]. At the same time, from the 6th week of gestation, fetal hematopoiesis appears in the liver and it remains the major hematopoietic organ during the 2nd trimester [6]. Besides the major cell lineage erythrocytes, cells from the granulocytic and megakaryocytic lineages are found in the fetal liver [6]. The final site for hematopoiesis during fetal development is the bone marrow. Bone marrow stroma appears in week 9-10 of gestation and about three weeks later, active hematopoiesis in the bone marrow begins [7, 8]. At week 22 of gestation all cell lines are represented in the bone marrow, indicating that the fetal bone marrow does not become the major hematopoietic organ until late in the 2nd or early in the 3rd trimester [9].

Fetal Immunology

The human fetus is protected from most pathogens, since the womb provides a physical barrier between the fetus and the outside world and the maternal immune system guards against infections of the mother and fetus. If this arrangement would be sufficient, it is possible that maturation of the fetal immune system could be delayed until the 3rd trimester of pregnancy. However, the fetus could need a functioning immune system earlier than that *e.g.* when the mother's immune system has failed to clear an infection and when the physical barrier to the outer world is disrupted. Previously, the early 2nd trimester fetus has been described as pre-immune, *i.e.* unable to mount an immunological response against donor cells [10-12]. This concept has been questioned and it is currently known that lymphocytes are found in the fetus as early as at the end of the 1st trimester [13-16]. Furthermore, 50% of the cells in the fetal thymus express common T-cell surface phenotypes in the 12th week of gestation [15] and T-cells can respond to allogeneic cells in mixed lymphocyte cultures (MLCs) *in vitro* already from the 8th to 12th gestational weeks [17]. Clinically successful outcomes after *in utero* transplantations (IUT) have only until recently been achieved in cases of immunodeficiency such as bare lymphocyte syndrome and severe combined immune deficiency (SCID) cases [10, 18-20]. In fetuses with hemoglobinopathies like sickle cell disease and thalassemia major, fetal liver cells are rejected [21]. Thus, the fetal immune system likely plays a vital role in the success or failure of IUT.

Stem Cells

Stem cells differ from other kinds of cells in the body. All stem cells, regardless of their source, have three general properties: they are capable of dividing and renewing

themselves for long periods, they are unspecialised and they can give rise to specialised cell types. Their self-renewal potential ensures that sufficient stem cells are available for the demands over a normal adult lifespan. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialised functions. However, unspecialised stem cells can give rise to specialised cells, like heart muscle, blood or nerve cells. Three major classes of stem cells are now recognised: totipotent, pluripotent and multipotent stem cells. Totipotent cells, like the fertilised oocyte, have total potential; they have the capacity to form an entire organism. Totipotent cells specialise into pluripotent cells, as for example embryonic stem cells that can give rise to most, but not all, of the tissues necessary for fetal development. Pluripotent cells undergo further specialisation into multipotent cells, like hematopoietic and mesenchymal stem cells, which are committed to give rise to cells that have a particular function.

Mesenchymal Stem Cells

Introduction

In addition to hematopoietic stem cells (HSCs), the bone marrow also contains mesenchymal stem cells (MSCs). These cells were first recognised by Friedenstein and colleagues more than 30 years ago, who identified a nonphagocytic, adherent, fibroblast-like population that *in vitro* could regenerate bone, cartilage, adipose and stroma [22]. The cells were thoroughly characterised in regard to their colony forming efficiency, strain generation and differentiation into bone, cartilage, adipose tissue and myelosupporting stroma. After transplantation of these cells under the kidney capsule, they engrafted in and differentiated into different connective tissue lineages [23], which indicates that a common progenitor cell exists that gives rise to these tissues.

Sources of MSCs

Human MSCs were first identified in postnatal bone marrow and later in a variety of other human adult tissues, including muscle, connective tissue, skin, adipose tissue, perichondrium, trabecular bone and placenta [24-30]. The quantity of MSCs in term cord blood and peripheral blood is still a subject of discussion. Some groups have identified them in term cord blood [31-34], peripheral blood [35] and growth factor-mobilised peripheral blood stem cells (PBSCs) [36], whereas others have not [37-41].
MSCs can also be isolated from several fetal tissues, such as human 1st trimester blood, bone marrow and liver [42] and from human 2nd trimester kidney, bone marrow, liver, lung, spleen, pancreas, blood, brain and amniotic fluid [40, 43-47].
MSCs represent a minor fraction in bone marrow and other tissues. The exact amount is difficult to calculate since different methods used to collect the bone marrow affect the harvest, but it is estimated that MSCs comprise 0.001% to 0.01% of the total bone marrow, hence about 10 fold less abundant than HSCs [48, 49]. Furthermore, the prevalence of MSCs declines with advancing age. In the marrow of a newborn, one MSC is found among 10,000 nucleated marrow cells, as compared to one MSC per 250,000 nucleated marrow cells in the adult bone marrow and one per 2×10^6 in a 80-year old [48]. In 1st trimester fetal blood, one MSCs is found among 3000 nucleated cells and frequency declined with advancing gestation. [42]. The amount of MSCs in different fetal tissues also varies. In 2nd trimester fetal tissues, one MSC is estimated to be present among 400 cells in the bone marrow, 1/700 in lung, 1/600 in spleen and

1/3500 in liver [45]. The decrease of circulating MSCs in fetal blood during gestation and the higher frequency of MSCs in the 2nd trimester bone marrow might be related to their migration from one hematopoietic site to another in the developing fetus. This is supported by the detection of maximal numbers of fibroblast colony-forming units in murine fetal liver, spleen and bone marrow at the time hematopoiesis begins at each site, suggesting the existence of a stromal stem cell migration [50].

MSCs are Multipotent Cells

MSCs can undergo over 25 passages *in vitro* (more than 50 cell doublings). This demonstrates a high capacity for self-replication; billions of MSCs can be generated from a small amount of starting material. During moderate *in vitro* expansion, they preserve their karyotype and show no sign of differentiation, but if cultured beyond 50 population doublings, signs of senescence and apoptosis appear [42, 51-53]. Therefore, the human MSCs may not be a true stem cell. However, MSCs isolated from rat is reported of being cultured beyond 70 population doublings without any signs of senescence or apoptosis [54].

MSCs are defined as multipotent cells that are capable of differentiating into several lineages of mesenchymal origin [49, 55] including bone [56], cartilage [56-58], tendon [59], muscle [60-62], marrow stroma [63] and adipose tissue [49] (Figure 1). Other reports show MSC differentiation into non-mesenchymal lineages as neuronal [64-70], endothelial [71] and possibly even hepatic [72].

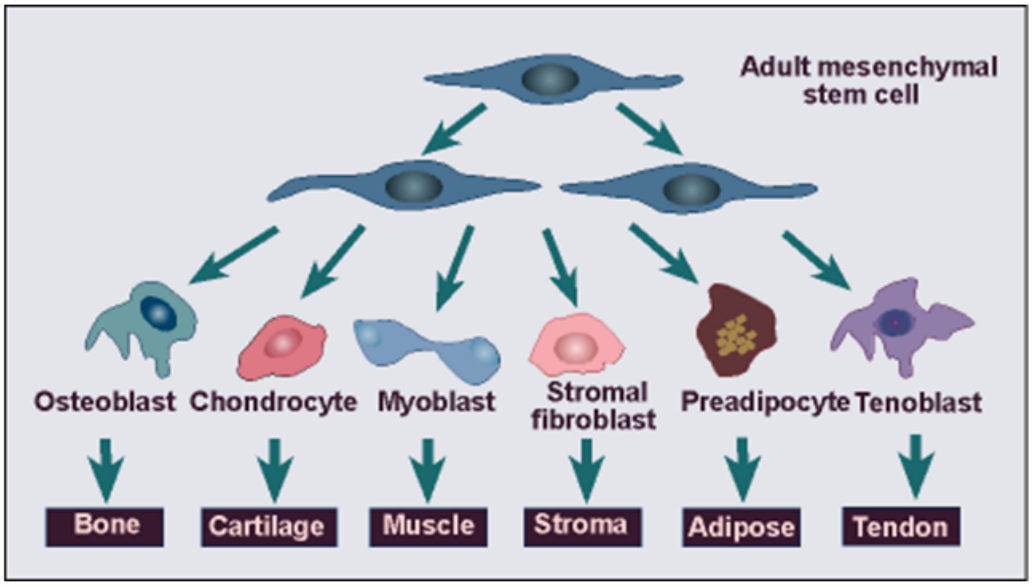

Figure 1.
Mesenchymal stem cells can differentiate into several mesenchymal tissues such as bone, cartilage, muscle, bone marrow stroma, adipose tissue and tendon.

One issue concerning MSC's differentiation capacity is the possibility of a subpopulation that proliferates and produces the desired results. To rule that out, MSCs were cloned using cloning rings and their differentiation potential determined [49]. The results showed that 50% of the cloned MSCs clearly were multipotent. Other studies demonstrates similar results [42, 73-76]. These results also show that the typical default pathway for most of the clones *in vitro* is bone, since all clones differentiated into

osteogenic cells. It is possible that the cloned MSCs loose some of their stem cell nature compared to the parent population since the cloned MSCs were expanded tremendously. This is investigated in two reports by Banfi et al., which showed that culture expansion causes MSCs to gradually loose their early progenitor properties [74] and that MSCs in culture undergo progressive replicative aging and osteogenic differentiation [77]. Further verification of their stem cell nature is the fact that single-cell colonies of MSCs co-express genes characteristic for the osteoblastic, chondrocytic, adipocytic, myoblasts, hematopoiesis-supporting stroma, endothelial, epithelial and neuronal lineages [78, 79], indicating that they can give rise to a broad range of cells. Furthermore, animal studies have shown that when infused intravenously, MSCs engraft in multiple tissues and demonstrate site-specific differentiation [80-82] and when injected into a blastocyst, they contribute to most tissues [83].

The differentiative capacity of fetal and adult MSCs may vary. D'Ippolito et al. examined the osteogenic differentiation of postnatal MSCs of different age and found that younger individuals exhibit increased osteogenic potential than older individuals [84]. Also, MSCs from various fetal tissues exhibit diverse differential potential; osteogenic differentiation was reduced in MSCs derived from liver and adipogenic differentiation was less in spleen-derived MSCs, compared to bone marrow and lung-derived MSCs [45].

Most of these data have been carried out *in vitro* and as of yet, MSCs have not been completely shown to be capable of regeneration or maintenance of a tissue *in vivo*. More experiments, like single cells transplantations and re-transplantations, needs to be performed to be able to name MSCs true stem cells.

Gene and Protein Expression by MSCs

Great effort has been applied to identify specific surface markers on MSCs for definition and identification of the cells *in vivo* and *in vitro*. The *in vivo* phenotype of MSCs has not been entirely established, however, MSCs expanded *in vitro* do not express the hematopoietic or endothelial surface markers CD11b, CD14, CD31, CD34 or CD45 but stain positive for CD29, CD44, CD73, CD105 and CD166 [49, 53, 56, 85]. Molecules suggested to be specific for primitive MSCs and their more differentiated progeny are STRO-1, CD63, CD49a and CD166 [86], and by using restriction fragment differential display PCR, a number of genes (βIG-h3, IGFbp3, and LOXL2) have been suggested to be upregulated in undifferentiated MSCs compared to osteoblast differentiated MSCs [87].

MSCs express human leukocyte antigen (HLA) class I, but not HLA class II and the expression of both molecules is upregulated following treatment with IFNγ [88, 89]. MSCs express a variety of cell adhesion molecules as integrins α1, α2, α3, α4, α5, α6, αv, β1, β3, β4 and β5 [53, 89]. Further characterisation show the expression of ligands for surface molecules present on cells of the hematopoietic lineage, including ICAM-1, ICAM-2, VCAM-1, LFA3 and CD72 [49, 53, 89], which could be important in cell binding and homing interactions between MSCs and other cell types. Several extra cellular matrix (ECM) molecules, as collagen, fibronectin, laminin and proteoglycans are also secreted by MSCs, implicating that they may have a central role in the organisation of the ECM [53, 90]. In addition, MSCs constitutively expressed mRNA for cytokines such as interleukin (IL)-6, -7, -8, -11, -12, -14, -15, leukemia inhibitory

factor (LIF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF) and *fms*-like tyrosine kinase-3 (Flt-3) ligand that are important for HSCs differentiation and support [63, 91].
Unfortunately, these antigens or molecules are expressed on a variety of other cell types and do not provide the specificity needed to exclusively identify MSCs. Characteristics of MSCs differ among laboratories and are probably due to differences in tissue origins, isolation methods and culture conditions.
To characterise human adult MSCs, the transcriptome of undifferentiated and differentiated MSCs has been examined by microarrays [79, 87, 92-96]. By serial analysis of gene expression (SAGE) a single cell-derived colony of MSCs was analysed, which revealed that the MSC colony simultaneously expressed transcripts characteristic of chondrocytes, myoblasts, osteoblasts, hematopoiesis-supporting stroma, endothelial, epithelial and neuronal cell lineages, reflecting the developmental potential of these stem cells [79]. Another SAGE-study compared MSCs with a library of $CD34^+$ cells and demonstrated that MSCs had increased expression of genes involved in the categories of cell adhesion, extracellular and development [94]. The same study showed that IL-11, -15, -27 and IL-10R, -13R and -17R and several transcripts for various growth factors and genes suggested to be enriched in stem cells were expressed in MSCs, indicative of various interactions with $CD34^+$ cells.
Different culture conditions naturally affect the protein production by MSCs. Incubating MSCs in serum-free medium for two to four weeks selects for a sub-population of cells, which survived and began to proliferate in serum-containing medium [97]. These cells had longer telomeres than the parent MSCs and the gene expression pattern indicated that serum-deprivation selected for early progenitor cells with enhanced expression of Oct-4 and several other genes typically expressed in embryonic stem cells.

Enhancement of Engraftment by MSCs

After chemotherapy prior to hematopoietic stem cell transplantation (HSCT), the marrow stroma is damaged and does not recover except in patients younger than five years [98]. MSCs represent a fundamental component of the stromal microenvironment that plays an important role in the regulation of hematopoiesis and the homing and engraftment of hematopoietic cells. Therefore, it is possible that transplantation of MSCs could reconstitute the stromal environment in the bone marrow and enhance hematopoiesis after HSCT. As described above, MSCs produce important hematopoietic cytokines and ECM components, and provide critical cell-cell interactions. They may also attract infused HSCs to the marrow by expressing homing receptors, and possibly play a role in the early maturation of T-cells [99, 100]. MSCs have been shown to maintain hematopoiesis in long-term cultures [91, 101], stimulate HSCs of GM-GSF-treated patients in colony assays [102] and support expansion of umbilical cord blood (UCB)-derived HSCs [29, 34, 42]. This data is suggestive of their use during progenitor cell expansion *in vitro*. Furthermore, fetal and adult human MSCs enhance the engraftment of UCB-derived $CD34^+$ hematopoietic cells in NOD/SCID mice [85, 103, 104] and fetal sheep [105, 106]. The MSCs support is not lineage restricted since it involves cells of lymphoid, myeloid and megakaryocytic lineages [107-109]. Other reported long-term, *in vitro* cultures with

MSCs show commitment and differentiation support of human HSCs to $CD19^+$ B-lineage cells [110, 111].
If transplanting smaller doses of HSCs in NOD-SCID mice, engraftment was only observed after co-infusion of MSCs [85, 104]. The mechanism behind the engraftment enhancement is not understood, but may not require homing of MSCs to the bone marrow since MSCs were not detected in the bone marrow of the transplanted mice [85]. Instead, it could be mediated by the release of cytokines that promote either homing or proliferation of the HSCs. The hematopoietic process involves more than 20 different adhesion receptors and 30 hematopoietic cytokines. This illustrates how complex this system is and that the mechanism regulating hematopoiesis needs to be further characterised.

MSCs Escape the Immune System

Using *in vitro* assays it has been demonstrated that adult MSCs escape recognition by the immune system; they fail to elicit a proliferative response from allogeneic lymphocytes [112-117], nor do they stimulate to IFNγ release [104], which suggests that they are not inherently immunogenic. If stimulated with IFNγ for full expression of HLA class II, the major transplantation antigen, they still escape recognition by alloreactive T-cells [88, 104, 115] and MSCs differentiated to osteogenic and adipogenic lineages are also non-immunogenic [88]. Furthermore, they escape lysis by cytotoxic T-cells and alloreactive killer inhibitory receptor mis-matched natural killer cells [118].
The immune phenotype of cultured MSCs is described as HLA class I positive and HLA class II negative, and with no expression of the co-stimulatory molecules CD40, CD40L, CD80 or CD86 [115, 119]. Treatment with IFNγ upregulates HLA class I and induces HLA class II expression [88, 115]. This phenotype is regarded as non-immunogenic and suggests that MSCs might be effective in inducing tolerance.
After IUT into pre-immune sheep fetuses, human adult MSCs engraft and demonstrate site-specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stroma and thymic stroma [81]. Surprisingly, there was long-term engraftment following IUT after the expected development of immunocompetence. In line with this observation is the finding that intravenous infusion of allogeneic, major histocompability (MHC) mismatched MSCs into mice and baboons was well tolerated in most animals and prolonged survival of skin allografts [113, 120, 121].

Immunomodulation by MSCs

MSCs exhibit immunosuppressive effects and inhibit T-cell alloreactivity induced by mitogens or in co-culture experiments *in vitro* [104, 112-116, 122]. Suppression occurs whether the T-cells are stimulated with mitogens or alloantigens, and the suppression is independent on HLA matching between the MSCs and the lymphocytes [113, 114, 116]. The MSC-induced suppression also appears to include both naïve and memory T-cells [116] and the suppressed T-cells do not become apoptotic or anergic, since they can be restimulated if the MSCs are removed [104, 113]. The mechanism governing the suppressive effect is not yet understood. It is likely to be mediated by soluble factor/factors since the suppression still occurs when the lymphocytes and MSCs are separated by a semi-permeable membrane [112, 115, 123, 124]. Studies indicate that MSCs do not constitutively secrete this factor since culture supernatants

do not suppress lymphocytes in MLCs, whereas cell-free supernatants from co-culture experiments with MSCs and lymphocytes are suppressive [104, 123, 124]. Several different suggestions on how MSCs exhibit the suppressive effect on lymphocytes have been proposed, but no conclusive evidence has been presented. It is likely that the mechanism is multifactorial and is more complex than first believed.
Little *in vivo* data is available, but the immunosuppressive effect of MSCs has been shown in immunocompetent baboons demonstrating that infusion of allogeneic MSCs prolongs 3rd party skin graft survival [113].

Clinical Applications of MSCs

One concern when expanding MSCs in culture is that their ability to home, engraft and differentiate could be altered. In order to investigate homing of MSCs, the fate of primary and cultured murine MSCs were compared in a syngeneic mouse model [125]. A remarkable difference in the homing to bone marrow and spleen between the two cell preparations was found, suggesting that *in vitro* propagation of MSCs dramatically decreases their homing ability. In another study, NOD/SCID mice were infused with human primary or culture-expanded fetal MSCs [85, 103]. Primary MSCs, but not culture-expanded MSCs, were detected in the mice, suggesting that the primary cells were able to home and that this capacity was lost during expansion. The engraftment-enhancing effect of HSCs was not affected by culture expansion.
In a fetal sheep model, human MSCs appear to be present in increased numbers in wounded or regenerating tissues, indicating that engraftment of MSCs might be better when the tissue is damaged. Another study supports this finding since MSCs infused into primates was found in injured tissues and a repair process was observed in several tissues [126].

In Utero Transplantation

Prenatal diagnosis offers the opportunity for early treatment of disease. There are several advantages of IUT: (1) The developing immune system of the fetus may not have the capacity to mount graft rejection of foreign tissue, (2) the greatly increased cell dosage given the size and weight of the fetus, (3) the rapid growth of the fetus provides an opportunity for engraftment and expansion of the donor cells, (4) the normal migration of stem cells to different anatomical compartments, (5) no need for myeloablation requirement, (6) early treatment of the disease is beneficial or critical for effective treatment and finally, (7) the far better psychosocial situation for the mother and father resulting from the birth of a child who has already been treated.
In humans, successful outcomes after IUT has only until recently been achieved in cases of immunodeficiency [10, 18-20, 127-129], while fetuses suffering from other disorders such as inborn errors of metabolism and hemoglobinopathies have clinically failed [21]. It has been speculated that the underlying disease with a defective immunological response might predispose to engraftment of allogeneic cells and that donor cells have selective competitive advantages over host cells [130, 131]. The success in engrafting SCID fetuses is not unexpected since SCID patients have also been transplanted postnatally with minimal or no cytoablation [132]. MSCs derived from human adult bone marrow have been transplanted *in utero* to fetal sheep where they differentiated into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells and thymic stromal cells [43, 47, 81, 133, 134]. Moreover, bone

marrow and fetal liver, both sources of HSCs and MSCs, were shown to engraft in the skeletal muscle of mice with muscular dystrophy [135].
The greatest challenge for IUT with MSCs, as for HSCs, is to obtain sufficient levels of chimerism to have a clinical impact on the disease. Successful IUT in disorders other than immunodeficiency will represent a major step forward in the management of patients with congenital metabolic and hematological disorders. Theoretically, this may be possible because MSCs seems to escape the immune system and also have immune modulatory effects. Further investigations are needed to determine the optimal setting for MSC transplants *in utero*.

Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) literally means "bone that is imperfectly made from the beginning of life". OI is a rare genetic disorder of type 1 collagen, the major structural protein of the ECM of bone and other connective tissues [136, 137]. Most patients with OI have a mutation in one of the two genes that encodes the α-chains of collagen type 1 (*COL1A1* and *COL1A2*), which disturb normal collagen fibril assembly [137, 138]. The result is brittle bones. Severity varies widely, ranging from intrauterine fractures and perinatal death to milder forms without fractures [139]. OI can be classified into four types [140]; type I includes patients with a mild disease and absence of major bone deformities. Type II is lethal in the perinatal period. OI type III is the most severe form in children surviving the neonatal period. Patients with mild to moderate bone deformities are classified as type IV OI. There is no cure for OI, nor is there any effective therapy.
After infusion of MSCs in a mouse model of OI, a small number of donor MSCs engrafted and normal collagen was detected in the bone [64]. The effect on the bone phenotype was small, but there were statistically significant increases of bone content of both collagen and mineral. Furthermore, studies in humans indicate a possible therapeutic effect of MSCs in OI. Five children with OI were transplanted with allogeneic bone marrow-derived HSCs and all had an increase in total body mineral content, compared to predicted values for healthy children [141, 142]. These improvements were associated with increased growth velocity and fewer bone fractures. In at later study, gene-marked allogeneic MSCs expanded from the original HSC-donor were infused to the same OI patients [143]. Engraftment of MSCs and an acceleration of growth velocity during the six months post transplantation could be demonstrated. These reports suggest that transplantation of MSCs could be beneficial in patients with OI.

MSCs for Inborn Errors of Metabolism

MSCs derived from adult bone marrow express arylsulfatase A and B, α-L-iduronidase, glucocerebrosidase and adrenoleukodystrophy protein at levels comparable to hematopoietic cells [144]. Another study showed high levels of arylsulfatase A, β-hexaminosidase A and B, β-hexaminosidase A and β-galactosidase in adult bone marrow-derived MSCs [145]. Furthermore, the MSCs secreted the enzymes into the media and enzyme-deficient fibroblasts were able to take up the enzyme from the media. These findings raised the possibility of transplanting MSCs in patients with lysosomal storage disorders.

Koç et al. investigated if donor derived MSCs are transferred to patients with lysosomal or peroxisomal storage disorders by transplantation of allogeneic bone marrow-derived HSCs. They found that MSCs isolated from the patients were not of donor origin despite successful donor HSCs engraftment [144].
Arylsulfatase A deficiency is the cause of metachromatic leukodystrophy (MLD) and α-L-iduronidase deficiency causes Hurler's syndrome. In a later study, Koç et al. infused expanded MSCs from the original HSC donors to patients suffering from MLD and Hurler's syndrome [146]. In most patients MSCs remained of host type and there was no major improvement in the overall health of the patients, but in four of six patients with MLD, there was a significant improvement in nerve conduction velocity.

Correction of Tissue Disorders

Given MSC's ability to self-renew and their multi-lineage differentiation, they could be used in repairing and regenerating damaged tissues of mesenchymal origin. A number of studies show the feasibility of MSC-transplantation for several purposes. MSCs have been demonstrated to have the ability to repair bone defects in canine femur [147], sheep long bones [148], as well in the murine craniofacial skeleton [149]. It has also been described that the delivery of MSCs to large tendon defects in rabbits significantly improved the biomechanics and structure of the tendon [59], as well as promoting regeneration of cartilage in a rabbit [150] and human study [151]. It has also been proposed that MSCs can migrate to wounded areas and participate in the healing of tissues. Ferrari et al. demonstrated that stromal cells from bone marrow transplanted in mice migrate to areas of induced muscle degeneration, undergo myogenic differentiation and participate in the regeneration of damaged fibers [60]. Other studies show the ability of MSCs to differentiate into cardiomyocytes [152, 153] and their capacity to repair infarcted regions in the heart of mice [154, 155], rats [117, 156] and humans [157]. Transplanted cells engrafted in infarcted myocardium and formed cardiomyocytes. MSCs have also been shown to engraft at high levels in lung tissue following exposure to bleomycin that induce lung injury and inflammation [158]. Sanchez-Ramos et al. evaluated MSC's ability to differentiate into neural lineages *in vitro* and also on their transplantation in mice and rats and came to the conclusion that they may be useful in the treatment of stroke, traumatic injury and Parkinson's disease [159]. Furthermore, it was recently demonstrated that adult bone marrow cells can enter the human brain and generate neurons after transplantation [160].
The selected data summarised here underlie much of the excitement and optimism about the use of MSC transplantation in the treatment of various tissue disorders.

Enhancement of Hematopoietic Engraftment

As previously described above, several *in vivo* animal studies show that MSCs support and enhance HSC engraftment. In an initial human study, bone marrow-derived MSCs were evaluated for the support of autologous hematopoietic engraftment in a phase I trial. It showed that MSCs could be isolated, cultured and infused without any adverse reactions [37]. In another study, autologous culture-expanded MSCs were infused in breast cancer patients to explore whether MSC's would enhance the engraftment of autologous PBSCs after myeloablative therapy [107]. There were no toxicities related to the infusion of MSCs and the hematopoietic recovery was quick. In a different study performed by the same group, patients suffering from MLD and Hurler's

syndrome were transplanted with culture-expanded allogeneic MSCs [146]. The results demonstrated slightly improved reconstitution of platelets and granulocytes. A patient with severe aplastic anemia received two infusions of allogeneic MSCs to improve the possibly defective bone marrow stroma and stimulate the HSCs [161]. The benefits of the MSC infusions were partial with improvement of the bone marrow stroma but not of the hematopoiesis.
These data suggests that infusion of MSCs is safe and may have a positive impact on hematopoiesis *in vivo*. Two prospective studies in recipients of hematopoietic stem cell transplants from HLA identical siblings or unrelated donors, where the patients are randomised to receive MSCs or not, are underway within the European group for Blood and Marrow Transplantation. In the first study the patients will receive MSCs from HLA identical sibling donor, in the second study MSCs will be expanded from haploidentical related donor.

Treatment of Graft versus Host Disease

The above described *in vitro* data on MSC immunosuppression of alloreactive lymphocytes are further supported by *in vivo* studies. In an initiative report, a 20-year-old woman with acute myelogenous leukemia received PBSCs combined with MSCs isolated and expanded from her HLA haploidentical father [162]. The patient engrafted rapidly, experienced no acute or chronic graft-versus-host-disease (GVHD) and was well 31 months after transplantation. In a pilot multicenter study, preliminary results on co-transplantation of HSCs and MSCs derived from HLA-identical donors in order to promote hematopoietic engraftment and limit GVHD show promising results with a reduction in acute and chronic GVHD [163]. Recently, our group reported on a boy with treatment resistant grade IV acute GVHD of the gut and liver [164]. The patient received two infusions of haploidentical MSCs isolated and expanded from his mother. There was no toxicity following infusion of the MSCs. After the transplantation of MSCs, his acute GVHD responded miraculously and fluorescence in situ hybridisation (FISH) analysis of the colon revealed female epithelial cells. It is not possible to evaluate if the female epithelial cells are derived from the HSC or MSC donor, since the HSC donor also were female. However, it is most likely that these cells are from the MSC donor, since studies show that MHC-mismatched MSCs engraft in the gastrointestinal tract after infusion in baboons [82] and MSCs are not transferred from the donor bone marrow to the patient after allogeneic bone marrow transplantation [144].
So far, the experience in using MSCs for the treatment of acute GVHD is limited to a few patients. The data described here show promising results, but controlled studies are needed, which are under way.

5 AIMS OF THE PRESENT STUDY

- To isolate and culture MSCs cells from human 1st trimester fetal livers.
- To characterise fetal MSCs in regard to their proliferation and differentiation potential.
- To explore if fetal and adult MSCs differentiated to adipocytes exhibit similar functional characteristics as mature adipocytes.
- To investigate fetal MSC's immunologic characteristics.
- To analyse the gene and protein expression by fetal MSCs compared to adult MSCs.
- To evaluate the ability of HLA mis-matched fetal MSCs to engraft, persist and differentiate after *in utero* transplantation in a patient with osteogenesis imperfecta.

6 MATERIALS AND METHODS

The materials and methods used in this thesis are described in detail in each paper or manuscript. In this section, I have discussed different aspects of them.

In Vitro Models (Papers I-V)

The experimental work in this thesis is mainly based on different *in vitro* methods. *In vitro* models have great potentials for the study of specific, isolated events that are integrated in a more complex biological process, where the assay mixed lymphocyte cultures is one example. However, *in vitro* data have to be interpreted with caution since the *in vivo* situation often is much more complicated and the results are not directly applicable to the clinical situation.

Materials (Paper I)

Human fetal MSCs were isolated from liver samples of aborted 1st trimester fetuses (range 6 to 11 weeks), where patients had volunteered to donate fetal tissue. The studies were approved by the Ethics Committee at Karolinska University Hospital (Dnr: 428/01). The abortions were performed with vacuum aspiration, as previously described in detail [165]. The women who donated fetal tissues were serologically screened for syphilis, toxoplasmosis, rubella, HIV 1, cytomegalovirus, hepatitis B and C, parvovirus and herpes simplex type 1 and 2.
For the isolation of adult MSCs, bone marrow aspirates were taken from the iliac crest of normal donors ranging in age from 5 to 47 years. The studies were approved by the Ethics Committee at Karolinska University Hospital (Dnr: 446/00).

Isolation and Characteristics of MSCs (Paper I)

Human fetal and adult MSCs were isolated and cultured in accordance with a technique reported elsewhere [166]. Briefly, mononuclear cells were collected through gradient centrifugation and cultured at $1.6x10^5$ cells/cm^2 in DMEM-LG supplemented with 10% fetal bovine serum (FBS) that had been specially selected for optimal growth and differentiation of MSCs. After three days, the non-adherent cells were discarded and the medium was changed every three to four days thereafter. At near confluence, the cells were detached using trypsin-EDTA and replated at a density of $4x10^3$ cells/cm^2.
Surface expression of CD29 (integrin β1 subunit that associates with CD49a in VLA-1 integrin), CD44 (mediates adhesion of leukocytes), CD73 (=SH3+4, dephosphorylates nucleotides to allow nucleoside uptake), CD105 (=SH2, endoglin receptor), CD166 (leukocyte cell adhesion molecule), HLA class I and II and the hematopoietic cell markers CD14 (lipopolysackarid receptor on monocytes), CD34 (on hematopoietic precursors and endothelial cells) and CD45 (leucocyte common antigen, on all hematopoietic cells) on MSCs were analysed by flow cytometry. Yet, no specific surface epitope has been identified on MSCs, making it important to analyse the expression of a whole panel of surface antigens to define MSCs.

Differentiation Assays (Papers I-III)

Even more important is to characterise the capacity of MSCs to differentiate along the osteogenic, adipogenic and chondrogenic lineages, since pluripotency is characteristic of stem cells. MSCs have been reported to differentiate into non-mesenchymal

lineages, as neurons, endothelial and hepatic, but differentiation into adipogenic, chondrogenic and osteogenic cells suffices to name them MSCs. The differentiations of MSCs were validated with stainings of lineage-specific molecules.
The ability of fetal and adult MSCs to differentiate and function as adipocytes were further explored. The adipocytic differentiation was performed using different concentrations of cortisol and dexamethasone to assess the optimal differentiation conditions. Functional assessment of adipocyte differentiation was determined by GPDH activity, glycerol release and secretion of adiponectin and leptin. Expression of proteins in the intracellular pathways of preadipocytes, as well as proteins implied in lipolysis were investigated by immunoblotting.

Gene and Protein Expression Analysis (Papers I-V)

Protein expression by cells was examined by several methods; flow cytometry, cell enzyme linked immuno-sorbent assay (ELISA), immunohistochemistry, immunoblotting and mRNA analysis by gene array. These methods analyse the presence of mRNA and proteins differently in regard of intracellular and cell surface expression. They possess different advantages and disadvantages, but by combining them, they become powerful tools.
Flow cytometry has the advantage of rapid simultaneous measurements of several parameters on one cell, *e.g.* determination and quantification of cellular features, organelles, cell structural components, analysis of the cell cycle and cell death, etc. Also, details on minor cell populations can be collected. The disadvantages are the possible cleavage of proteins when the cells are trypsinised prior to the analysis and that MSCs are not in their natural adherent form when analysed.
Immunohistochemistry combines anatomical, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction. This method makes it possible to visualise the distribution and localisation of specific cellular components within a cell or tissue. The quality of the immunolabelling highly depends on the specificity and affinity of the primary antibody and on proper digestion of the tissue. Problems can arise when the antigen of interest has been denatured or altered during the fixation process, especially when using paraffin-embedded tissue sections, such that recognition by the primary antibody is diminished or completely obliterated.
Immunoblotting is a sensitive technique that provides information about presence, molecular weight and quantity of an antigen by combining protein separation via gel electrophoresis with specific recognition of antigens by antibodies. Limitations are nonspecific binding and cross-hybridisation of antibodies, the need for antibodies that recognise the denatured form of the antigen, degradation of the protein of interest and additionally, not being able to distinguish intracellular proteins from surface expressed proteins.
The advantages of using *cell ELISA* for MSCs are that the cells exist in their natural adherent form when they are analysed and that they are minimally manipulated. One disadvantage is that the data is of a population of cells, one will not know which individual cell produced a particular measurement.
With *microarray analysis*, one studies the expression of mRNA and not the actual proteins, which is the big drawback of this method. The analysis is obviously also

restricted to genes present on the microarray. We used the Affymetrix setting, which contains oligonucleotide probes for 22,215 transcripts and approximately 14,500 genes. It is a well validated method with standardised protocols where the data is obtained in a short period of time. On the other hand, it is not so flexible and does not provide absolute values, only changes (up/down) in gene expression.

Immunological Assays (Papers I, II, V)

To study how fetal and adult MSCs affect lymphocyte alloreactivity, one-way *Mixed Lymphocyte Cultures* (MLCs) were performed. MLCs is a method to detect HLA class II differences and reactivity in MLCs is correlated with risk for rejection and graft survival [167]. In MLCs, lymphocytes or fetal liver cells (FLCs) from two individuals are cultured together where one individual serves as stimulator and is prevented from proliferating by treatment with irradiation. The proliferative response is measured by ^{3}[H]-labelled thymidine uptake by dividing cells. In our study, irradiated MSCs were added to the MLCs to observe how they affect the proliferating lymphocytes and FLCs. Proliferation assays are reliable and easy to perform and have been widely used to assess overall immunocompetence. A disadvantage of this method is that it only detects dividing cells instead of measuring the true effector T-cell function. Using *mitogens* to stimulate T-cells is a more straight forward approach and is a "cleaner" setting than MLCs. Concanvalin A (ConA) and phytohemagglutinin (PHA) directly activate T-cells, whereas pokeweed mitogen (PWM) is a T-cell dependent B-cell mitogen and protein A from *Staphylococcus Aureus* (SpA) stimulates B-cells.

Cell proliferation in response to external stimuli is a very complex process often involving delivery of a signal or set of signals to the cell membrane, activation of intracellular enzymatic pathways that are not well understood, activation and transcription of multiple genes, DNA and protein synthesis and finally, cell division. Combining different proliferation protocols in one experiment can be helpful, since they measure different features of cell proliferation. In both MLCs and mitogen assays, the proliferative response should only be used as a general indicator of T-cell reactivity. They provide an estimate of DNA synthesis and cell proliferation in an entire cell population, but not information on the proliferation of individual cells.

In Utero Transplantation of Fetal MSCs to a Patient With Osteogenesis Imperfecta (Paper V)

A 26-year-old women in her first pregnancy underwent an ultrasound examination in the 15th week of gestation. Femur length of the fetus was below the 5th percentile. An amniocentesis revealed a normal female karyotype. Ultrasound examinations in weeks 24-27 showed that all the limbs were below the 5th percentile, with angulated and fractured femur bones. A tentative diagnosis of OI was made. In the 30th week, the responsible physician raised the possibility of an IUT with MSCs. The parents chose this therapeutic option, aware that the fetal treatment was highly experimental and that the outcome was uncertain. The transplantation procedure was approved by the Ethics Committee of Karolinska University Hospital (Dnr: 91:157) and written consent was obtained from the patient's parents.

MSCs were isolated from one male fetal liver (10 weeks) aborted in the 1st trimester where the woman had volunteered to donate fetal tissue. The study was approved by the Ethics Committee at Karolinska University Hospital (Dnr 428/01) and written

consent was obtained from the patient. The cells were isolated, cultured and characterised on expression of cell surface molecules as well as the differentiation potential as described above. At passage two, the cells were harvested. Aliquots of cells were assayed for sterility, HIV antigen, HTLV I and II, hepatitis B and C and mycoplasma. Harvested cells were counted, their viability evaluated and concentrated to a volume of two ml in sterile NaCl.

Engraftment Monitoring Assays (Paper V)

Using *Fluorescence in situ Hybridisation* (FISH) one can detect one copy of a gene. It is a fast, reliable, sensitive and quantitative method where multiple fluorochromes can be used to discriminate different targets simultaneously. A key advantage of FISH is its ability to target only those genetic sequences of interest and at the same time observe cell morphology stainings. When working with tissues, it can be difficult to digest the tissue properly to perform the analysis and problems also occur with cutting the epitope of interest away, resulting in a false-negative or lower engraftment quantity.

Polymerase chain reaction (PCR) is a straightforward method for the specific and sensitive detection and amplification of nucleic acids. Detection is based on the presence or absence of a PCR product. When studying engraftment by PCR, we used primers for either the Y-chromosome or a patient specific HLA class II DRB1 allele. With these methods it is possible to detect, at least, one donor cell in 10,000 recipient cells. The major problems with PCR are poor primer pair constructs and contamination of the DNA sample leading to false-negative/positive results. In the specific analysis performed in this thesis, controls were as required but we could not detect any donor cells, leaving these issues unwarranted.

7 RESULTS AND DISCUSSION

Isolation of MSCs From Fetal Livers (Paper I)

MSCs were successfully isolated and *in vitro* expanded from human 1st trimester fetal livers. These cells had the morphological characteristic features of adult MSCs.

The cell doubling time of fetal MSCs was significantly shorter than that of adult MSCs. As an example, from passage three to passage four, the number of adult MSCs increased 2.6 times whereas the number of fetal MSCs increased 12.5 times. The fact that fetal MSCs show a considerable ability to grow *ex vivo* makes them ideal for transplantation purposes.

In Vitro Differentiation of Fetal MSCs (Paper I-III)

When induced, fetal MSCs differentiated into osteogenic, chondrogenic and adipogenic lineages, showing that the MSCs we have isolated from human fetal livers share the multi-lineage potential of MSCs present in adult bone marrow [49] and other fetal tissues [42, 45].

It has been unknown whether MSC-derived adipocytes exhibit the functional characteristics of mature fat cells. Using the standard (1 μM) dexamethasone concentration, the adipogenic conversion rate reached approximately 50% in fetal MSCs and almost 100% in adult MSCs. The expression of proteins implied in the intracellular pathways (JNK, p38 and p44) and lipolysis (β2-AR, α2A-AR and hormone-sensitive lipase (HSL)) of preadipocytes appear to be identical in MSC-derived adipocytes.

Functional assessment of adipocyte differentiation was determined by GPDH activity, glycerol release and secretion of adiponectin and leptin. Fetal and adult MSC-derived adipocytes display both similarities and differences when compared to preadipocytes. Like human preadipocytes, MSC-derived adipocytes display a pronounced effect of the antilipolytic α2A-AR, which appears to play a unique role in human adipocytes. Differentiated human adipocytes have lipolytic ability, stimulated primarily by β1- and β2- and inhibited by α2A-adrenoceptors. It is evident that both fetal and adult MSC-derived adipocytes had lower overall lipolytic activity than adipocytes derived from preadipocytes. Adipocytes secrete the adipocyte-specific proteins leptin and adiponectin, which presence was measured in conditioned media from fetal and adult MSC-derived adipocytes. The low differentiation conversion using cortisol was reflected in the low adiponectin and leptin secretion, whereas higher levels were measured when using dexamethasone.

The ability to differentiate into the adipogenic lineage declined after more than six passages in culture, which is similar to that reported by others [42, 53]. It has been observed that the numbers of adipocytes in the bone marrow increases in parallel with a decrease in the number of osteoblasts in a variety of types of osteoporosis [168] and that the volume of adipose tissue in bone increases with age [169]. Based on this and similar observations, it has been suggested that bone loss in age-related osteoporosis could be caused by a shift in MSC differentiation from the osteoblastic to the adipocytic pathway. This could explain why fetal MSCs differentiate more poorly into adipogenic compared to osteogenic lineages than adult MSCs do.

Human preadipocytes constitute a standard model system in adipocyte research. However, the limited amount of cells that can be obtained from one individual, the

short life span and failure to survive freeze/thaw procedures of the preadipocytes are major drawbacks. Awaiting a human-derived adipocyte cell line, MSCs appear to be an efficient and reliable source for cell biology research in this field, since MSCs cells can be differentiated into adipocytes that display morphological and functional characteristics of mature fat cells.

Phenotype of Fetal MSCs (Paper I+II)

Fetal MSCs cultured for two to ten passages consisted of a phenotypically homogeneous cell population when examined by flow cytometry. Fetal MSCs were positive for CD29, CD44, CD73, CD105 and CD166 and negative for CD14, CD34 and CD45, exhibiting similar expression pattern of these proteins as adult MSCs [49]. This confirms the findings about fetal MSCs reported by others [42, 45].

By flow cytometry, immunoblotting and immunohistochemistry, HLA class I and II expression in cultured and differentiated fetal MSCs were analysed.

Fetal MSCs have no HLA class II on the cell surface or intracellularly and surface expression requires seven days of treatment with IFNγ. Adult MSCs express slightly higher levels of HLA class I than fetal MSCs [88]. Intracellular levels of HLA class II can be detected in untreated adult MSCs and surface expression is induced by stimulation with IFNγ for one day. These results are of interest since the allogeneic response in transplantations is usually HLA mediated and it is expected that transplantation with cells that do not express HLA class II antigens may minimise the risk for alloreactivity.

Effects of Undifferentiated MSCs on Proliferating Lymphocytes and Fetal Liver Cells (Paper I)

To determine whether fetal and adult MSCs induce a proliferative response by allogeneic cells, peripheral blood lymphocytes (PBLs) and fetal liver cells (FLCs) were cultured with fetal and adult MSCs. No increase in lymphocyte proliferation was seen after addition of 1% fetal MSCs to PBLs. However, slight lymphocyte proliferation was seen when higher concentrations of fetal MSCs were present in the culture. The addition of adult MSCs to allogeneic lymphocytes induced little, if any, proliferation [114]. When fetal and adult MSCs were added to FLCs, proliferation increased in a dose-dependent way. MSCs constitutively secrete a large number of cytokines [63, 91] and support the expansion and engraftment of HSCs *in vitro* and *in vivo* [42, 85, 91, 105]. Thus, the increase in cell proliferation seen after addition of MSCs to FLCs may reflect proliferation of hematopoietic cells and hepatocytes rather than an immune response.

To determine whether fetal and adult MSCs affect the proliferation of lymphocytes in response to alloantigens, PBLs were stimulated with PBLs from another person.

No suppression of proliferative responses was seen after adding fetal MSCs. In contrast, suppression of proliferation in MLCs occurred in a dose-dependent fashion when adult MSCs were added [114].

We thereafter examined if fetal or adult MSCs could modulate fetal alloreactivity against adult lymphocytes. When FLCs were stimulated with irradiated adult lymphocytes, there was no inhibition, but rather an increase in fetal MLCs after adding fetal or adult MSCs.

The addition of fetal MSCs to lymphocytes stimulated with ConA, PHA, SpA and PWM suppressed the mitogenic response in a dose-dependent fashion. The most marked inhibitory effect was seen with PHA and SpA, which activates T-cells [170] or B-cells [171], respectively. Less suppression occurred after activation with the T-cell mitogen ConA [170] and T-cell dependent B-cell mitogen PWM.
Adult bone marrow-derived MSCs possess unique immunomodulatory properties and inhibit T-cell proliferation induced by non-specific mitogens, cognate peptide as well as alloreactivity induced in MLCs [112-115]. The mechanism behind the immunosuppressive effect of MSCs is unknown, but it appears to be mediated by a soluble factor, since MLCs carried out with allogeneic lymphocytes and MSCs separated by a semi permeable membrane still are suppressed [104, 123, 124].
This discrepancy of fetal MSCs with an ability to suppress lymphocyte proliferation induced by mitogens but not allogeneic MLCs could be due to immunologic immaturity during the first trimester. Recently, we have found that there are different mechanisms of immunosuppression by MSCs depending on T-cell stimulation [172].

Effects of Differentiated Fetal MSCs on Lymphocyte Alloresponses (Paper II)

To determine whether differentiated fetal MSCs induce a proliferative response by allogeneic lymphocytes, PBLs were cultured with fetal MSCs that had been differentiated to adipogenic and osteogenic cells. No alloreactivity was seen. IFNγ-stimulated undifferentiated and differentiated fetal MSCs also escaped recognition by alloreactive lymphocytes.
Fetal MSCs exposed to IFNγ for seven days, for full surface expression of both HLA class I and II, inhibited lymphocyte proliferation at a magnitude similar to that seen with adult MSCs. In spite of the up-regulation of class II alloantigens, other concurrent events induced by IFNγ appear to enhance the antiproliferative effect fetal MSCs exert on lymphocyte proliferation. Thus, fetal MSCs do not escape alloreactivity and suppress lymphocyte alloreactivity due to the lack of HLA class II antigens.

Differences in Gene and Protein Expression between Fetal and Adult MSCs (Paper IV)

The gene expression by fetal and adult MSCs was analysed by gene array chip experiments. Only unique transcripts that had ≥twofold significant increased or decreased expression in fetal MSCs compared to adult MSCs were selected and grouped into functional categories.
Transcripts involved in the promotion of the cell cycle, DNA repair and especially chromatin regulation were more abundant in fetal MSCs and genes that regulate the cell cycle negatively had a decreased expression, compared to adult MSCs. The immunological inertness of fetal MSCs is further illustrated here. We could only detect three transcripts implied in immune recognition that were increased in fetal MSCs, whereas eight showed reduced expression. In the group of developmental genes, expression of transcripts involved in differentiation to more mature cells, *e.g.*, keratinocytes and smooth muscle were decreased and genes that are involved in limb, germ plasm and brain development had an increased expression in fetal MSCs.

The expression of antigens was analysed by cell ELISA, flow cytometry and immunoblotting and also by searching the gene array data for specific genes. Overall, fetal and adult MSCs exhibit a similar pattern of antigen expression.
The surface expression of the adhesion molecule VCAM-1 was higher in adult than fetal MSCs. VCAM-1 expression on fetal MSCs seems to increase during gestation in comparison to 1st and 2nd trimester and UCB-derived MSCs [31, 42, 44]. It is possible that the VCAM-1 production could be specific to developmental stages [173].
Fetal MSCs expressed more ICAM-1 on the cell surface when analysed by cell ELISA and HLA-G could only be detected intracellularly by immunoblotting in fetal MSCs.
The presence of recognised and suggested embryonic stem cell (ES) markers was analysed by browsing the data. The expression of these genes by MSCs could be important in the search for characteristic MSC-markers. The majority of the ES-specific genes were not expressed by either fetal or adult MSCs, but fetal MSCs expressed a few more of the proposed ES genes, which could illustrate the more immature state of the fetal MSCs compared to adult MSCs.
Fetal and adult MSCs are similar in many respects, but differ in several ways, *e.g.*, in their proliferative and differentiative capacity and in some of their immunologic properties. It is possible that the divergence in gene expression between fetal and adult MSCs presented here could be explained by their difference in age rather than by their diverse origins: 1st trimester fetal liver vs adult bone marrow.

Identification of Donor Fetal MSCs in a Patient with Osteogenesis Imperfecta (Paper V)

Transplantation of Fetal MSCs and Clinical Course

After ultrasound examinations of a fetus, a tentative diagnosis of severe OI was made and IUT with male HLA mismatched fetal MSCs was performed in week 32. At 35 weeks, the baby girl was delivered by cesarean section. She was small, typical of severely affected OI neonates [136], but well and was discharged three weeks later. Due to osteopenia and new compression fractures of the spine, treatment with pamidronate [174] was instituted at four months. Three fractures have been clinically suspected, but otherwise the two years of life were otherwise uncomplicated, without hospitalisation.

Engraftment and Identification of MSCs

Osteogenesis imperfecta is a heterogeneous disorder and is caused by a mutation in either of the two genes encoding type I collagen [175]. The patient was found to be heterozygous for a miss-sense mutation of the *COL1A2* gene. This mutation has not been previously reported in other OI patients, however, similar mutations are associated with a severe, nonlethal phenotype [176, 177].
At nine months, a bone marrow biopsy showed normal bone histology with no fibrosis or apparent signs of healing or remodelling. The amount and distribution of osteocytes and the ossification were normal for her age.
In bone marrow biopsy slides hybridised with probes against the whole male and female genome, Y-positive cells were detected in the patient. A centromeric XY chromosome-specific probe identified 0.3% cells containing XY. Using a probe that recognises multiple parts of the whole male genome, we detected a median of 7.4%

Y chromosome-positive cells (range 6.8-16.6%). The probability of finding the centromeric region in sliced samples is lower than that of finding any region of the Y genome and could explain the lower number of Y-positive cells found with the centromeric-specific probes.
DNA isolated from umbilical cord fibroblasts harvested at birth and from skin fibroblasts and bone marrow MSCs expanded in culture harvested at nine months of age was analysed by PCR, detecting no donor DNA. The negative results for MSCs derived from the bone marrow aspirate could be explained by the very low sample volume and subsequently isolation and culture of MSCs. Also, detection of donor MSCs has proved difficult with cultured bone marrow aspirates from both human and animals, because MSCs are preferentially located in the endosteum [121, 143, 161]. Another explanation is loss of proliferation potential by expanded MSCs after intravenous infusion [125].
From the time of transplant to the age of two years, the girl grew appreciably. Pamidronate treatment does not stimulate growth in OI children, instead the bone mineralization is improved [178]. In children with OI transplanted with MSCs from an HLA-identical sibling donor, the presence of only 1-2% donor MSCs lead to clinical improvements [143]. Thus, low levels of MSC engraftment may be sufficient to produce a shift in the balance between the synthesis of mutated and normal pro-alpha chains. In the patient described here, it was not possible to determine if the presence of 0.3-7.4% donor-derived cells had any influence on the OI phenotype. Controlled studies are needed to fully evaluate the therapeutic effect of donor MSC therapy.

Immune Response

Blood samples for MLCs were collected before transplantation, at birth and at seven and nine months of patient age. PBLs from the patient at the time of transplant proliferated against allogeneic lymphocytes *in vitro*, demonstrating immuno-competence of the fetus. Postnatally, lymphocytes isolated from the child at birth and at seven and nine months of age continued to respond to allogeneic lymphocytes. In contrast, alloreactivity against donor MSCs was not detected prior to transplant or at any other time, indicating that no immune reaction had occurred against the transplanted cells. This indicates a lack of immune reactivity against the transplanted allogeneic MSCs; however, to definitely demonstrate a lack of allo-response to the donor, one would need to test donor tissues other than the MSCs against the recipient lymphocytes. This was not possible since no other donor tissue was available.

This is the first demonstration of donor MSC engraftment in a human fetus with normal immunologic function. *In vitro* studies indicate that engraftment occurred because MSCs are immune-privileged cells. The data presented here suggest that allogeneic mis-matched MSCs can be safely transplanted *in utero* to a patient with severe OI, where the cells engraft in bone. This represents a step forward in the treatment of patients with various congenital disorders.

8 CONCLUSIONS

- MSCs can be isolated and expanded from human 1st trimester fetal livers.
- Fetal MSCs have a shorther cell doubling time if compared to adult bone marrow-derived MSCs.
- Fetal MSCs differentiate into osteogeneic, chondrogeneic and adipogeneic lineages.
- Fetal and adult MSCs differentiated along the adipogeneic lineage are fully functional and display characteristics unique to mature adipocytes.
- Fetal MSCs do not induce proliferation of peripheral blood lymphocytes or 1st trimester fetal liver cells *in vitro*.
- Fetal MSCs inhibit proliferating of mitogen activated allogeneic peripheral blood lymphocytes *in vitro*.
- Fetal MSCs inhibit allogeneic peripheral blood lymphocytes in co-culture experiments *in vitro* if previously stimulated with IFNγ for seven days.
- Fetal MSCs express less mature cell differentiation and more pro-proliferation gene transcripts if compared to adult MSCs, showing their less mature nature as stem cells and their high proliferation capacity.
- Allogeneic HLA mis-matched MSCs of fetal liver origin migrated from the intravascular space to bone, following intravenous transplantation *in utero* and persisted for extended times in a patient with severe osteogenesis imperfecta.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Stamceller skiljer sig från andra celler i kroppen. Alla stamceller, oberoende av deras ursprung, har tre generella egenskaper: de kan förnya sig själva genom delning under en lång period, de är ospecialiserade och de kan ge upphov till specialiserade celltyper. Deras förmåga till självförnyelse garanterar att det finns tillräckligt med stamceller under en normal livslängd. En av grundprinciperna hos en stamcell är att den inte har några vävnadsspecifika egenskaper som gör att den kan utföra specialiserade funktioner. En stamcell kan inte arbeta med dess granne för att pumpa blod genom kroppen (som en muskelcell), den kan inte transportera syremolekyler genom blodomloppet (som en blodcell) och den kan inte skjuta iväg elektrokemiska signaler till andra celler som gör att kroppen rör sig (som en nervcell). Men ospecialiserade stamceller kan ge upphov till specialiserade stamceller, som till exempel muskel-, blod- och nervceller, som kan utföra sådana funktioner.
Det finns tre klasser av stamceller; totipotenta, pluripotenta och multipotenta. Totipotenta celler, som det befruktade ägget, har total potential, de har kapaciteten att bilda en hel organism. Totipotenta celler specialiserar sig till pluripotenta celler, till exempel embryonala stamceller som kan ge upphov till många, men inte alla, vävnader nödvändiga under fosterutvecklingen. Pluripotenta celler genomgår ytterligare specialisering till multipotenta celler som ger upphov till celler som har en speciell funktion i kroppen, som blodstamceller eller de celler som jag studerat; mesenkymala stamceller.

Benmärgen består av två sorters stamceller; blodstamcellerna som bildar blodceller och mesenkymala stamceller (MSC) som bildar bindväv. MSC kan bland annat mogna ut till ben, brosk och fett. Omogna MSC ökar produktionen av blodceller genom att bilda tillväxtfaktorer och styra var i märghålan blodstamcellerna finns.
Från ett benmärgsprov eller fostervävnad odlar och förökar vi MSC på laboratoriet till flera miljoner celler. Cellerna förändrar sig inte under odlingen och behåller förmågan att producera tillväxtfaktorer. Genom olika tillsatser vid odlingen bildar de ben, brosk och fett.
Eftersom blodbildningen är störd vid blodcancer, har vi på laboratoriet undersökt hur MSC påverkar blodstamcellerna och immunförsvarets celler (vilka tillväxtfaktorer de tillverkar och vad de har för ämnen på cellytan som är viktiga). MSC från vuxen benmärg, men inte från fostervävnad, hämmar immunförsvarets celler men det är okänt hur. Vi studerade det i olika experiment samt hur många MSC som behövs för hämningen.
Vi deltar i en internationell studie med samtransplantation av blodstamceller och MSC som behandling av blodcancer. Detta verkar minska bristen av vita blodkroppar och blodplättar hos patienten efter transplantationen av blodstamceller och dämpar transplantat-kontra-värd-reaktionen som kan uppstå och ge svåra komplikationer. Stamcellstransplantation blir då en mindre farlig behandling med färre infektioner och minskat behov av immunhämmande medicin.
Vår grupp har även transplanterat foster-MSC till en flicka innan hon föddes eftersom man fann att flickan hade fått benbrott redan i mammans mage. Hon har en bensjukdom, osteogenesis imperfecta, som gör att benen är sköra och bryts väldigt lätt. Vi tror att transplantation av MSC kan hjälpa personer med dessa och liknande problem

eftersom MSC mognar ut till bland annat ben. Idag, snart tre år efter transplantationen, kan flickan gå och mår troligtvis bättre än om hon inte fått cellerna.
Mitt arbete har bestått av att karakterisera och beskriva MSC från fostervävnad. Ett sätt att göra det på är att jämföra dem med vuxna MSC från benmärgen och undersöka skillnaderna mellan dem. Många av olikheterna kan förklaras av cellernas olika ålder, foster MSC är inte lika mogna som de vuxna MSC. Att beskriva skillnaderna mellan foster och vuxna MSC är viktigt för en framtida klinisk användning, som till exempel vid transplantation av MSC, eftersom man då kan välja att använda foster eller vuxna MSC vid olika sjukdomar på grund av cellernas olika egenskaper.

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