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Exploring Hurler syndrome through the study of disease-specific multipotent and pluripotent stem cells

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A noi tre...

che abbiamo sempre superato

ogni difficoltà con un sorriso!

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Chapter 1

General Introduction

1. Lysosomal Storage Disorders

The Lysosomal Storage Disorders (LSDs) represent a group of at least 41 distinct genetic diseases, belonging to the metabolic disorders, each of which is the result of a specific defect in a lysosomal enzyme. Except for red blood cells, lysosomes are contained in all cells of the organism, thus the metabolic disorder may affect different organs and systems at the same time. The reduced or absent enzyme activity results in the lysosomal accumulation of glycosaminoglycans (GAGs) that would normally be degraded. GAGs are important constituent of the extracellular matrix, joint fluid, and connective tissue throughout the body. Progressive accumulation of GAGs within the cells of various organs ultimately compromises their function.

The LSDs are classified according to the quantity and type of accumulated substance; in general, the disease is named after the type of undegraded substrate, i.e, lipid storage disorders, mucopolysaccharidoses and glycoproteinoses. Most LSDs have an autosomal recessive inheritance pattern, with the exception of Mucopolysaccharidosis type II, Fabry disease and Danon disease which show X-linked recessive inheritance.

In the late 1950s and early 1960s, De Duve and colleagues, using cell fractionation techniques, cytological studies and biochemical analyses, identified and characterized the lysosome as a cellular organelle responsible for intracellular digestion and recycling of macromolecules. This was the scientific breakthrough that would lead to the understanding of the physiological basis of the LSDs.

With the advent of molecular biology and the characterization of many of the LSD genes, it is now recognized that the range of severity may in part be ascribed to different mutations within the same gene. However, genotype-phenotype correlations do not always hold. Other factors, including genetic background and environmental factors, presumably play a role in disease progression.

Although each LSD results from mutations in a different gene and consequent deficiency of enzyme activity or protein function, all LSDs share a common biochemical characteristic in that the disorder results in an accumulation of normally degraded substrates within lysosomes. The particular substrates stored and the site(s) of storage vary, although the substrate type is used to group LSDs into broad categories, including mucopolysaccharidoses, lipidoses, glycogenoses, and oligosaccharidoses [1]. These categories show many clinical similarities within groups as well as significant similarities between groups.

From a clinical point of view, the biochemical alteration turns into a gradually deteriorating clinical picture: coarse facial features, hepatosplenomegaly, skeletal anomalies; various degrees of mental retardation prevail in Mucopolysaccharidoses, Mucolipidoses and Glycoproteinoses, while the remaining LSDs are mainly characterized by an involvement of the central nervous system, associated, in some forms, with hepatosplenomegaly [2]. Many of the conditions show significant similarities. Indeed, disruption of a single metabolic pathway can lead to a number of related diseases, depending on where in the pathway the defect occurs.

Most LSDs are characterized by their progressive course, often resulting in severe disease manifestations and early death. Individually, the LSDs are rare; overall, their incidence has been estimated as 1 in 7000 to 1 in 8000 live births [3].

The prognosis is very serious in most LSDs and great effort has always been made to find treatment options fit to face the underlying causes. A successful therapeutic approach to LSDs should ensure an available source of the deficient enzyme, thus helping the degradation of the accumulated metabolites in the various organs, and, at the same time, preventing their further deposition.

1.1 Therapeutic strategies for LSDs

Different therapeutic approaches were used in the past to face the underlying causes of the diseases: infusion of plasma or plasma fractions, intravenous injection of exogenous enzymes extracted from human tissues, infusion of leukocytes and implantation of skin fibroblasts or amniotic cells. Though theoretically correct, all these first therapeutic attempts resulted in a poor efficacy from a clinical point of view, and their use in patients was impractical. However, they led to the innovative therapies, such as bone marrow transplantation (BMT) and infusion of recombinant enzymes, laying the foundation for gene therapy as well [2].

Hematopoietic Cell Transplantation (HCT). In the early 1980s Hobbs et al. [4] published the first results concerning the use of HCT in two patients affected by Hurler syndrome. The rationale of such approach was the possibility of providing a patient with a permanent source of the defective enzyme. Through HCT, hematopoietic stem

cells of the donor colonize the bone marrow of the recipient, where they differentiate into the various hematopoietic lines. The monocyte-macrophage system is the basic mechanism of the therapeutic action, as it is based on the capability of the circulating monocytes to escape from the vessels and migrate inside the organs where they turn into macrophages. When reaching the different sites, the macrophages secrete the defective enzyme, which is internalized by the surrounding affected cells; then the enzyme reaches the lysosomes and degrades the stored, undigested material. The results described by Hobbs and coworkers were strikingly encouraging; straight afterwards, HCT became a choice therapy for many patients affected by different LSDs and various severity of symptoms. Nowadays, more than 500 patients affected by lysosomal storage disorders have been treated with allogenic stem cell transplantation with variable success [5] [6].

Enzyme Replacement Therapy (ERT). In 1964 De Duve first suggested that LSDs could be treated by replacing the defective enzyme, but only with the advent of molecular genetic techniques, therapeutic amounts of the defective enzymes could be synthesized and ERT is now available for several LSDs. [7] [8]. Gaucher disease was the first LSD treated with recombinant human α-glucocerebrosidase; non-neuronopathic type 1 Gaucher patients experience significant improvements from baseline in hematological measures, organomegaly measures and bone manifestations in response to ERT. At the moment there are specific drugs for the treatment of Fabry disease, Hurler-Scheie (MPS IH-S) and Scheie (MPS IS) syndromes, Hunter syndrome (MPS II), Maroteaux-Lamy (MPS VI) syndrome, Pompe disease, Niemann-Pick B disease,

Metachromatic leukodystrophy and α-mannosidosis. Studies carried out so far have proved a consistent positive effect of ERT treatment in several LSDs modifying the natural progression of the disease. In particular, it has been shown a reduction of neuropathic pain, improvement of renal, myocardial and nerve fiber functions, an increase of growth rate, a decreasing hepatosplenomegaly. The results of ERT vary considerably from disease to disease. Important considerations are the age of onset, rapidity of progression and the presence or absence of neurological involvement. Mildly affected patients are the most likely to respond. It is convenient to point out that the use of ERT is advisable only in the types of LSDs without mental retardation, since the exogenous enzyme does not cross the hemato-encephalic barrier and so would be uneffective in patients with mental retardation (Hurler syndrome, Sanfilippo syndrome, Tay-Sachs syndrome, etc.).

Other Therapeutic Approaches. An alternative approach to the treatment of some LSDs is the use of substances able to inhibit the storage of specific metabolites, by depriving the lysosomes of the undegraded substance. In particular, this therapeutic strategy, called substrate reduction therapy (SRT), was first used in Gaucher disease, and subsequently it has been tested in Fabry disease and GM1 and GM2 Gangliosidoses, as well [9] [10]. The main idea is that patients who have significant residual enzyme activity can gradually clear the lysosomal storage material. The drugs used in this approach aim at retarding the formation of the lysosomal substance to a rate at which the residual enzyme activity can catabolize stored and incoming lysosomal substance.

Finally, in the last few years, many studies have been carried out *in vitro* as well as on animal models to evaluate the effectiveness of gene therapy in LSDs. This therapeutic strategy is based on the idea of directly transferring the normal gene into the defective cells in order to supply the active enzyme and, consequently, reduce the intralysosomal undegraded substances. This can be achieved by either *ex vivo* or direct *in vivo* gene therapy strategies. One advantage of gene therapy is the potential for overexpression of enzyme, which may achieve better results than conventional BMT, which is only likely to result in enzyme expression at donor levels. For example, in the mouse model of metachromatic leucodystrophy, stem cell retroviral-based gene therapy did not achieve significant CNS correction [11]. However, over-expression of the enzyme, using *ex vivo* lentivirus-mediated transduction and transplantation, achieved superior results in both the central and peripheral nervous system [12].

1.2 The endosomal-lysosomal system

The lysosome is just one component of a series of seemingly unconnected intracellular organelles, collectively known as the endosomal–lysosomal system. The various components of the system were described over 30 years ago by Novikoff (1973). The principal components of this system are the early endosome, situated at the cell periphery, the late endosome, which tends to be perinuclear, and the lysosome. They form a chain that is responsible for the trafficking and digestion of endocytosed molecules. It is now known that endosomes also participate actively in sorting and recycling.

The final compartment of this system is the lysosome. The term 'lysosome' was first coined by De Duve et al (1955). It is characterized by the presence of a membrane, a low internal pH, and vesicles containing many hydrolytic enzymes. The membrane contains transport systems that carry particles between lumen and cytosol, and an electrogenic proton pump called the vacuolar proton pump [13]. It also contains several membrane proteins of uncertain function [14]. It is in the lysosome that substrate breakdown occurs. However, it is far from being a 'dead end' compartment. For example, lysosomes are capable of secreting their contents after fusion with the plasma membrane [15]. Lysosomes also have other functions. For example, calcium-regulated exocytosis of lysosomes is important for membrane repair [16].

Synthesis and trafficking of lysosomal enzymes (the "synthetic" pathway). The various steps involved in the synthesis of lysosomal enzymes are summarized in Fig 1. They are glycoproteins that are synthesized in the rough endoplasmic reticulum (ER). At this early stage they are inactive. They then translocate through the ER membrane with the help of N-terminal signal sequences.

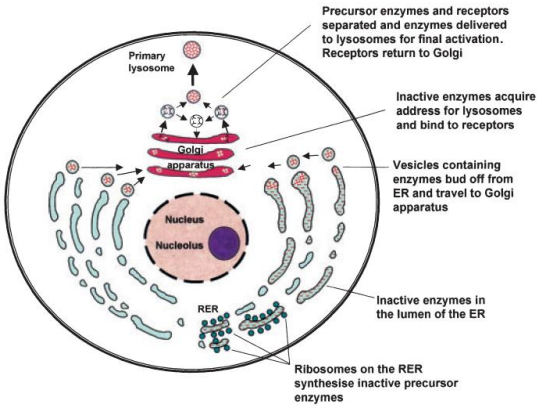

Figure 1. Making the lysosomal enzymes (Vellodi A, 2005)

Once in the lumen of the ER, they undergo N-glycosylation and lose the signal sequence. They then move to the Golgi compartment, and at this stage they acquire a mannose 6-phosphate (M6-P) ligand. This process requires the sequential action of two enzymes, a phosphotransferase and a diesterase. It is important to understand these two steps because it is the acquisition of the M6-P marker that separates glycoproteins that are destined for the lysosome from secretory glycoproteins. Failure of acquisition of this marker results in mistargeting of lysosomal enzymes; they will not enter the lysosome and substrate breakdown will not occur. This is precisely what happens in Mucolipidosis I and Mucolipidosis III. These patients lack the enzyme responsible for the first step, the phosphotransferase. Consequently, all enzymes requiring the M6-P marker fail to enter the lysosome; these patients have high plasma levels of all such enzymes.

The receptor–protein complex then moves to the late endosome, where the low pH causes it to dissociate [17]. The hydrolase moves on into the lysosome and the receptor then is recycled either to the Golgi to pick up another ligand, or to the plasma membrane. The final steps in the maturation of the lysosomal enzyme include proteolysis, folding and aggregation.

Transport of macromolecules to the lysosome (the endocytotic pathway). The basic steps of the endocytotic pathway are summarized in Fig 2. The material to be broken down in lysosomes may be extracellular or intracellular. Extracellular materials enter the cell either by endocytosis or phagocytosis, depending on the nature of the molecule. Receptor-mediated endocytosis is the process by which most biologically important extracellular substances are internalized;

this occurs by binding to specific cell surface receptors [18]. Ligands are first delivered to early endosomes, and then transported to late endosomes, probably by multivesicular bodies. Finally they are delivered to the lysosomes. Phagocytosis is the route of entry into the cell for microorganisms and cellular debris. Such particles are incorporated into phagosomes, which fuse with primary lysosomes to form secondary lysosomes. Finally, intracellular materials undergo autophagy. Although a small amount of hydrolysis takes place in endosomes, the bulk of it takes place in the lysosome. This is because it is only in the acid milieu of the lysosome that hydrolases are active. The low pH of the lysosome is maintained by the vacuolar proton pump.

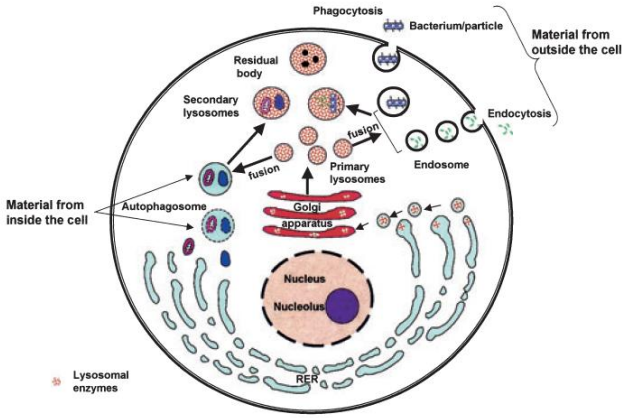

Figure 2. The endocytic pathway (Vellodi A et al, 2005)

1.3 Mucopolysaccharidoses

The mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders caused by deficiency of enzymes catalyzing the degradation of glycosaminoglycans (mucopolysaccharides).

Depending on the enzyme deficiency, the catabolism of dermatan sulfate, heparan sulfate, keratan sulfate, chondroitin sulfate, or hyaluronan may be blocked, singly or in combination. Lysosomal accumulation of glycosaminoglycan molecules results in cell, tissue, and organ dysfunction. GAG fragments generated by alternative pathways are excreted in urine. The stepwise degradation of GAGs requires four exoglycosidases, five sulfatases, and one nonhydrolytic transferase. Endoglycosidases also participate in the degradation. The genes and cDNAs encoding most of these enzymes have been cloned, leading to elucidation of their primary structure, to production of recombinant enzymes, and to identification of mutations causing disease [19].

There are eleven such diseases, each caused by genetic deficiency of a different lysosomal enzyme involved in the degradation of GAGs. The diseases are numbered from MPS I to MPS IX and named after the physicians who first described the syndromes or discovered the underlying enzyme deficiency [20]. All MPSs except MPS II (Hunter syndrome; X-linked recessive) are inherited in an autosomal recessive manner. There are geographical variations in incidence of the different MPS types, with MPS II (Hunter) syndrome more common in Israel and MPS IV Morquio more common in Northern Ireland [21].

The MPSs share many clinical features, although in variable degrees. These include a chronic and progressive course, multisystem involvement, organomegaly, dysostosis multiplex, and abnormal facies. Hearing, vision, airway, cardiovascular function, and joint mobility may be affected. Profound mental retardation is characteristic of MPS IH (Hurler syndrome), the severe form of MPS II (Hunter

syndrome), and all subtypes of the MPS III (Sanfilippo syndrome), but normal intellect may be retained in other MPSs. The bony lesions of MPS IV (Morquio syndrome) are specific to that disorder. There is clinical similarity between different enzyme deficiencies, and, conversely, a wide spectrum of clinical severity within any one enzyme deficiency.

Number	Eponym	Enzyme deficiency	Storage product
I	Hurler/ Scheie	α-L-iduronidase	Heparan sulfate/dermatan sulfate
II	Hunter	Iduronate 2-sulfatase	Heparan sulfate/dermatan sulfate
III-A	Sanfilippo type A	Sulfamidase	Heparan sulfate
III-B	Sanfilippo type B	α-*N*-acetylglucosaminidase	Heparan sulfate
III-C	Sanfilippo type C	Acetyl-CoA; α glucosaminide N-acetyltransferase	Heparan sulfate
III-D	Sanfilippo type D	*N*-acetylglucosamine 6-sulfatase	Heparan sulfate
IV-A	Morquio type A	Galactose 6-sulfatase	Keratan sulfate/chondroitin 6-sulfate
IV-B	Morquio type B	β-galactosidase	Keratan sulfate/chondroitin 6-sulfate
VI	Maroteaux–Lamy	N-acetylgalactosamine 4-sulfatase	Dermatan sulfate/chondroitin 4-sulfate
VII	Sly	β-glucuronidase	Heparan sulfate/dermatan sulfate/chondroitin 4-sulfate/chondroitin 6-sulfate
IX		Hyaluronidase	Hyaluronic acid

Table 1. Enzyme deficiencies and storage products in MPSs. (Ousseren E et al, 2011).

Mutations underlying any one MPS are very heterogeneous, but one or a few mutant alleles may predominate in specific populations. Most are point mutations or small changes in the gene, although major DNA rearrangements and large deletions occur in MPS II. Correlation of disease severity with genotype is sometimes possible, but the effect of missense mutations is generally difficult to predict.

Enzyme assays are available for diagnosis, including prenatal diagnosis, of all the MPSs. Identification of heterozygotes on the basis of enzyme activity is generally difficult or insufficiently accurate. Because of the heterogeneity of mutations, diagnosis and carrier testing by DNA analysis requires knowledge of the mutant alleles in the family under consideration.

There are numerous animal models of MPSs. In addition to the models derived from mutations that have occurred naturally in dogs, cats, rats, mice, and goats, there are several mouse models created by targeted disruption of the corresponding mouse gene. The biochemical and pathologic features of these animal models is generally quite similar to those of their human counterparts, but the clinical presentations may be milder [19].

The MPSs have long been considered potentially amenable to therapy by exogenously supplied enzyme. The success of HCT in altering the course of some MPSs demonstrated that enzyme from donor hematopoietic cells could reduce glycosaminoglycan storage in somatic tissues of the recipient. Unfortunately, the variable neurologic benefit and the high risk associated with bone marrow transplantation limit the value of this form of therapy. Enzyme replacement therapy is under active development, while gene therapy is at a much earlier stage [19].

Of the MPSs, mucopolysaccharidosis type I is by far the most common type.

2 The Hurler Syndrome

2.1 Mucopolysaccharidosis type I

Mucopolysaccharidosis type I (MPS I) is a rare, inherited lysosomal storage disorder caused by the mutations in the *idua* gene, which encodes for the lysosomal enzyme alpha-L-iduronidase. This enzyme is a glycosidase that removes non-reducing terminal α-L-iduronide residues during the lysosomal degradation of the glycosaminoglycans heparan sulphate and dermatan sulphate. The disease is inherited in an autosomal recessive manner. The alpha-L-iduronidase deficiency results in an inability of the lysosome to break down GAGs, particularly dermatan sulfate and heparan sulfate. This process is essential for normal growth and homeostasis of tissues. In this disease, GAGs progressively accumulate in the lysosomes, ultimately causing cell, tissue, and organ dysfunction by largely unknown pathophysiological mechanisms.

The *idua* gene has been mapped to chromosome band 4p16.3. *idua* is approximately 19 kb with 14 exons; the cDNA open reading frame is about 2 kb [22] and encodes a peptide of 653 amino acids. To date more than 110 *idua* mutations have been identified in individuals with MPS I. Known mutations include nonsense, missense, and splice site mutations, small deletions, and insertions [23]. Although genotype-phenotype correlations do not always hold, the presence of a nonsense mutation on both *idua* alleles causes severe MPS I in all cases [24]. These nonsense mutations are believed to result in a complete lack of residual enzyme activity, causing rapid accumulation

of lysosomal GAGs storage with early onset of clinical signs and rapid disease progression. Two of these mutations, W402X and Q70X, are particularly common in patients of European ancestry, accounting for up to 70% of MPS I disease alleles, although considerable differences in frequency are seen across the various countries [25] [26].

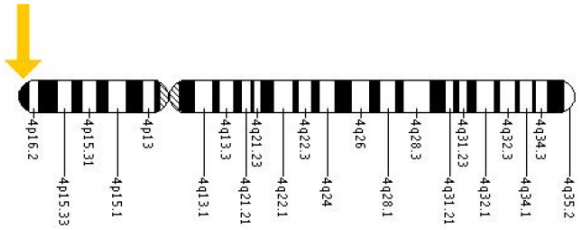

Figure 3. An ideogram of G-banded chromosome 4 (Scott HS et al, 1995)

Historically, MPS I has been broadly categorized into 3 clinical subtypes: Hurler (severe), Scheie (mild/attenuated), and Hurler-Scheie (intermediate). This classification is based on clinical factors, including the age of onset, the rate of functional deterioration, and the range of affected organs (e.g. Central Nervous System involvement). There are, however, no precisely defined clinical or biochemical criteria that can reliably distinguish among these subtypes. It has been acknowledged that these 3 subtypes represent a broad continuous spectrum of clinical phenotypes, caused by a high degree of genotype heterogeneity and associated with various degrees of residual enzyme activity [27]. Lifespan in MPS I ranges from death in early childhood in the most severe form to adulthood in the least severe variant.

The diagnosis of MPS I relies on the demonstration of deficient activity of the lysosomal enzyme α-L-iduronidase in peripheral blood leukocytes, cultured fibroblasts, or plasma. Increased

GAGs (heparan and dermatan sulphate) urinary excretion is a useful preliminary test. Molecular genetic testing of *idua*, the only gene in which mutations are currently known to cause MPS I, is clinically available. Sequence analysis is expected to identify both *idua* mutations in most individuals with MPS I. Prenatal diagnosis can be done by enzymatic assay in cultured chorionic or amniotic cells.

2.2 The Hurler Syndrome

Hurler Syndrome (MPS IH) is the most severe phenotype in the spectrum of MPS I, with incidence estimates approximately 1:100,000 new born [28]. The severely deficient, or absent, IDUA enzyme activity in MPS IH patients results in a progressive accumulation of the incompletely degraded GAGs dermatan and heparan sulphate within the lysosomes. Eventually, widespread progressive cellular, tissue, and organ failure will originate, presenting clinically as progressive and ultimately fatal multi-system deterioration [23]. The exact pathogenesis of the tissue and organ failure in MPS IH patients is still poorly understood. As the nondegraded GAGs accumulate, the number, as well as the volume, of the lysosomes increases, eventually causing mechanical and chemical disruption of the cell. In addition to this direct (primary) effect of accumulating GAGs on cellular function, secondary pathways are thought to be triggered as well, including inflammatory processes (macrophage/cytokine release) [29] and the accumulation of GM2 and GM3 gangliosides in the brain [30] [31].

The clinical phenotype and the detection of an increased amount of urinary GAGs, showing predominant excretion of heparan and dermatan sulphate, often suggest the diagnosis. Definitive diagnosis of MPS IH is confirmed by severely decreased or absent IDUA enzyme activity in peripheral blood leukocytes, filter paper blood spots or fibroblasts, and subsequent mutation analysis [23, 32].

MPS IH patients usually have a normal appearance during the first 6 months of life, although certain, mostly non-specific, symptoms are often already present in this initial period. These include mild facial dysmorphisms, persistent rhinitis, recurrent upper respiratory tract infections, hepato-splenomegaly, recurrent hernias, and mild thoracolumbar kyphoscoliosis [33] [34]. After 6 months of age, MPS IH patients will begin to develop progressive multi-system morbidity including progressive psychomotor retardation, impaired hearing and vision, organomegaly, severe musculoskeletal manifestations, and cardiovascular and pulmonary failure with death in early childhood [23]. Based on an MPS I registry, excluding patients with a known family history and prenatal diagnosis, diagnosis in MPS IH patients (n=137) was made at a median age of 10 months with a median interval from symptom onset until diagnosis of 4 months. This underscores the difficulties of diagnosing patients with an evolving phenotype [35].

2.3 Clinical aspects of the Hurler Syndrome

Clinical manifestations of Hurler syndrome show a chronic multisystemic and progressive course. The main clinical features of MPS IH patients are outlined below.

Facial dysmorphism or coarsened facial features. Coarsening of the facial features is usually the first abnormality detected. These features often first become apparent at age 3-6 months and may become progressively more evident. The head is large with bulging frontal bones. The skull is often scaphocephalic secondary to premature closure of the metopic and sagittal sutures. The nasal bridge is depressed with broad nasal tip and anteverted nostrils. The cheeks are full. The lips are enlarged, and the mouth is usually held open, particularly after age 3 years. Chronic nasal discharge is present. Eyes may be widely spaced, and eye sockets may be shallow, causing the eyes to slightly protrude.

Corneal clouding. As a result of GAGs storage, progressive corneal clouding is common in MPS IH and can begin as early as the first year of life. Clouding of the cornea has a ground-glass appearance and may lead to blindness. Retinal degeneration is also common in MPS I.

Visceral involvement. Progressive hepatosplenomegaly is common in MPS I. GAG storage in the liver and spleen does not lead to organ dysfunction; however, organ size may be massive. Loose stools and diarrhea are episodic problems for some patients. Inguinal and umbilical hernias are common in MPS IH. They are occasionally present at birth or develop within the first several months of life and are often one of the first clinical signs noted.

Skeletal involvment. Progressive skeletal dysplasia (dysostosis multiplex) involving all bones is seen in all individuals with severe MPS I. Children have significant early bone involvement. Mild dysostosis, particularly of the hip, as well as thickening of the

ribs, can be detected on radiographs at birth. Gibbus deformity (dorsolumbar kyphosis) often becomes clinically apparent within the first 14 months [36]. By age three years linear growth ceases. Defective ossification centers of the vertebral bodies lead to flattened and beaked vertebrae and subsequent spinal deformity. Complications may include spinal nerve entrapment, acute spinal injury, and atlanto-occipital instability. The clavicles are short, thickened, and irregular. Long bones are short with wide shafts; the knees are prone to valgus and varus deformities. Endochondral growth plates are thickened and disordered. Typically, the pelvis is poorly formed. The femoral heads are small and coxa valga is common. Involvement of the femoral heads and acetabula leads to progressive and debilitating hip deformity. Progressive arthropathy leading to severe joint deformity is universal; joint stiffness is common by age two years. Phalangeal dysostosis and synovial thickening lead to a characteristic claw hand deformity. Carpal tunnel syndrome and interphalangeal joint involvement commonly lead to poor hand function.

Cardiopulmonary. Cardiac involvement is evident by echocardiography much earlier than observed clinically. Progressive thickening and stiffening of the valve leaflets can lead to mitral and aortic regurgitation, which may become hemodynamically significant in the later stages of disease [23]. As lysosomal storage continues in the heart, cardiomyopathy, sudden death from arrhythmia, coronary artery disease, and cardiovascular collapse may occur. A small subset of individuals with severe MPS I has an early-onset fatal endocardiofibroelastosis.

Frequent upper and lower respiratory tract infections are common. Respiratory obstruction occurs secondary to enlargement of tonsils and adenoids, chronic hearing loss, and enlarged tongue.

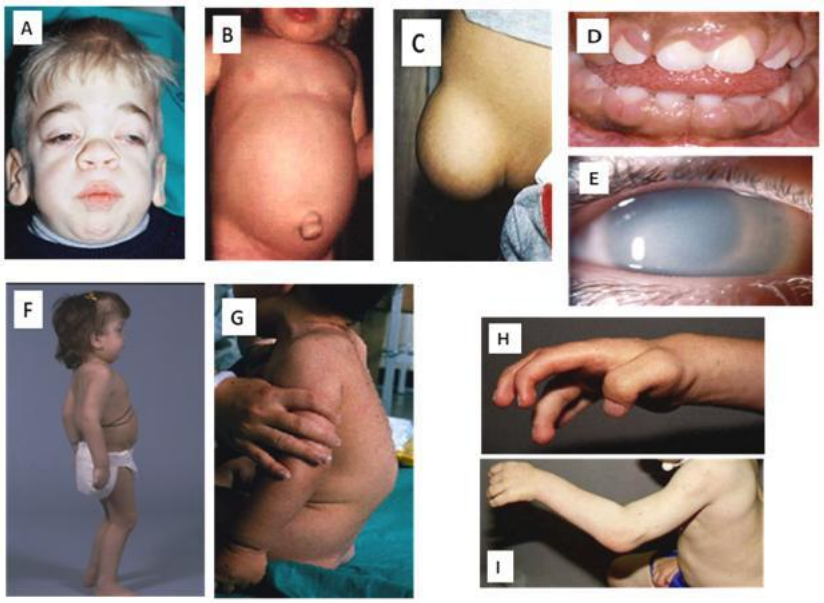

Figure 4. Clinical features of MPS IH patients: (A) facial dysmorphism, (B) hepatosplenomegaly, (C) umbilical hernia, (D) peg-shaped tooth and hypertrophic gingiva, (E) corneal clouding, (F) short stature and varus knees, (G) gibbus deformity, (H) claw hand deformity and (I) joint stiffness

Development. In severe forms of MPS I, developmental delay is often apparent by age 12-24 months, with a maximum functional age of 2-4 years, followed by progressive deterioration. Most children develop limited language because of developmental delay.

Others. The neck is short, and odontoid hypoplasia is noted. Vertebral subluxation with cord compression can occur. Body hair may be coarser than usual, and the skin may be thicker.

2.4 Therapeutic approaches for the Hurler Syndrome

Because of the multisystemic involvement, the treatment of patients with MPS I is multidisciplinary and encompasses both the curative and palliative elements.

Hematopoietic Stem Cell Transplantation (HCT). The first stem cell transplantation for MPS IH was performed more than 30 years ago [4]. Biochemical improvement as well as a dramatic reversal of the clinical manifestations in a 1 yr old boy with MPS IH was demonstrated and this case was a landmark ‘‘proof-of-principle’’ experiment. HCT is still the only treatment available that can prevent the disease progression. Following successful HCT, the donor-derived stem cells provide a continuous endogenous source of the deficient enzyme. The various peripheral tissues (including the liver, spleen, lungs, and heart) presumably benefit from both enzyme secretion by infiltrated (donor) macrophages as well as enzymes secreted into the bloodstream by (donor) leukocytes. Because freely circulating enzymes are not able to cross the blood brain barrier, the CNS relies on infiltration by macrophages alone. In the CNS, these macrophages differentiate into microglia, which secrete the deficient enzyme for recapture by the surrounding neurons.

HCT for MPS IH accounts for the majority (approximately 40%) of HCTs performed in lysosomal storage disorders. After engraftment, significant improvement in the clinical course of MPS IH patients has been observed, although there is wide variation among the various tissues and organ systems, as well as among MPS IH patients. The outcome of the various tissues and organ systems after HCT is briefly discussed below. Within 3 months after successful HCT, the leukocyte

enzyme activity (IDUA), increases to values within the expected range of the donor (heterozygote carrier or unaffected homozygote). At the same time, the previously increased GAGs excretion is reduced to the upper limit of age-matched control values [37] [38] [39] [40]. After engraftment, the metabolism and clearance of GAGs is dramatically improved in the highly perfused visceral organs, such as the liver, spleen, adenoid, tonsils, heart, and lungs. A major reduction of the hepato-splenomegaly as well as relief of the obstructive airway symptoms, including persistent rhinorrhea and obstruction of the upper airways, is seen in all patients within the first 3-6 mos after HCT [37] [39] [40] [41]; [42]. Long-term follow-up of MPS IH patients after HCT showed that when transplantation is performed early enough in the disease process, ie before extensive cerebral damage has occurred, the neurocognitive development improved or stabilized in most children, preventing progressive mental deterioration [37] [39] [40] [43] [44] [45]. After successful HCT, hearing is either gradually normalized, improved, or stabilized in most MPS IH patients. Linear growth is often maintained for several years after HCT. However, long-term follow-up results (3-16 yrs post-HCT) showed that this growth gradually fell to -1 standard deviation in most patients (67-71%) and even below -2 standard deviations in several patients (29-40%; all 6 yrs post-HSCT) [37] [39]. Except for odontoid dysplasia, which showed progressive improvement after successful HCT [46] [47], increasing genu valgum, progressing acetabular dysplasia and the development of carpal tunnel syndrome were not prevented by HCT, despite continued engraftment [48] [47]. In addition, MPS IH patients often showed progression of the

thoracolumbar kyphosis, although the rate of progression varied among patients. As a consequence, orthopedic and neurosurgical interventions were still necessary in several MPS IH patients. The fact that the musculoskeletal manifestations can deteriorate after successful HCT is presumably related to the relative avascularity of the ground substance of the musculoskeletal tissues, precluding enzyme penetration into these tissues [48]. Life expectancy has been significantly improved after successful HCT. This is illustrated by survival, extending into the third decade of life after HCT [49] [43] [50], compared to a median survival of less than 5 years in untreated patients and only rare survivors beyond 10 years. This greater lifespan of MPS IH patients after HCT is mainly the result of the prevention of cardio-pulmonary complications as well as the prevention of severe developmental deterioration [51].

HCT should only be indicated in MPS IH patients under the age of 2 yrs and with no or only minimal cognitive impairment (MDI>70). Although HCT in MPS IH patients has clearly shown to be effective, for several important clinical outcome parameters, is variable among MPS IH patients and there are still some major limitations, including:

- incomplete response: successful engraftment does not prevent disease progression in all organ systems
- morbidity, mortality and graft failure due to the procedure itself

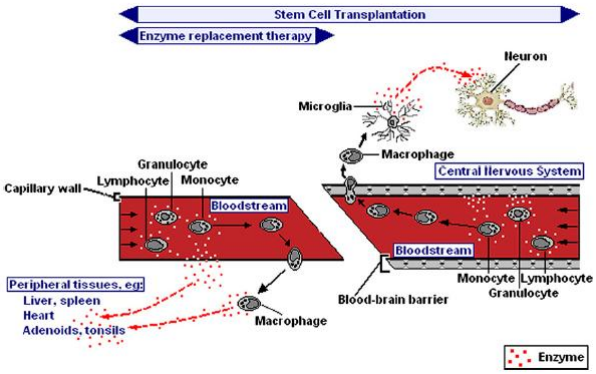

Figure 5. Enzyme delivery to the peripheral tissues and the central nervous system after stem cell transplantation.
In the peripheral tissues, the donor monocytes are able to cross the capillary wall, after which they differentiate into tissue-macrophages and secrete the deficient enzyme for delivery to the various cells. In addition, these peripheral tissues will presumably also benefit from the freely circulating enzymes, secreted by the donor leucocytes, because these enzymes are able to cross these capillary walls as well. Likewise, in enzyme replacement therapy, the intravenously administered enzymes are able to cross the capillary wall of these peripheral tissues.
In the central nervous system (CNS), the donor monocytes cross the blood-brain barrier and differentiate into microglia. These 'CNS macrophages' deliver the deficient enzyme to the neurons and other cells in the CNS. As freely circulating enzymes in the bloodstream are not able to cross the blood-brain barrier, the brains depend on these donor monocytes alone. This explains why enzyme replacement therapy is not beneficial for the disease manifestations involving the CNS in contrast to a successful stem cell transplantation (Aldenhoven M et al, 2008).

Enzyme Replacement therapy (ERT). Replacing the deficient enzyme may improve symptoms and delay disease-induced complications. The effectiveness of ERT depends on the ability of recombinant enzymes (supplied intravenously) to enter cells and to localize to the lysosome, the appropriate intracellular site [52] [53].
The cloning of complementary DNA encoding α L iduronidase led to the production of human recombinant IDUA by recombinant DNA technology. Laronidase is a polymorphic variant of the human enzyme

α-L-iduronidase. Laronidase (Aldurazyme®) received marketing approval in 2003 and is currently licensed in the US, Europe, and Canada for use in treating non-CNS manifestations of MPS I. The current dose regime involves premedication with an anti-inflammatory and antihistamine drugs and intravenous weekly infusion of 100 U/kg of Aldurazyme® over four hours. Laronidase is unable to cross the blood brain barrier in significant quantity, and is therefore not effective in preventing cognitive decline in severely affected MPS IH patients.

Several clinical trials have demonstrated that intravenous ERT with laronidase has proven to be safe and effective in MPS I patients across a wide range of ages (young children to adults) and phenotypes (severe and attenuated), significantly ameliorating some aspects of clinical disease. The findings resulted in clinical and biochemical improvement, such as the normalization of liver and spleen size, improvement of pulmonary function and walking ability and decreased urinary glycosaminoglycan excretion [54] [55] [53]. These improvements are sustained with continued treatment. Long-term ERT effectively treats several somatic signs and symptoms of MPS I, resulting in improved health-related quality of life [54] [55] [53].

Although most patients developed IgG antibodies to laronidase, infusion-related reactions (such as flushing, fever, headache, or rash) are generally manageable with slowing of the infusion rate and/or administration of medication (antipyretic agents or antihistamines). These antibodies may, however, hinder therapeutic benefit by promoting more rapid clearance of the enzyme. Follow-up of

individuals who were part of clinical studies indicates that immune tolerance is eventually reached [56] [57].

Because laronidase does not cross the blood-brain barrier in any appreciable amount at the labeled dose, it is unlikely to improve cognitive or central nervous system function in patients with MPS I. Like HCT, laronidase may not correct preexisting cardiac valvular disease or skeletal abnormalities, although it can improve or preserve joint mobility. Early initiation of therapy, before irreversible damage, may be more effective in slowing disease progression.

Notably, one of the key clinical observations is that various aspects of MPS I respond differentially to enzyme replacement therapy and have different time courses of response. These differences presumably reflect the complex underlying pathophysiology of MPS I, as well as the variable biodistribution of enzyme in tissues. The chronology of responses (months for urinary glycosaminoglycan, liver volume, and sleep apnea; years for endurance, mobility, and activities of daily living) is important for guiding treatment expectations.

Laronidase therapy has a positive safety and tolerability profile, with low associated risks.

Gene therapy. Gene therapy represents a potential alternative therapy by supplying a functional copy of the gene. This could result in a constant delivery of the missing enzyme to all tissues and organs or to targeted organs, for example the musculoskeletal system or CNS [58].

The benefits of different gene therapy approaches were established in MPS I animal models. Intravenous delivery of viral vectors, which can establish a tissue source for systemic enzyme distribution, was

effective in controlling disease manifestations in MPS I animal models upon neonatal treatment [59] [60] [61] [62]. Moreover, has been demonstrated that lentiviral vectors constitute a valuable alternative to oncoretroviral vectors, enabling more efficient marking of murine and human hematopoietic stem and progenitor cells (HSPCs) and robust, long-term transgene expression in their progeny. Recently, has been published a study in which a lentiviral vector was used to transduce HSPCs in order to obtain direct IDUA expression in all tisues in the MPS I mouse model. This study demonstrate that gene-corrected cells are capable of robust, effective delivery of the functional IDUA enzyme to diseased tissues, including the CNS, where supranormal enzymatic activity was measured. The efficient delivery of IDUA to diseased sites was associated with metabolic correction of the affected tissues, as shown by the clearance of accumulated GAGs within hematopoietic and nonhematopoietic cells. This finding suggests the occurrence of active secretion of the functional enzyme by the gene corrected progeny of the transplanted cells and its reuptake by the resident populations. Importantly, the efficient clearance of the storage material achieved by gene therapy allowed to obtain a complete correction of the MPS I–associated phenotype [63]. To date, this study demonstrate that lentiviral-mediated HSC gene therapy allows efficacious enzyme delivery to all MPS I affected tissues, resulting in a complete correction of disease manifestations, including neurologic and skeletal abnormalities, that are refractory to correction by other therapeutic approaches.

Although gene therapy performed in MPS I animal models did seem encouraging, further research is needed before it could be clinically

useful for MPS IH patients. The gene therapy represents an effective, applicable therapeutic opportunity for MPS I patients warranting future development toward clinical testing.

3 Multipotent mesenchymal stem cells

3.1 Mesenchymal stem cells (MSCs)

In the early 1970's, the pioneering work of Friedenstein and colleagues demonstrated that the rodent bone marrow had fibroblastoid cells with clonogenic potential *in vitro* [64] [65]. Friedenstein flushed out the whole bone marrow into plastic culture dishes, and, after discarding the non-adherent cells a few hours later, isolated spindle-like cells adherent to the plastic, which were heterogeneous in appearance but capable of forming colonies (Colony-forming unit fibroblastic, CFU-F). These cells could also make bone and reconstitute a hematopoietic microenvironment in subcutaneous transplants. Moreover, Friedenstein demonstrated that they could regenerate heterotopic bone tissue in serial transplants, thus providing evidence in support of their self-renewal potential. Over the years, numerous laboratories have confirmed and expanded these findings by showing that cells isolated according to Friedenstein's protocol were also present in the human bone marrow, and by demonstrating that these cells could be sub-passaged and differentiated *in vitro* into a variety of cells of the mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts [66] [67] [68] [69] [70]. Friedenstein had thus isolated from the bone marrow what later on would have been renamed by Caplan and

colleagues “mesenchymal stem cell” or MSCs [67]. MSCs are rare in bone marrow, representing ~ 1 in 10,000 nucleated cells. Recently, several studies have reported that MSCs can also differentiate into cells from unrelated germline lineages in a process known as transdifferentiation [69] [71]

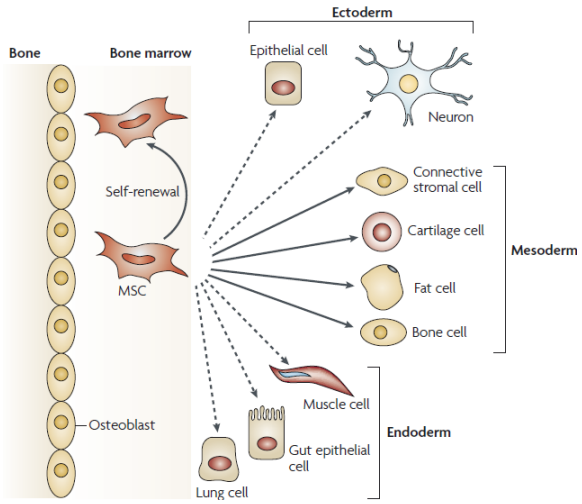

Figure 6. Ability of the MSCs in the bone marrow cavity to self-renew (curved arrow) and to differentiate (solid arrows) towards the mesodermal lineage. The reported ability to transdifferentiate into cells of other lineages (ectoderm and endoderm) is shown by dashed arrows, as transdifferentiation is controversial *in vivo*. (Uccelli A et al, 2008)

MSCs or MSC-like cells are not a unique feature of the bone marrow, as they also are found in tissues such as fat, umbilical cord blood, amniotic fluid, placenta, dental pulp, tendons, synovial membrane and skeletal muscle, though the complete equivalency of such populations has not been formally demonstrated using robust scientific methods [72] [73] [74] [75] [76] [77] [78] [79] [80]. Much effort has been invested both in expanding and phenotypically

characterizing these cells in vitro and in identifying factors that might keep them in undifferentiated state, in order to then transplant them back in vivo for the purpose of repairing specific tissues such as bone and cartilage [81] [82] [83] [84] [85] [86]. Therefore, the knowledge of MSCs is virtually entirely based on the characterization of cultured cells, and the definition of MSCs is indeed an "operational" definition based on the potential to self-renew and differentiate *in vitro*. Interestingly, no evidence of asymmetric cell division, which is considered a property of self-renewing cells in some settings [87], has been yet provided for MSCs.

Little is known about the phenotypic characteristics of MSCs *in vivo*, their developmental origin, their contribution to organogenesis and postnatal tissue homeostasis normally, and their anatomical localization. Moreover a faithful assay that would rigorously test for their ability to self-renew *in vivo*, and would thus prove their "true stemness", is still missing.

An *in vivo* characterization of MSCs could allow for either pharmacological or genetic manipulations of this cellular pool *in vivo*, and it could make possible the isolation of a more enriched population for tissue engineering applications with potentially better capabilities of self-renew and proliferate upon *in vitro* expansion and in *in vivo* transplants.

In vitro studies over many years established the essential role of bone marrow stromal cells for the development and differentiation of hematopoietic cells *in vitro*. The distinct lineage origin of such stromal cells was elegantly demonstrated by Simmons and colleagues, who showed that these cells isolated from patients with functioning

sex-mismatched but HLA-identical allografts were exclusively of host genotype [88]. This finding clearly indicated that stromal cells supporting hematopoiesis are a population distinct from hematopoietic cells. An important implication of this discovery was that mesenchymal stem cells also give rise to stromal cells supporting hematopoiesis, were thus likely to be distinct from the hematopoietic stem cells. The current model indeed is that there are at least two types of stem cells in the bone marrow, hematopoietic stem cells (HSCs) and MSCs. In this formulation, HSCs would give rise to hematopoietic cell types and to cells that resorb bone (osteoclasts), whereas MSCs would generate CFU-Fs and differentiate into a variety of mesenchymal lineages such as chondrocytes, adipocytes and osteoblasts *in vitro.*

Over the years, it has also become progressively clear that MSCs could be the basis for an extremely powerful "natural system of tissue repair" [89] [90].

MSCs have been demonstrated, upon exogenous administration, to serve as effective therapeutic agents in a variety of experimental models of tissue injuries [91] [92] [93] [94] [89]. Curiously, in the vast majority of these studies the therapeutic efficacy did not correlate with the efficiency of engraftment, which was in general low [90]. This finding suggests that the ability to repair was very likely secondary not to transdifferentiation of MSCs into the appropriate cell phenotype or to cell fusion, but rather to the secretion by MSCs of soluble factors that altered the tissue microenvironment [90]. In other terms, MSCs may thus provide what Caplan and colleagues define as "trophic activity". In this regard, extensive proteomic analyses have

indeed revealed that MSCs in vitro produce a variety of factors that influence a broad range of biological functions, including angiogenesis, and secrete neuroregulatory peptides and cytokines with critical roles in inflammation and repair [67].

A greater understanding of the biology of MSCs, particularly in the *in vivo* setting, will probably provide novel and critically important insights into the cellular mechanisms of bone development, hematopoiesis, vasculogenesis and angiogenesis. Surprisingly, despite a variety of very elegant studies that correlate the number of CFU-Fs to bone mass [95] [96], rigorous evidence that MSCs are the skeletal stem cells has not been provided so far.

Already, the abundance of *in vitro* studies has generated important information concerning the use of MSCs for tissue engineering applications. Indeed, investigators have identified efficient modalities of expanding MSCs isolated from bone marrow or adipose tissue aspirates, while maintaining their multipotency [67, 90]. These cells have been used with appropriate scaffolds to form tissues such as bone and cartilage, upon transplantion at specific sites in experimental animal models. However, no human MSC-based technology is currently available.

3.2 The Niche of MSCs

In absence of specific and unique markers that would allow for a proper identification of MSCs *in vivo*, a histological localization of these cells is virtually impossible to identify and is clearly lacking.

An extensive literature has pointed to pericytes as a potential source of MSCs [80] [97] [98] [99] [100]. Bianco and colleagues have

recently reported that MCAM/CD146 (+) subendothelial cells in the human bone marrow are the only cell population that is both clonogenic *in vitro* and capable of transferring a hematopoietic microenvironment in subcutaneous transplants [101]. These cells, which reside in the wall of the sinusoidal blood vessels of the bone marrow, are also positive for angiopoietin-1, a critical regulator of vascular remodeling. The findings by Bianco and colleagues represent the first rigorous attempt to histologically localize and phenotypically define MSC-like cells, or at least a subpool of this population. Notably, a recent paper by Crisan and colleagues suggests that multipotent MSCs with perivascular localization exist in numerous human organs [80].

Whether the vascular setting provides a true niche for pericytic MSC-like cells and is the main source of MSCs *in vivo*, remains to be established. In this regard, however, it is important to note that an increasing amount of evidence has recently linked angiogenesis to osteoblastogenesis, suggesting that blood vessels could be a source of osteoprogenitors or of MSCs with osteogenic potential [102]. A possible implication of these exciting findings is that MSCs could be the skeletal stem cells that contribute to the physiological processes of bone modeling and remodeling *in vivo* [100].

The developing hematopoietic cells are retained in a quiescent state in the bone marrow until, after the appropriate stimulation, they differentiate and are then released in the sinusoidal vascular system. In the bone marrow, the niche stromal cells surround the hematopoietic stem cells and their progeny. Two types of niche have been described in rodents. The ‘endosteal niche’ is formed by osteoblasts that line the

endosteal surface of the trabecular bone, and the ‘vascular niche’ is composed of endothelial cells and CD146+ sub-endothelial stromal cells that lie at the abluminal side of bone-marrow sinusoids. Stromal cells in both types of niche provide a sheltering microenvironment that supports the maintenance and self-renewal of HSCs by shielding them from differentiation and apoptotic stimuli that would otherwise challenge stem-cell reserves. Moreover, the niche also controls the proliferation and differentiation of HSCs and the release of mature progeny into the vascular system. The regulation of HSC quiescence, through the maintenance of HSCs in the G0 phase of the cell cycle in the endosteal niche, and the control of HSC proliferation, differentiation and recruitment in the vascular niche can be ascribed to MSCs [103].

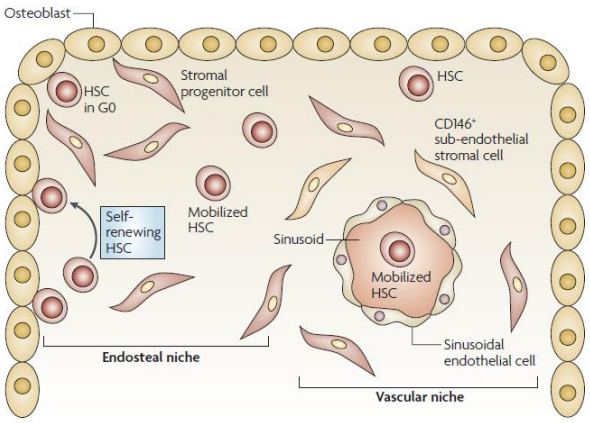

Figure 7. In the bone marrow MSCs at different stages of maturation contribute to the formation of the haematopoietic stem cell (HSC) niche. In the endosteal niche, MSCs, together with osteoblasts, contribute to the maintenance of HSCs in a quiescent state. Around sinusoids, sub-endothelial stromal cells, perivascular stromal cells and sinusoidal endothelial cells also regulate HSC maintenance and control HSC proliferation, differentiation and recruitment to the vascular niche. The endosteal niche also contains self-renewing (dividing) HSCs and mobilized HSCs

that are recruited to the vascular niche following proper activation. (Uccelli A et al, 2008)

3.3 MSCs for Tissue Repair

The fact that MSCs can be differentiated into several different cell types in vitro, their relative ease of expansion in culture, and their immunologic characteristics clearly make MSCs and MSC-like cells a promising source of stem cells for tissue repair and gene therapy. However, compared with in vitro characterization, there is less information on the in vivo behavior of MSCs. The studies that have been performed can be split into observations following site-directed or systemic administration of these cells.

Site-directed delivery of MSCs has shown their engraftment in several tissues, particularly after injury. Several groups have used bone marrow stromal cells to repair infarcted myocardium [104] [105]. Hofstetter and colleagues injected rat MSCs into the spinal cords of rats rendered paraplegic 1 week after injury. They found that MSCs formed bundles bridging the epicenter of the injury and guided regeneration through the spinal cord lesion, thus promoting recovery [106]. This implies that the beneficial effect of MSCs in sites of injury may not necessarily involve their differentiation into the regenerating tissue type but rather the local production of growth or other factors or physical attributes such as forming guiding strands in the injured spinal cord.

Some reports showed that when MSCs are transplanted into fetal or neonatal animals, they engraft and contribute to many different tissues [107]. The signals they respond to in the fetus or

neonate will be very different from those in the adult animal, and hence MSCs may be capable of differentiating into more cell types in the embryo than in the adult.

Systemic delivery of MSCs has been reported by several groups. Barbash and colleagues investigated whether cultured MSCs could be successfully delivered to the infarcted myocardium with a view to repair [108]. They found that early infusion of MSCs resulted in significantly higher uptake in the heart; MSCs were preferentially attracted to, and retained in, the ischemic tissue but not in the remote or intact myocardium. This suggests that injured tissue might express specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury, but these may be downregulated a fairly short time after injury occurs.

Despite the fact that MSCs can get trapped in the lungs, evidence has accumulated to show that MSCs are capable of homing to injured tissues after intra-venous delivery. Systemic infusion of allogeneic MSCs has also led to encouraging results in patients with cancer who underwent high-dose chemo therapy, through the acceleration of bone-marrow recovery [109]. However, the *in vivo* immunosuppressive effect of infused MSCs has only been successfully shown so far in acute, severe Graft versus Host Disease (GvHD) [110], for which the effect was probably due to the inhibition of donor T-cell reactivity to histocompatibility antigens of the normal tissues of the recipient. However as histocompatibility antigens are also expressed by leukaemia cells, MSCs might impair the therapeutic graft-versus-leukaemia effect, as shown by the recent report of effective prevention of GvHD but higher incidence of relapses in

patients with leukaemia who were co-transplanted with MSCs and MHC-identical allogeneic HSCs [111]. Modulation of host alloreactivity led to accelerated bone-marrow recovery in patients cotransplanted with MSCs and haploidentical HSCs [112]. Cultured MSCs have also been administered systemically to humans to treat several conditions, including osteogenesis imperfecta (OI), a disease in which osteoblasts produce defective type I collagen, which leads to osteopenia, multiple fractures, bone deformities, and shortened stature. Horwitz and colleagues used bone marrow transplant (HCT) after ablative chemotherapy to treat children with severe deforming OI. After 3 months, there was new dense bone formation, an increase in total body bone mineral content, an increase in growth velocity, and reduced frequency of bone fracture in all patients [113]. This study demonstrates that mesenchymal progenitors in transplanted marrow can migrate to bone in children with OI and then give rise to osteoblasts whose presence correlates with an improvement in bone structure and function. However, with increasing time post-transplantation, growth rate slowed and eventually reached a plateau, so it was hypothesized that additional therapy using isolated MSCs without marrow ablative therapy would safely boost responses. They infused culture-expanded MSCs into children who had previously undergone conventional HCT and found that some cells engrafted in defective bone and differentiated to osteoblasts capable of extending the clinical benefits of HCT [114]. Thus, allogeneic MSCs can be safely transplanted to children with OI without provoking an immune response, and some cells home to the bone marrow.

There is much evidence to support the theory that MSCs can home to tissues, particularly when injured or inflamed, involving migration across endothelial cell layers. The mechanism by which MSCs home to tissues and migrate across endothelium is not yet fully understood, but it is likely that injured tissue expresses specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury, as is the case with recruitment of leukocytes to sites of inflammation. Chemokine receptors and their chemokine ligands are essential components involved in the migration of leukocytes into sites of inflammation, and it has recently been shown that MSCs also express some of these molecules. In addition, some of the adhesion molecules known to be involved in migration of leukocytes across the endothelium are also reported to be expressed on MSCs. Harnessing the migratory potential of MSCs by modulating their chemokine-chemokine receptor interactions may be a powerful way to increase their ability to correct inherited disorders of mesenchymal tissues or facilitate tissue repair in vivo.

The properties of MSCs make these cells potentially ideal candidates for tissue engineering.

3.4 MSCs and bone remodeling: the RANKL/OPG/RANK pathway

Bone is a dynamic tissue that is constantly formed and resorbed in response to changes in mechanical loading, altered serum calcium levels and in response to a wide range of paracrine and endocrine factors. The dynamic nature of the skeleton is achieved by a process

of remodeling; this is the co-ordinated actions of osteoclasts (cells that destroy bone) and osteoblasts (cells that form bone) as well as osteocytes within the bone matrix and osteoblast derived lining cells that cover the surface of bone. The co-ordinated actions of these cells is described as the "Basic Multicellular Unit". Within the BMU, cellular activity is matched (or "coupled"), a principle that the amount of bone destroyed by osteoclasts in the BMU is equal to the amount of bone formed by osteoblasts. Remodeling commences with the initiation of osteoclast formation, osteoclast-mediated bone resorption, a reversal period, and then a long period of bone matrix formation mediated by osteoblasts, followed by mineralisation of the matrix. Imbalances of remodelling can result in gross perturbations in skeletal structure and function, and potentially to morbidity and shortening of lifespan [115]. Whitin bone, osteoblasts and osteoclasts are able to act independently.

Osteoblasts are derived from mesenchymal progenitors via a process that requires the sequential action of the transcription factors Runx2 and osterix. Matrix-synthesizing osteoblasts are recognized by their cuboidal shape, their location on newly formed bone matrix, and the expression of relatively osteoblast-specific genes such as osteocalcin. During the process of bone formation, matrix-synthesizing osteoblasts have at least three potential fates: (1) Some of them become embedded within the bone matrix and are thereafter referred to as osteocytes, which continue to live and function within the mineralized tissue; (2) at the end of the remodeling cycle, some osteoblasts become flattened and then remain as quiescent lining cells

at the bone surface; and (3) some matrix-synthesizing osteoblasts die by apoptosis [116].

The osteoclast is a tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursors cells at or near the bone surface. The mature, multinucleated osteoclast is activated by signals, which leads to initiation of bone remodeling. The differentiation of mature osteoclast from precursors is dependent on their interactions with osteoblasts because the ablation of mature osteoblasts is sufficient to abrogate osteoclastogenesis in vivo. The osteoblast produces the key osteoclastogenic cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kB ligand (RANKL), which play an essential instructive role in the commitment of monocytic cells into osteoclast precursors. Once formed, osteoclasts have the unique ability to destroy (resorb) mineralised bone as part of bone modelling in early life, or as part of remodelling during adult life. This process occurs in three stages: osteoclast attachment and polarisation, initiation of resorption and cessation of resorption (perhaps followed by cell death). Osteoclasts are highly motile cells and migrate from one site of resorption to a new site. The capacity of the cells to fulfil this role is made possible by the ability to disassemble and reassemble their podosomes quickly. [117]. Recent breakthroughs in understanding of osteoclast differentiation and activation have come from the analysis of a family of biologically related tumour necrosis factor (TNF) receptor (TNFR)/TNF-like proteins: osteoprotegerin (OPG), receptor activator of nuclear factor (NF)-kB (RANK) and RANK ligand (RANKL), which together regulate osteoclast function [118].

In contrast to all other TNF receptor superfamily members, OPG lacks transmembrane and cytoplasmic domains and is secreted as a soluble protein. OPG mRNA was found to be expressed in a number of tissues, including lung, heart, kidney, liver, stomach, intestine, brain and spinal cord, thyroid gland, and bone. Because the major biologic action of OPG described to date has been to inhibit osteoclast differentiation and activity, the potential role of OPG in these other tissues remains to be established. OPG functions as a decoy receptor for RANKL [118].

RANKL is identified as an essential factor for osteoclastogenesis. RANKL mRNA is expressed at highest levels in bone and bone marrow, as well as in lymphoid tissues (lymph node, thymus, spleen, fetal liver, and Peyer's patches). RANKL is released from the cell surface as a soluble molecule following proteolytic cleavage by matrix metalloproteinases (MMPs) such as MMP-14. Both the soluble and membrane-bound RANKL forms function as agonistic ligands for RANK; however, the membrane-bound RANKL functions more efficiently than soluble RANKL. Its major role in bone is the stimulation of osteoclast differentiation, activity, and inhibition of osteoclast apoptosis. Indeed, in the presence of low levels of M-CSF, RANKL appears to be both necessary and sufficient for the complete differentiation of osteoclast precursor cells into mature osteoclasts. In addition, it is clear that RANKL has a number of effects on immune cells, including activation of c-*Jun* N-terminal kinase (JNK) in T cells, inhibition of apoptosis of dendritic cells, induction of cluster formation by dendritic cells, and effects on cytokine activated T cell proliferation. Osteoblasts and MSCs are

thought to be the major cell types that express RANKL in support of osteoclastogenesis. However, because RANKL is expressed by several different cell types in both bone and bone marrow, including osteoblasts, osteocytes, and MSCs, the actual major source of RANKL *in vivo* is as yet unclear [119].

The receptor for RANKL is RANK, a type I transmembrane protein, similar to others members of the TNF receptor family. RANK mRNA is highly expressed by isolated bone marrow-derived osteoclast progenitors and by mature osteoclasts *in vivo.* It is expressed primarily on cells of the macrophage/monocytic lineage, including preosteoclastic cells, T and B cells, dendritic cells, and fibroblasts [118]. Ligation of RANK with RANKL results in the commitment of monocyte/macrophage precursor cells to the osteoclast lineage and the activation of mature osteoclasts, leading to a cascade of intracellular signaling proteins that trigger the formation and sustained activity of mature bone-resorbing osteoclasts.

Expression of RANKL and OPG in bone remodeling process is therefore coordinated to regulate bone resorption and density positively and negatively by controlling the activation state of RANK on osteoclasts. RANKL is critical for the differentiation, fusion into multinucleated cells, activation, and survival of osteoclastic cells. OPG puts a brake on the entire system by blocking the effects of RANKL. A number of proresorptive cytokines, such as TNF-α and IL-1, modulate this system primarily by stimulating M-CSF production (thereby increasing the pool of preosteoclastic cells) and by directly increasing RANKL expression. In addition, a number of other cytokines and hormones, such as TGF-β (increased OPG

production), PTH (increased RANKL/decreased OPG production), 1,25-dihydroxyvitamin D3 (increased RANKL production), glucocorticoids (increased RANKL/decreased OPG production), and estrogen (increased OPG production) exert their effects on osteoclastogenesis by regulating osteoblastic/stromal cell production of OPG and RANKL [118]. There are several levels of control of the RANK signalling pathway that enhance or dampen osteoclastogenesis and activation driven by RANKL. The RANK signalling pathway is negatively controlled by OPG *in vitro* and *in vivo*. The T cell is also an important source of RANKL in the bone. Activation of T cells *in vitro* and *in vivo* leads to increased osteoclastogenesis and bone resorption, suggesting that acute and chronic inflammatory states, and certain leukaemias, contribute to pathologic bone loss.

In humans, mutations in RANK, RANKL, and OPG have been identified in patients with bone disorders, such as familial expansile osteolysis, autosomal recessive osteopetrosis, and Juvenile Paget's disease, respectively.

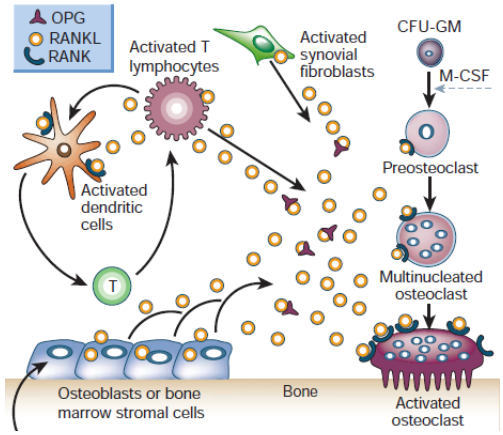

Figure 8. RANKL expression is induced in osteoblasts, activated T cells, synovial fibroblasts and bone marrow stromal cells (MSCs), and subsequently binds to its specific membrane-bound receptor RANK, thereby triggering a network of TRAFmediated kinase cascades that promote osteoclast differentiation, activation and survival. Conversely, OPG expression is induced by factors that block bone catabolism and promote anabolic effects. OPG binds and neutralizes RANKL, leading to a block in osteoclastogenesis and decreased survival of pre-existing osteoclasts (Boyle WJ et al, 2003).

4 Induced pluripotent stem cells

4.1 iPSCs: origin and features

In 2006 Yamanaka and colleagues made a breakthrough discovery that added another dimension to the stem cell field. This group found a combination of defined transcription factors (Oct4, Sox2, Klf4 and cMyc) that when introduced into murine fibroblasts by retroviruses, would induce reprogramming of the cell to a pluripotent state, resembling embryonic stem cells (ESCs) [120]. These cells were named induced pluripotent stem cells or iPSCs.

iPSCs are morphologically similar to ESCs: they grow as a colony, mainly on top of a monolayer of mitotically inactivated mouse embryonic fibroblasts and in the absence of serum. They express pluripotency markers such as TRA-1-60, TRA-1-80, SSEA-3, SSEA-4, Oct4, Sox2 and Nanog. In addition, they pass the hallmark test of pluripotency; when injected into immunocompromised mice, they form teratomas, showing their potential to differentiate *in vivo* into the three embryonic germ layers. Moreover, they also contribute to germ line transmission [121]. Furthermore, iPSCs have pluripotent

differentiation potential, independently of the tissue of origin. Various reports show differentiation into several committed functional lineages: blood cells displaying hematopoietic colony activity [122] [123]; motor neurons that are electrically active [124]; cardiomyocytes that beat rhythmically and respond to cardioactive drugs [125] and adipocytes [126] among others. DNA methylation analysis showed that on a global scale the methylomes of ESCs and iPSCs are highly similar, however at higher resolution substantial differences exist [127]. Nowadays, iPSCs are not only derived from fibroblasts. Different groups generated iPSCs from other somatic cells providing evidence that is possible to reprogram cells of different origins, such as keratinocytes, mesenchymal cells, oral mucosa cells, dental pulp cells, peripheral blood and cord blood, in addition to skin fibroblasts. Similarly, iPSCs have been generated from several species: mouse, rat, monkey, pig, dog, rabbit and human.

Initially, all the iPS cell lines have been generated transducing the four factors used by Yamanaka. However, it is possible to induce pluripotency in somatic cells by using only some of them. It has been reported that neural stem cells, which endogenously express Sox2, cMyc and Klf4, can be reprogrammed using only one factor, Oct4. This system offers the advantage of avoiding transfection of the oncogenes cMyc and Klf4 that could contribute to tumorigenesis in offspring. However, the reprogramming efficiency is very low [128] [129]. Alternative and integration-free methods have been reported in order to avoid viral integration for generating iPSCs. Two groups have shown that it may be feasible to induce iPSCs without viral integration. Stadtfeld et al. generated iPSCs from mouse hepatocytes

using adenoviruses carrying the four reprogramming factors [130]. In a other study, a different group generated iPSCs from mouse embryonic fibroblasts using plasmids [131]. Recently, iPSCs have been generated by genomic integration of the four reprogramming factors using plasmids [132], lentiviruses [133], or transposons [134], followed by transgene removal using Cre-mediated excision or re-expression of transposase. However, the efficiency of iPS cell generation using adenoviruses or plasmids is extremely low. Another way to avoid viral integration is to generate iPSCs using chemicals or small molecules. Several groups have already identified chemicals that can replace one or two reprogramming factors during iPS cell generation [135] [136]. More recently, has been shown that direct delivery of synthetic mRNA also generated iPSCs at high efficiency. The mRNA sustained high and relatively long expression of encoding reprogramming factors by using modified ribonucleotides [137].

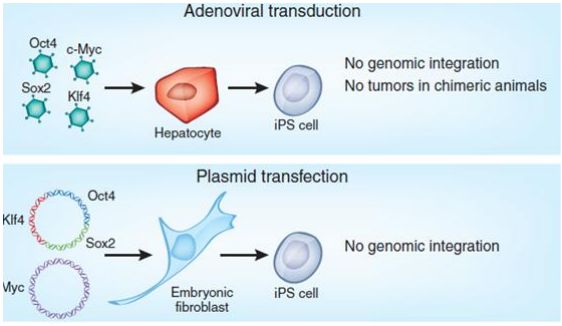

Figure 9. Different strategies to generate iPSCs: these new reprogramming approaches rely on transient expression of the reprogramming factors without the integration of ectopic DNA into the genome (Lowry WE et al, 2008)

4.2 iPSCs: short-term applications

One of the most valuable functions of iPSCs is modeling human diseases, above all those that are difficult to recreate in animals because of their restricted representation of human pathophysiology. Some of the limitations that animal models have, are due to their genomic differences, in terms of genetic background and number of chromosomes [138] [139]. A comprehensive report published by Park et al. in 2008 showed for the first time the feasibility of generating iPSCs from fibroblasts of patients with complex genetic disorders including Huntington and Parkinson's disease, diabetes mellitus and Down syndrome, among others. Subsequently, other groups successfully showed that it is possible to model diseases using iPS cell technology. One example is spinal muscular atrophy (SMA), a neuromuscular disorder caused by mutations in the SMN1 gene resulting in the degeneration of selected motor neurons. iPSCs established from a SMA patient maintain the disease phenotype and are capable of differentiation into motor neurons initially, but when compared to those derived from the patient's unaffected mother, they degenerate with time [140]. Another example is familial dysautonomia (FD), a rare but fatal peripheral neuropathy caused by a point mutation in the IKBKAP gene, involved in transcriptional elongation. The disease is characterized by the depletion of autonomic and sensory neurons. Neural crest precursors derived from FD-iPS cells show defects in IKBKAP splicing, neurogenesis and migration compared to those from non-affected control-iPS cells [141].

Patient specific-iPSCs promise to be a powerful system for drug discovery as well. Two recent reports show the response of

functionally active differentiated iPSCs to certain drugs. In the first study, iPS-SMA derived motor neurons are compared to those derived from a healthy donor after exposure to valproic acid and tobramycin, two compounds known to induce the expression of the survival motor neuron 1 (SMN1) protein. iPS-SMA derived motor neurons carry the SMN1 gene mutation that leads to reduced levels of its protein, however they respond to the drugs by increasing SMN1 protein expression in the same fashion as controls [140]. The second report shows that when neural crest precursors derived from familial dysautonomia iPSCs, that have a point mutation in the IKBKAP gene are treated with kinetin, the missplicing of IKBKAP is corrected [141]. In both reports, the drug treatment compensates the failure in the cellular mechanism of the disease showing the feasibility of identifying drug candidates using iPS cell models.

In addition to their use in modelling diseases and drug screening, the most promising purpose of iPSCs will be their potential application in regenerative medicine. The ultimate goal of this technology would be the transplantation of a lineage restricted progenitor cell, derived from a patient-specific iPS cell that does not trigger any immune response, does not promote tumor formation and that could recover the target-damaged tissue.

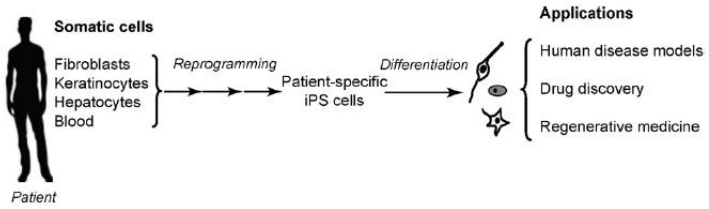

Figure 10. Schematic diagram showing the generation of iPS cells and their application (Vitale AM et al, 2011)

4.3 iPSCs and LSDs

Most lysosomal storage diseases are life-threatening genetic diseases. The pathogenesis of these diseases is poorly understood. Induced pluripotent stem cell technology offers new opportunities for both mechanistic studies and development of stem cell–based therapies. The paucity of mechanistic studies of LSDs is due largely to the absence of faithful in vitro disease model systems. iPSCs offer the opportunity to establish such in vitro models of LSDs, because they can be generated from skin fibroblasts of patients and theoretically can differentiate into multiple cell types in vitro. In addition, the embryonic stem cell–like properties of iPSCs permit to study the disease in a culture system at a very early stage of embryogenesis that is difficult or nearly impossible to study *in vivo*, especially in humans. This unique capability provides an invaluable tool for investigating that abnormal early embryonic development that may occur in these genetic disorders.

To date only few studies have been described to use the isolation of iPSCs for the study of a LSD. Meng and collaborators generated iPSCs from mouse models of Fabry disease, globoid cell leukodystrophy (GLD) and mucopolysaccharidosis VII (MPSVII), demonstrating defects in disease-specific enzymes and the accumulation of substrates of their enzymes. Fabry-iPS and GLD-iPSCs were efficiently differentiated into disease-relevant cell types in vitro. MPSVII-iPSCs exhibited a decreased ability to form embryoid bodies, suggesting possible abnormal embryogenesis in MPSVII [142]. Similarly, iPSCs were isolated from the fibroblasts of a murine model of Pompe disease. The generated Pompe-iPSCs were

successfully differentiated into skeletal muscle cells and accumulated massive glycogen in lysosomes as a Pompe disease model [143]. In a human setting, Tolar and colleagues described the isolation of iPSCs from patients with mucopolysaccharidosis type I (Hurler syndrome). They differentiated iPSCs-MPS IH into a hematopoietic progeny of gene-corrected autologous cells via lentiviral transduction [144]. More recently, iPSCs have been generated from the skin fibroblasts of children with mucopolysaccharidosis type IIIB (Sanfilippo syndrome type B). The NAGLU enzyme deficiency affected the proliferation of emerging iPS clones, necessitating compensation of the genetic defect through enzyme replacement for efficient isolation. The isolated iPSCs rapidly exhibited prominent cell pathology consisting of intracellular vacuoles and severe disorganization of Golgi complex structure, once exogenous enzyme supply was halted. The examination of patients' pluripotent stem cells and their neural and neuronal progenies revealed for the first time the existence of Golgi defects in a lysosomal disorder [145].

4.4 Gene targeting of iPSCs ZFNs-mediated

iPSCs are considered a potential source of patient-specific autologous effector cells in regenerative medicine and gene therapy, as they preclude the risk of transplant rejection and, in the case of iPS-derived hematopoietic stem cells, the development of graft-versus-host disease [146] [147]. To answer specific questions in biomedical research or to generate therapeutically relevant effector cells for regenerative medicine, technologies for the precise modification of

iPSC or ESC genomes are highly warranted. Various approaches have been used to alter the genome of pluripotent stem cells.

Some genome engineering protocols involved the use of retroviral vectors or transposons, which in many instances yield high integration efficiencies and stable transgene expression [148] [149]. However, both retrovirus and transposon- based vector systems insert their cargo more or less randomly throughout the genome of the host cell, thus precluding the precise manipulation of prespecified target genes [150] [151]. Moreover, randomly inserted transgene cassettes are subject to chromosomal position effects that often lead to partial or complete gene silencing [152].

Gene targeting by homologous recombination (HR) circumvents these problems and the technology has successfully been applied in mESCs since the late 1980s for generating knockout mice [153]. However, conventional gene targeting in mESCs is a painstaking and time-consuming process because the frequency of HR in mammalian cells (10^{-5} to 10^{-8}) is ~1000 times lower than illegitimate recombination.

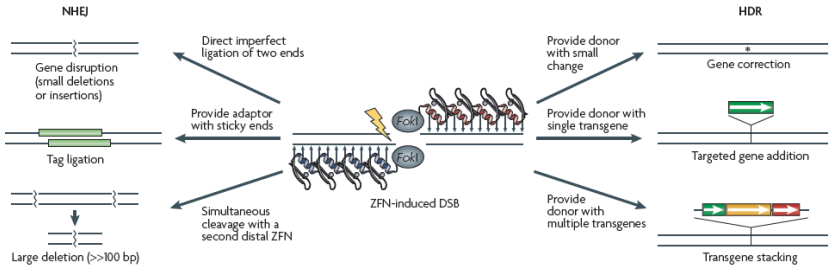

Figure 11. Types of genome editing made possible using Zinc Finger Nucleases (Urnov FD et al, 2010)

Higher HR efficiencies are achieved using a technology based on zinc-finger nucleases (ZFNs). ZFNs are molecular scissors that induce DNA double-strand breaks (DSBs) at pre-specified genomic sites. ZFNs consist of a specific DNA-binding domain fused to the non-specific, catalytic domain of the FokI endonuclease [154]. The DNA-binding domain usually consists of three or four custom designed Cys_2-His_2 zinc-finger motifs, oriented as tandem repeats. Each of the zinc-finger motifs recognizes about three nucleotides of the target DNA. Because the nuclease domain is only active as a dimer [155], two ZFN subunits have to bind the DNA in correct orientation and spacing to achieve cleavage. Depending on the number of zinc-fingers per subunit, the target site comprises 18 or 24 bp. Upon dimerization, the nuclease domain cleaves the DNA at the desired site and activates the cellular DNA damage response.

The enzymatically-induced DSB can be repaired either by error-prone non-homologous end-joining (NHEJ), leading to short insertions or deletions [156], or by homology-directed repair (HDR), based on HR between an exogenous donor DNA and the target locus [157]. NHEJ can be exploited for creating functional gene knockouts [158]. Furthermore, two ZFN pairs targeting adjacent sites on a chromosome can be used to delete the interjacent sequence [159] [160]. On the other hand, ZFN-triggered HDR can be harnessed for targeting the integration of transgenes, like for example the knock-in of reporter cassettes to produce isogenic reporter cell lines. Finally, a donor DNA can be designed to correct inborn mutations in therapeutic applications. Typically, a ZFN-induced DSB increases the frequency of HR several 1000-fold [161] [162].

Overall, the ZFN technology has been successfully applied in more than 10 organisms, including Drosophila, plants, zebrafish, rat, and mouse or human pluripotent stem cells [163] [164] [165] [166].

Since the initial reports on the ability of ZFNs to stimulate HDR, the technology has improved significantly by implementing several important changes: (i) increasing the DNA-binding specificity of ZFNs based on novel design platforms, (ii) optimizing the architecture of the nuclease domain to decrease the frequency of off-target events, and (iii) using a set of matched interdomain linkers to augment target site selectivity.

First successful ZFN-mediated genome editing experiments in pluripotent stem cells were reported by Lombardo et al. in 2007. Aiming to insert an eGFP expression cassette into the CCR5 locus of human ESCs, the authors obtained a targeting frequency of 3.5% in the absence of selection. Two more recent studies provided further evidence of ZFN-stimulated gene targeting in human ESCs and human iPSCs [164] [165].

Recently, a few studies have reported the *AAVS1* site, as a ‘‘neutral site’’ for transgene integration in the human genome. ZFNs have been designed and used to target the *AAVS1* locus [167] [164]. This locus is commonly targeted for long-term stable transgene expression in a number of cell types including hESCs. Integration of adeno-associated virus (AAV) disrupts the *PPP1R12C* gene on chromosome 19, commonly referred to as the *AAVS1* locus; however, this targeting event is not associated with any pathophysiology [168]. Both hESCs and hiPSCs with a disrupted *PPP1R12C* gene, either through conventional gene targeting or ZFN-driven gene addition,

proliferate normally, have a normal karyotype, express pluripotency markers, and maintain pluripotency [167] [164] [168]. Thus, the *AAVS1* locus is designated a safe harbor as there are no deleterious effects following its disruption and stable and prolonged expression of the transgene in many cell types with both promoterless and promoter-containing cassettes. In addition, delivery to a safe harbor can achieve isogenic settings for analysis due to the specificity of ZFN-mediated gene addition at this site and hence the avoidance of random integrations in human iPSCs. Two studies, from Hockemeyer et al. and Dekelver et al. demonstrated that a number of different expression cassettes can be targeted to and integrated into the *AAVS1* locus in hESCs and hiPSCs in a single step using plasmid DNA constructs. Robust and long-term expression of the transgene can be achieved either with the native *PPP1R12C* promoter or exogenous promoters in the inserted expression cassette, with no effect on cell growth.

5 Scope of the thesis

The scope of the present PhD project was focused on the isolation and characterization of two different stem cell populations, multipotent and pluripotent, derived from patients affected by Mucopolysaccharidosis type I (Hurler syndrome). We aim to use Mesenchymal Stem Cells (MSCs) and induced Pluripotent Stem Cells (iPSCs) as a tool to explore still unknown disease mechanisms involved in the genetic metabolic disorder of our interest.

The first chapter provides a general introduction on pathologic features of Hurler disease, as well as on the peculiar characteristics of the stem cell sources used in this study, supplying basic literature elements to better understand the developed project.

The second chapter presents our recently published study focused on the characterization of MSCs isolated from bone marrow of Hurler patients. The MSCs were characterized for their expansion rate, phenotype, telomerase activity, IDUA activity and differentiation capacity towards adipocytes, osteoblasts, chondrocytes and smooth muscle cells *in vitro.* Interestingly, affected MSCs displayed increased capacity to support osteoclastogenesis according to the upregulation of the RANKL/RANK/OPG molecular pathway in Hurler MSCs.

The third chapter describes the isolation of iPSCs from fibroblasts of Hurler patients. The generated cell lines were fully characterized for their pluripotency markers, gene expression profile, viral copy number integration and differentiation potential both *in vitro* and *in*

vivo. As a proof of principle, we are attempting to gene correct patient-derived iPSCs with an alternative and safer method than viral vectors, using a Zinc Finger Nucleases-mediated approach for gene targeting of pluripotent cells.

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Chapter 2

Hurler disease bone marrow stromal cells exhibit altered ability to support osteoclast formation

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Abstract

Mucopolysaccharidosis type I (MPS IH; Hurler syndrome) is a rare genetic disorder, caused by mutations in the IDUA gene, resulting in the deficiency of α-L-iduronidase enzyme activity and intra-cellular accumulation of glycosaminoglycans. A characteristic skeletal phenotype is one of the many clinical manifestations in Hurler disease. As the mechanism(s) underlying these skeletal defects are not completely understood, and bone and cartilage are mesenchymal lineages, we focused on the characterization of mesenchymal cells isolated from bone marrow (BM) of five Hurler patients. IDUA mutated BM stromal cells (BMSC) derived from MPS I patients, exhibited decreased IDUA activity, consistent with the disease genotype. The expansion rate, phenotype, telomerase activity, and differentiation capacity towards adipocytes, osteoblasts, chondrocytes and smooth muscle cells *in vitro* of MPS I BMSC lines were similar to that of BMSC from age matched normal control donors. MPS I BMSC had also similar *in vivo* osteogenic capacity as normal BMSC. However, MPS I BMSC displayed increased capacity to support osteoclastogenesis which may correlate with the upregulation of the RANKL/RANK/OPG molecular pathway in MPS I BMSC compared to normal BMSC.

Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive metabolic disorder caused by mutations in the IDUA gene, leading to inactivity of the lysosomal enzyme α-L-iduronidase. This causes a progressive intracellular accumulation of nonmetabolized glycosaminoglycans (GAGs), dermatan and heparan-sulfate [1,2]. This accumulation disrupts cell functions and gives rise to multiorgan dysfunction. MPS I is a highly heterogeneous disorder with a wide spectrum of clinical manifestations. MPS IH or Hurler disease is the most severe form of the disease, affecting approximately 50% of all patients with MPS. The condition is marked by hepatosplenomegaly, obstructive airway disease complicated by respiratory infections, cardiac failure, and progressive mental retardation. The array of musculoskeletal abnormalities observed in Hurler syndrome is known as *dysostosis multiplex*, and underlies the typical dwarfism and gargoyle-like features. These abnormalities arise from a disarray of skeletal remodelling, disordered endochondral and intramembranous ossification, and infiltration of GAGs into ligaments, tendons, joint capsules, and other soft tissue structures. This leads most often to gibbus deformity, prominent sternum, bulging forehead, slowing of physical growth, joint contractures, kyphoscoliosis and subluxation of the hip joints [3]. Although allogeneic hematopoietic stem cell transplantation can alleviate some of the systemic complications of MPS IH, it cannot - or only partially can - prevent and correct the skeletal anomalies associated with the disease [4-6]. A possible explanation for the lack of improvement in the skeletal defects after transplant is poor penetration of α-L-iduronidase, produced by the

donor leukocytes into host skeletal tissues [7], despite the fact that bone is a highly vascularized tissue. The cellular and molecular mechanisms underlying the skeletal disease in MPS I remain largely unknown. Studies in the MPS I mouse model revealed aberrant bone remodelling, as well as growth plate development and maturation with abnormalities in the early cortical bone structure [8]. In young mice, the growth plate appeared thickened with a high number of chondrocytes, and a disorganized structure was observed in the hypertrophic zone. Arrangement of the primary trabecular bone was abnormal and an increased presence of cartilage within the woven bone was observed, suggesting difficulties in cartilage resorption during endochondral ossification. Wilson *et al.* have recently demonstrated that high concentrations of GAGs in MPS I may inhibit the collagenolytic activity of cathepsin K, a lysosomal cysteine protease highly expressed by osteoclasts and responsible for a significant part of total bone resorption. These studies suggest that an impairment of cathepsin K activity may contribute to the deranged bone remodelling in MPS I [9]. In another study, Simonaro *et al.* reported that GAGs storage in MPS leads to inflammation, apoptosis of cartilage cells, and synovial hyperplasia, resulting in poorly organized and metabolically abnormal connective tissue matrices [10]. In addition, they reported that the Toll-like receptor 4 (TLR4) signalling pathway may have a significant role in the pathogenesis of MPS bone and joint disease, as treatment of MPS VI rats with an anti-TNF-α drug dramatically reduced the inflammation in the joints, which was associated with improved joint pathology [11].

Bone marrow stromal cells (BMSC) contain a sub-population of multipotent stem cells capable of differentiating toward multiple skeletal cell types, including osteoblasts (bone), chondrocytes (cartilage), adipocytes (BM fat), fibroblasts (periosteum), and adventitial reticular cells (BM stroma) [12]. The involvement of BMSC in skeletal disorders is well known, such as in fibrous dysplasia of bone, Paget's disease, hyperparathyroidism, osteoporosis and osteoarthritis [13-17]. The evaluation of the biological and functional characteristics of BMSC from patients affected by these diseases has provided additional insights into some of the pathogenetic mechanisms underlying the disorders. One study analyzed BMSC from MPS I transgenic mice. No differences were found in the basic features (clonogenicity, expansion and differentiation capacity) of transgenic BMSC compared to wild-type [18]. To study a possible involvement of BMSC in the complicated skeletal abnormalities affecting Hurler patients, we isolated BMSC from pediatric patients with MPS IH disease and evaluated their properties in comparison with BMSC from healthy pediatric donors. Aside from characterizing the IDUA molecular mutations and IDUA enzyme activity, we evaluated the phenotype and differentiation ability of MPS I BMSC into chondrogenic, osteogenic, adipogenic, and smooth muscle lineages. As stromal cells are also involved in the activation as well as differentiation of osteoclasts, we also assessed the ability of MPS I BMSC to stimulate osteoclastogenesis *in vitro.* Receptor activator of nuclear factor-κB (RANK), osteoprotegerin (OPG) and the RANK ligand (RANKL) have been identified as key partners of a system that directly regulates osteoclast differentiation

[19]. In particular, RANKL binds to RANK present on osteoclast precursors, inducing their differentiation into osteoclasts. OPG is a decoy receptor, which acts by neutralizing and preventing RANKL from binding to RANK, resulting in decreased osteoclast recruitment. Alterations in the balance of the OPG/RANK/RANKL molecular triad are associated with numerous bone diseases [20]. Here we show that MPS I BMSC display excessive osteoclastogenetic capacity probably related to dysregulation of RANKL and OPG expression.

Materials and Methods

Isolation and culture of human BMSC

Human BMSC were obtained from BM of pediatric healthy donors (n=4; aged 7 months, 1 year, 7 years, 9 years) and MPS IH patients (n=5; aged 9 months, 11 months, 11 months, 13 months, 20 months), with informed consent per institutionally approved protocols. BMSC were isolated and cultured as previously described [21]. Briefly, a Ficoll-gradient separation of BM cells was performed and mononuclear cells (BMMNC) were seeded at a density of 2×10^5 cells/cm^2 in basal growth medium, containing DMEM-LG (Invitrogen, San Giuliano Milanese, Italy), 10% fetal bovine serum (FBS) (Biosera, Ringmer, UK), 1% Penicillin-Streptomycin (Invitrogen) and 1% L-glutamine (Invitrogen). After 48 hours, medium was changed and the resulting adherent fibroblast-like cells were cultivated at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, medium was replaced twice a week. Cells were maintained in basal medium until they reached 70% of confluence and harvested with 0.05% trypsin (Invitrogen). For subsequent expansion,

cells were plated at a density of 2×10^3cells/cm^2 and maintained in culture for several passages.

Colony-Forming Unit-Fibroblast (CFU-F) assays

The number of BMSC progenitors was determined in 3 patients by the colony-forming-unit fibroblast (CFU-F) assay, as previously described [22]. Briefly, BMMNC were seeded in a P100 dish (Nunc, Rochester, MN) at serial dilutions from 1.5×10^6cells/dish to 1×10^4cells/dish and maintained for 14 days in basal medium. To enumerate CFU-F, dishes were washed 3 times with PBS, cells fixed with methanol, and stained with the Giemsa solution. CFU-F were scored at 20X magnification. The experiment was performed in triplicate for each sample.

Proliferation kinetics of BMSC

The population doublings (PDs) were calculated as follows: PDnew=PDinitial+[log(C_0/C_1)]/log2, wherein C_0=cell number initially seeded and C_1= cell number harvested. The PDs of cells from P2 to P7 were determined. Three sets of cultures were repeated for each sample.

Flow cytometric analysis

BMSC at passage 3 were labelled with phycoerythrin-(PE) or fluorescein isothiocinate-(FITC) conjugated antibodies against CD14, CD34, CD45, CD90, CD73, CD105, CD146, HLA-ABC, and HLA-DR. Isotype antibodies were used as control (all antibodies were purchased from BD Biosciences, Franklin Lakes, NJ). Flow cytometric analysis was performed on 20,000 events with the use of a FACScan cytometer, and data were analyzed using the CellQuest software (BD Biosciences).

Cell cycle analysis

BMSC at passage 4 were harvested 48, 96 and 144 hrs after seeding and washed twice with ice-cold PBS (Gibco, Grand Island, NY). Cells were resuspended in GM saline solution and permeabilized with ice-cold 70% ethanol, followed by incubation with 2ml of a solution containing 10μg/ml propidium iodide plus 25μl 1mg/mL RNAse 10.000 Units overnight [23]. Flow cytometric analysis was performed on 20,000 events by using a FACS Calibur, and the cell cycle phase distribution were calculated as percentages by a Gaussian-modified method [24].

Detection of telomerase activity

Proteins were extracted from $1x10^6$ BMSC at passage 6 and telomerase activity determined on 1μg of protein extract per sample using the TRAPeze RT telomerase detection kit (Chemicon, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. This method measures the incorporation of telomeric repeats (TTAGGG) to the 3'end of a substrate oligonucleotide. The extension products are amplified and measured by quantitative RT-PCR (Q-RT-PCR) using Ampifluor® Primers and the produced fluorescent emission is directly proportional to the amount of telomeric repeats (= copy numbers).

α-L-iduronidase enzyme assay

IDUA activity was determined according to Clements *et al.* [25]. Briefly, cells were resuspended in 150 mM NaCl and freeze-thawed 6 times. Protein extracts were assayed for total protein content using the Lowry assay. 5 μg of protein was added, in triplicate, to a solution containing 8 mM D-Saccharic acid 1,4-lactone and 2 mM 4-

methylumbelliferyl-α-L-iduronide in 0.1 M sodium formate buffer, pH 3.2. Samples were incubated for 1 hr at 37°C before stopping the reaction by adding 1 ml of 0.5 M carbonate buffer, pH 10.7. The cleaved substrate was quantified on a Perkin Elmer fluorometer. The enzyme activity was calculated from a reference curve obtained by using 4-methylumbelliferone and was expressed as nmoles/hr/mg.

***In vitro* Differentiation of BMSC**

Differentiation potential of cultured BMSC was assessed using standard methods [21, 26-27].

For chondrogenic differentiation, BMSC at passage 2 were seeded in a 15 ml conical tube at a density of $2.5x10^5$ cells/tube and resuspended in Chondrogenic Differentiation Medium consisting of DMEM-High glucose (Invitrogen) supplemented with ITS+premix (Collaborative Biomedical Products, Bedford MA, USA), 1 mM pyruvate (Sigma), 50 μg/ml 2-phosphate–ascorbic acid (Fluka), 100 nM dexamethasone (Sigma), and 10 ng/mL TGF-β3 (Sigma). Cells were grown as pellets for 3 weeks at 37°C, 5% CO_2. For histology, the resulting tissues were embedded in paraffin and stained with Hematoxilin-Eosin (Sigma). Sulfated GAGs were visualized by staining with Alcian Blue (Sigma). For Collagen type II immunolocalization, sections were deparaffinized and exposed to 3% hydrogen peroxide for 30 minutes. Monoclonal anti Coll type II antiserum (Abcam, Cambridge, UK) was diluted 1:100 and applied over-night at 4°C. The reaction was developed by using an indirect immunoperoxidase protocol. For the assessment of chondrogenic expression profile, transcript levels for *type II collagen (COL2A1), type X collagen (COL10A2), SRY-BOX9 (SOX 9) and aggrecan (ACAN),* were analysed by Q-RT-PCR at day 21 of culture.

For osteogenic differentiation, BMSC at passage 3 were seeded at a density of $6x10^3$ cells/cm^2 in basal medium. After 48 hours, medium was switched to Osteogenic Induction Medium, consisting of DMEM-Low glucose (Invitrogen), supplemented with 10% FBS (Biosera), 100 nM dexamethasone (Invitrogen), 10 mM B-glycerol-phosphate (Invitrogen) and 0.05 mM 2-phosphate-ascorbic acid (Invitrogen). The osteogenic differentiation was assessed through the Alizarin Red S (Sigma) staining on day 21 of differentiation. In parallel experiments, transcript levels for *alkaline phosphatase (ALP), type I collagen (COL1A2), osteonectin (SPARC), runt-related transcription factor 2 (RUNX2), osteopontin (SPP1), osteocalcin (BGLAP), receptor activator for nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG)* were analyzed by Q-RT-PCR at day 0, day 7, day 14 and day 21 of culture.

For adipogenic differentiation, BMSC at passage 3 were seeded at a density of $2x10^4$ cells/cm^2 in basal medium. After 24 hours, medium was switched to Adipogenic Induction Medium, consisting of DMEM-High glucose (Invitrogen), supplemented with 10% FBS (Biosera), 1μM dexamethasone (Sigma), 1μM indomethacin (Invitrogen), 500 μM 3-isobutyl-1-methylxantine (IBMX, Sigma) and 10 μg/ml human recombinant insulin (Sigma). For the detection of adipogenic differentiation, intracellular lipid droplets were stained by Oil Red O solution (Sigma) on day 18 of differentiation. At day 21, transcript levels for *fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPARG)* were analysed by Q-RT-PCR.

For smooth muscle cell (SMC) differentiation, BMSC at passage 3 were plated at 5×10^3 cells/cm^2 on 100 ng/ml fibronectin-coated (Sigma-Aldrich) wells or cover glasses. After 24 hours, differentiation was induced in serum-free basal medium (Invitrogen) supplemented with 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN). On day 0 and day 6 of differentiation, the expression of SMC specific proteins, alpha smooth muscle actin (α-SMA) and calponin (CALP), was assessed by immunofluorescence. Microscopy analysis was performed with confocal laser microscopy (Radiance 2100; Biorad Laboratories, Hercules, CA, USA). At day 6, transcript levels for *alpha smooth muscle actin (ACTA2), calponin (CNN1) and transgelin (TAGLN)* were analysed by Q-RT-PCR.

RNA isolation and Q-RT-PCR reaction

Total RNA was extracted using TRIZOL® reagent (Invitrogen), following the manufacturer's protocol. 1 μg of RNA was then reverse transcribed with the use of a SuperScript® II Reverse Transcriptase kit (Invitrogen) in the presence of random hexamers. Quantitative real-time polymerase chain reaction assays were performed in triplicate on an ABI 7900 Real-Time PCR system thermal cycler with the qPCR Mastermix (Applied Biosystems). All TaqMan Gene Expression assays were provided by Applied Biosystems, see supplemental table 1. The relative expression of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as reference gene. Total mRNA levels were quantified using the comparative threshold cycle method. Data are represented as DDCt, calculated considering the baseline observation (expression levels of each group at day 0) as reference for cases and controls.

Coculture of BMSC and CD34+ purified cells

BM cell suspensions were centrifuged on Ficoll-Paque (Pharmacia Biotech, Piscataway,NJ) to isolate BMMNC. CD34+ cells were purified using the MidiMACS CD34 isolation kit (Miltenyi Biotec, Auburn, CA). CD34+ cell purity was 95-97% by flow cytometry. BMSC at passage 5-7 were seeded at $1.5x10^3$cells/cm^2 and cultured in basal medium until 80% confluence was reached. Aliquots of CD34+ enriched cells ($3x10^4$cells/cm^2) were plated on the monolayers of BMSC and cocultured at 37°C in 95% air-5% CO_2 for up to 3 weeks. Every 3 days, half the culture medium was carefully removed to minimize loss of non-adherent cells and was replaced with an equal volume of fresh medium.

Tartrate-resistant acid phosphatase (TRAP) staining

The medium was removed from BMSC-C34+ cell cocultures and the cells fixed by addition of 60% acetone solution in citrate buffer (pH 5.4) for 30 sec. Cells were washed twice with distilled water and air dried. TRAP-positive (TRAP+) cells were detected using a commercial TRAP staining kit (Sigma Chemical Co). TRAP+ mononucleated and multinucleated cells were counted manually in representative areas of each culture using an inverted-phase contrast microscope (Axiovert 40 C, Zeiss). In some experiments, cells were counterstained after TRAP staining with 49,6-diamidino-2-phenylindole (Molecular Probes, Inc., Eugene, OR) to visualize the nuclei.

Elisa for the quantitative determination of free human RANKL and OPG

Human sRANKL and human OPG ELISA (Biomedica, Wien, A) were performed on supernatants of cultured BMSC and osteogenic differentiated BMSC, according to the manufacturer's instructions. The samples for OPG testing were diluted 1:5.

***In vivo* transplantation**

In vivo transplantation of human BMSC was performed as reported [28,29]. All animal procedures were approved by the animal ethics committee of Biomedical Science Park, University La Sapienza, Rome. Briefly, $2x10^6$ BMSC at passage 3 were loaded onto 40 mg of hydroxyapatite/tricalcium phosphate particles (100–200 μm; Zimmer, Warsaw IN) and embedded in a fibrin gel to generate carrier-cell constructs. The constructs were transplanted subcutaneously into 8- to 15-week old female SCID/beige mice (Harlan-Sprague Dawley, Indianapolis, IN). Samples were harvested after 8 weeks, fixed in 4% formaldehyde, decalcified in 10% EDTA pH 7.2 and embedded in paraffin. Deparaffinized and rehydrated sections were stained with hematoxylin and eosin as described previously [30]. Transplants of carrier alone were used as control. Histomorphometry measurement of bone volume/total volume (BV/TV, %) was performed on routinely processed samples by using a Leica Leitz DM RB image analyzer.

Statistical Analysis

Continuous data are described by mean and standard deviation (SD). The presence of clustering due to the repeated measurements on each case/control is taken into account in the calculation of SD. One sample T-test was used to compare the mean counts of colony-forming

efficiency (CFE) in controls to the average counts on a single case considered as a reference. Differences between groups were tested by the nonparametric rank sum test for clustered data [31] or by an unpaired 2-tailed T-test. The Mann-Whitney test was used when normality test failed. All tests were one-sided at a significance level of 5%. The package R version 2.10.0 (2009 The R Foundation for Statistical Computing) was used for statistical analysis. P-values <0.05 were considered significant.

Results

Expansion capacity and telomerase activity is similar for BMSC isolated from MPS IH or healthy donors

BMSC were isolated from five patients affected by MPS IH (hereafter named MPS I BMSC) before they were treated with hematopoietic stem cell transplantation. Four pediatric healthy donors (hereafter named HD BMSC) served as controls. MPS I patients were aged 11 months (female, patient n°1), 13 months (female, patient n°2), 20 months (female, patient n°3), 9 months (male, patient n°4) and 11 months (female, patient n°5). All patients had biochemical and molecular confirmation of the diagnosis and presented with coarse facies, multiple dysostosis including dorsolumbar spine kyphosis and hip dysplasia and other typical clinical signs. BM cells harvested from MPS IH patients formed discrete colonies similarly to cultures of control donors. BMSC lines could be established from all MPS IH patients. We quantified the colony-forming efficiency (CFE), which is the number of CFU-F formed after seeding a clonogenic concentration of BMMNC from 3 MPS IH patients and 4 HD. The CFE was

decreased in MPS I BMMNC (P=0.02) in comparison with healthy pediatric donors (Figure 1, panel A).

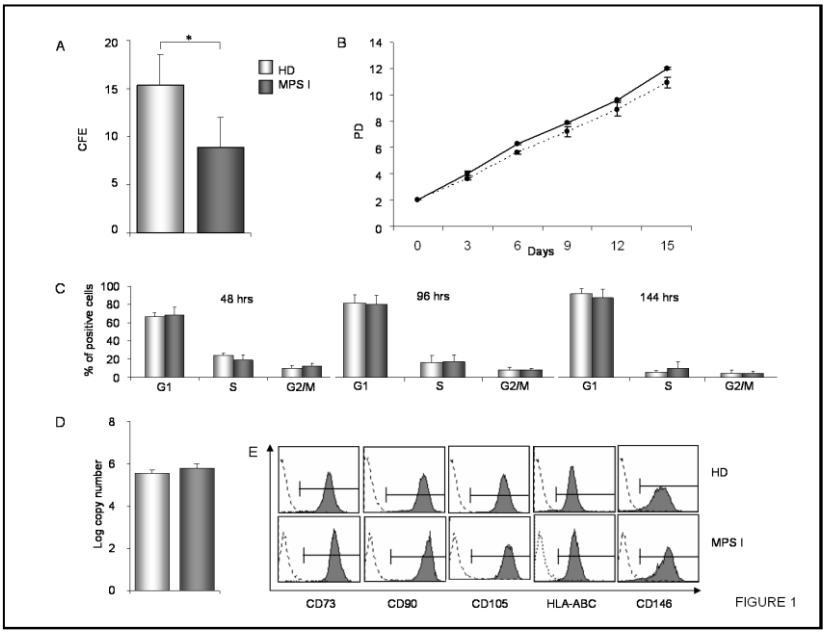

Figure 1. Clonogenicity, expansion capacity, telomerase activity and immunophenotype of MPS I BMSC.

(A) CFU-F in cultures from MPS I and HD BMMNC. The dark grey bar represents three MPS I patients and the light grey bar the CFE values of age-matched groups of four normal donors (both groups performed in triplicate). **(B)** Expansion curve of MPS I BMSC and HD BMSC. The graph represents the mean ± SD of 3 HD subjects (dotted line) *versus* 3 MPS-I patients (continuous line). PD=population doubling **(C)** Cell cycle analysis. The percent cells in the different phases of the cell cycle was assessed by propidium iodide (PI) staining in flow cytometry. PI incorporation was evaluated on at least 20,000 cells per sample 48, 96 and 144 hrs after replating. The graph represents the mean ± SD of the three different subjects (3 HD *versus* 3 MPS I). **(D)** Telomerase activity. Telomerase activity of BMSC measured at passage 6 of culture using the TRAPeze assay. Results are shown as the average logarithm of the copy number (=incorporated telomeric repeats). The graph represents the mean ± SD of 3 HD subjects *versus* 3 MPS I patients. **(E)** FACS analysis of MPS I and HD BMSC at passage 3. Isotype control staining (white area under dotted line) *versus* the specific antibody staining profile (grey area) is shown. The upper panels represent healthy donor (HD) BMSC and the lower panels an MPS I BMSC population. *P<0.05.

No differences in morphology of MPS I and HD BMSC could be detected (data not shown). Although it has been reported that MPS I fibroblasts proliferate more quickly than their normal counterparts [32], we found no differences in PDs between MPS I and HD BMSC (Figure 1, panel B). To confirm that MPS I BMSC expansion capacity was similar to that of HD BMSC we performed cell cycle analysis at different time points. The cell cycle distribution pattern was similar for MPS I and HD BMSC (Figure 1, panel C). For both MPS I and HD BMSC, the majority of cells were in the G0/G1 phase of the cell cycle (69±9% for MPS I BMSC *versus* 66±5% for HD BMSC) at 48h after seeding, and the remaining cells were similarly distributed in SG_2M phase. Likewise, no differences in cell cycle analysis could be detected between MPS I and HD BMSC at 96 and 144 hours after seeding. We could also not detect differences in telomerase enzyme activity, as evaluated by the telomeric repeat amplification (TRAP) method, between HD and MPS I BMSC cells at passage 6 (Figure 1, panel D).

Immunophenotype of MPS I and HD BMSC is similar

To further characterize MPS I BMSC, surface protein expression was examined at passage 3 by flow cytometry. BMSC did not express the hematopoietic antigens, CD45, CD14 and CD34. More than 95% of MPS I BMSC expressed CD73, CD90, CD105, CD146. In one MPS I and one HD BMSC line, 74.4 and 77.3% of the cells was CD146 positive, whereas the other cell lines were 98.3±0.5 CD146+. More than 70% of the BMSC derived from all donors expressed HLA-ABC, however none of the BMSC expressed HLA-DR. Selected antigens

representing an MPS I BMSC line and an HD BMSC line are depicted in Figure 1, panel E.

Mutation analysis and iduronidase activity in MPS I BMSC

The disease-specific genotype of MPS I BMSC was compared with that of their primary somatic counterparts. Sequence analysis of the IDUA gene in the three MPS I BMSC lines revealed the previously identified mutations (Figure 2, panel A).

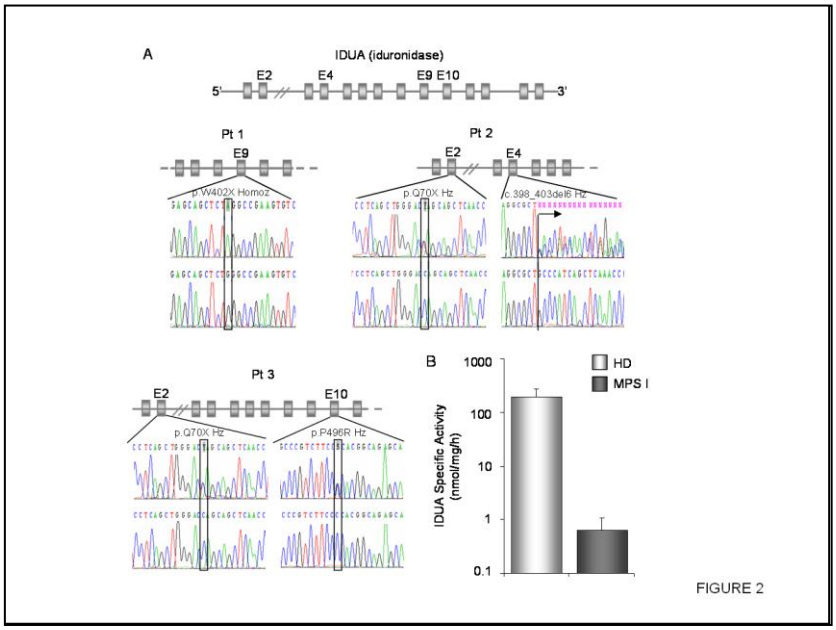

Figure 2. Mutation analysis and iduronidase activity in MPS I BMSC.
(A) Molecular analysis of IDUA mutation. Automated sequencing of the IDUA coding region has identified the mutations in the genomic DNA of MPS I patients. The panel shows the positions of mutations (electroferograms higher) compared with the wild-type sequence (electroferograms lower) in BMSC derived from the three different patients. **(B)** IDUA activity evaluation. IDUA enzymatic activity was measured in the extracts of BMSC at passage 3. Activity was expressed as nmoles of enzyme normalized against protein content (mg) and incubation time (hr). Each bar represents the mean ± SD of 3 subjects.

BMSC derived from MPS IH patient n°1 (Pt 1) were homozygote for the p.W402X mutation in exon 9. Molecular analysis of MPS I patient n°2 (Pt 2) revealed a compound heterozygosity for the p.Q70X mutation in exon 2 and the c.398_403del6 mutation in exon 4, which is a deletion of 6 nucleotides (TGGGCA) that causes an in frame deletion of the aminoacids M133 e G134. MPS I BMSC from patient n°3 (Pt 3) revealed a compound heterozygosity for p.Q70X and p.496R mutations. The p.Q70X (c.208C>T) and p.W402X (c.1205G>A) introduce a premature STOP codon at position respectively 70 and 402 of protein chain; the p.P496R missense mutation in exon 10 (c.1487C>G), resulting in a non conserved aminoacid change (Proline > Arginine) at position 496 of protein chain. In addition, we evaluated the specific IDUA enzymatic activity of MPS I BMSC and compared this with the enzymatic activity of HD BMSC. As shown in figure 2 (panel B), the mean IDUA activity of MPS I samples (0.63±0.45) was reduced to less than 1% of HD BMSC IDUA activity (192.73±80.66).

Differentiation potential of MPS I BMSC to chondrocytes, osteoblasts, adipocytes and smooth muscle cells *in vitro* is similar to that of HD BMSC

BMSC cultured in the presence of TGF-β_3 generate a solid three-dimensional tissue structure that can be harvested and processed for histology and molecular analysis. MPS I BMSC generated typical pellets when cultured as micromasses under previously standardized chondrogenic conditions for 21 days. As negative control, MPS I BMSC pellets cultured in the absence of TGF-β_3 did not form typical cartilage structure. Cartilage matrix stained with alcian blue with

typical cartilage morphology (Figure 3, panel A). The pellets also stained diffusely positive for type II collagen (Figure 3, panel A). The morphology and histology of MPS I and HD pellets was highly similar. In addition, gene expression studies on MPS I and HD pellets confirmed no significant differences in the transcript levels of *type II collagen (COL 2), type X collagen (COL 10), SOX-9* and *aggrecan* (Figure 3, panel E), typical chondrogenic genes. MPS I and HD BMSC were grown for 3 weeks in osteoblastic and adipocytic differentiation medium. BMSC cultured under osteogenic differentiation conditions were stained with Alizarin Red, to reveal mineral deposits (Figure 3, panel B) and cells cultured in adipogenic medium were stained with Oil Red O, to detect intracytoplasmic lipid droplets (Figure 3, panel C). In order to analyse the osteogenic differentiation at the molecular level, expression of selected genes involved in bone formation [*osteopontin (OPN), runt-related transcription factor 2 (RUNX2), osteocalcin (OTC), osteonectin (OTN), alkaline phosphatase (ALP)* and *type I collagen alpha II chain (COL1A2)]* was analyzed at 7, 14 and 21 days by Q-RT-PCR. An increase in all analysed transcripts at each time point was detected (Figure 3, panel E). No significant differences were seen in the levels of these differentiation markers between MPS I and HD BMSC. Likewise, the expression of adipogenesis-related genes *[fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPARG)]* at 21 days after adipogenic induction was similar in both groups (Figure 3, panel E).

As Hurler disease is characterized by multiorgan lesions of connective tissue, we also examined the smooth muscle cell (SMC)

differentiation capacity of BMSC derived from MPS I *versus* HD by culturing with TGF-β_1. Transcript levels of SMC contractile apparatus–specific genes, including *α-SMA, SM22α* and *CALPONIN* were analysed. As shown in Figure 3 (panel E), culture of all MPS I and HD BMSC populations with TGF-β_1 resulted in a comparable increase in SMC transcript levels by day 6 compared with undifferentiated BMSC. Immunofluorescence for α-SMA and CALPONIN of MPS I BMSC and HD BMSC confirmed these results (Figure 3, panel D).

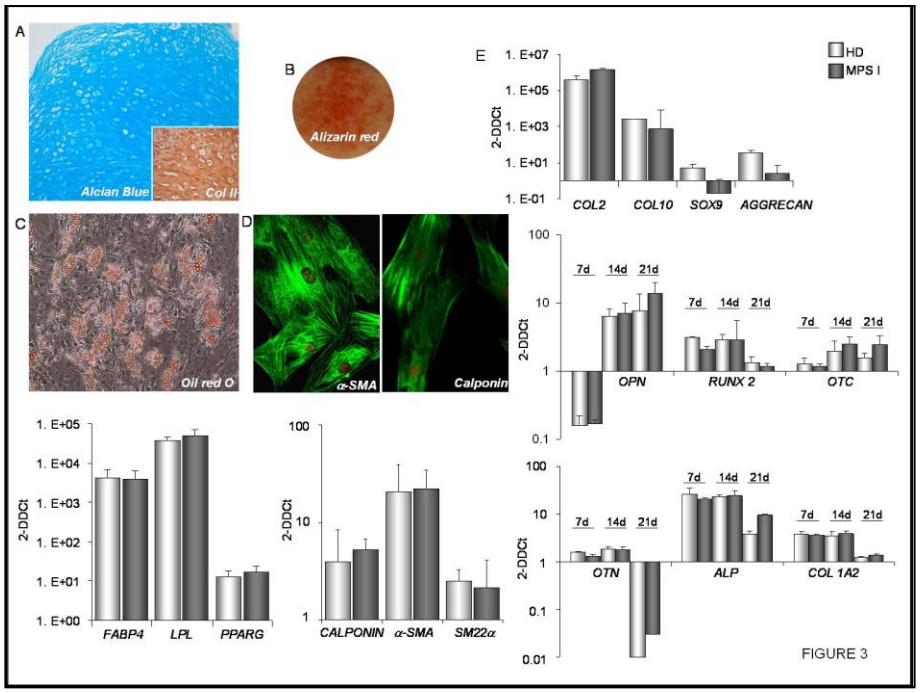

Figure 3. Multilineage differentiation capability of MPS I BMSC.
(A) Chondrogenic differentiation. Histological sections stained for alcian blue (magnification 10x) and type II collagen (inset, magnification 20x) consistent with cartilage formation. **(B)** Osteogenic differentiation. The staining with Alizarin red S solution revealed calcium-rich deposits in progeny of BMSC from MPS I. **(C)**

Adipogenic differentiation. Adipocytes differentiated from MPS I BMSC and stained with Oil Red O (magnification 10x). **(D)** Smooth muscle cells differentiation. Confocal microscopy images (green) for α-SMA and calponin; nuclei were stained with propidium iodide (red) (magnification 40x). **(E)** The data were confirmed by Q-RT-PCR for the chondrogenic genes *type II collagen (COL2), type X collagen (COL10), SOX 9 and aggrecan* on day 21, for the osteogenic genes *osteopontin* (*OPN*), *runt-related transcription factor 2* (*RUNX2*), *osteocalcin* (*OTC*), *osteonectin* (*OTN*), *alkaline phosphatase (ALP) and collagen type1 alpha 2 chain (COL1A2)* on day 7, 14 and 21 following osteogenic induction, for smooth muscles genes *calponin, alpha smooth muscle actin (α-SMA) and transgelin (SM-22 α)* on day 6 and for the adipogenic genes *fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPARG)* on day 21. Bars are the means DDCt ± SEM of 3 HD subjects *versus* 3 MPS IH patients; each subject has been tested in three independent experiments.

In vivo osteogenic differentiation capacity of MPS I BMSC is normal

An *in vivo* transplantation assay was then used to assess the bone formation capacity of MPS I *versus* HD BMSC. MPS I and HD BMSC were loaded onto hydroxyapatite/tricalcium phosphate particles, embedded in a fibrin gel and transplanted subcutaneously into immunocompromised mice. In both groups, analysis of transplants harvested at 8 weeks (Figure 4, panel A) revealed abundant newly formed bone tissue containing fully differentiated osteoblasts and osteocytes. MPS I and HD BMSC generated heterotopic bone with a similar amount and distribution (Figure 4, panel B), suggesting a similar osteogenic capacity for MPS I and HD BMSC.

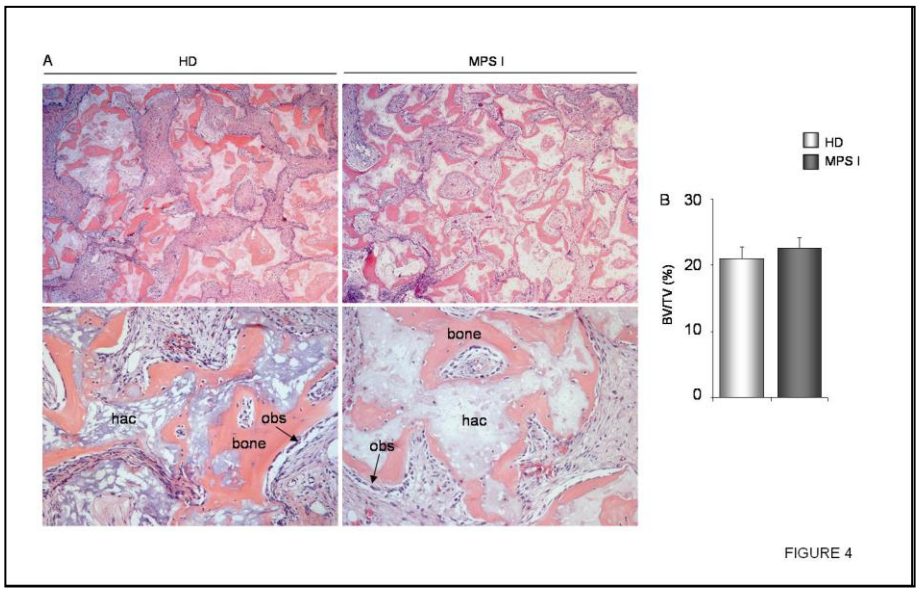

Figure 4. ***In vivo*** **transplantation of MPS I BMSC** ***versus*** **HD BMSC.**
MPS I and HD BMSC were loaded in hydroxyapatite/tricalcium phosphate particles, embedded in a fibrin gel and transplanted subcutaneously into immunocompromised mice**.** After 8 weeks, grafts were harvested. **(A)** Histological analysis of the sections (4µm, hematoxylin/eosin) revealed new bone formation along the hydroxyapatite carrier (hac). Bone tissues contain fully differentiated osteoblasts (obs). Left panels represent HD grafts, right panels represent MPS I grafts; upper panels, magnification 10x, lower panels, magnification 20x. **(B)** Analysis of bone volume by histomorphometry. Each column shows the mean ± SD of 3 HD BMSC sections and 3 MPS I grafts.

MPS I BMSC have significantly greater pro-osteoclastogenetic capacity than HD cells

To investigate the osteoclastogenic capacity of BMSC derived from MPS IH patients, we established a coculture system between MPS I and HD BMSC and CD34+ HD BM derived cells (purity>95%, by flow cytometry), as previously described by Mbalaviele *et al*. [33], to evaluate the formation of osteoclast-like cells (OCs). After 3 weeks, we observed multinucleated cells in both groups of cocultures (Figure

5, panel A) and TRAP staining was performed to identify differentiated osteoclasts and mononuclear precursors thereof.

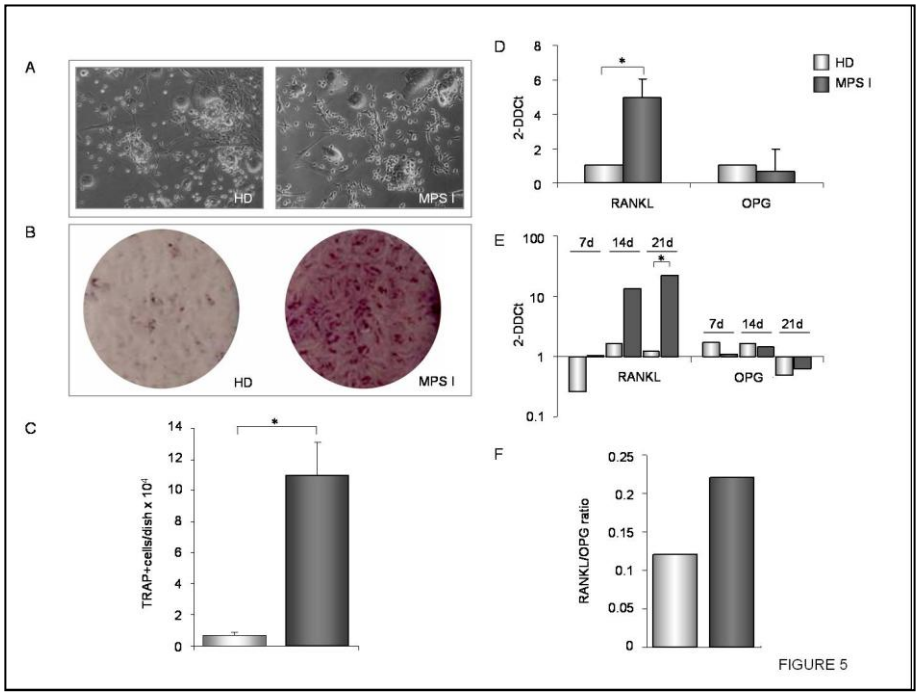

Figure 5. MPS I BMSC induce increased osteoclast formation and express higher levels of *RANKL*.

CD34+ cells were isolated from BM of HD and cocultured for 3 weeks with MPS I and HD BMSC. **(A)** Phase-contrast morphology of osteoclast precursors and osteoclasts after co-culturing of HD BMSC (HD) or MPS I BMSC (MPS I) and HD CD34+ cells. **(B)** Osteoclast formation revealed through staining for tartrate-resistent acid phosphatase (TRAP) of mononuclear and multinucleated cells after 3 weeks of co-culture. **(C)** Means ± SD of TRAP+ cells induced by BMSC from three MPS I and HD subjects. **(D)** *RANKL* and *OPG* expression levels were measured through Q-RT-PCR in basal BMSC and **(E)** in osteogenic inducted BMSC cultures at three different time points (DDCt±SD)**; (F)** RANKL/OPG ratio of osteogenic-progeny from MPS I and HD BMSC at 21 days. Each bar represents the mean results of 3 subjects evaluated; three independent sets of experiments have been done. *P<0.05.

The number of TRAP+ cells in each dish, counted under an inverted-phase contrast microscope (Figure 5, panel B), was significantly increased in cocultures with MPS I BMSC compared to HD BMSC (p= 0.01) (Figure 5, panel C).

Increased osteoclastogenesis of MPS I BMSC and osteoblasts is associated with increased production of RANKL

RANKL and OPG are key regulators of osteoclastogenesis and bone resorption, mediating the interactions between osteogenic cells and hematopoietic osteoclast precursors. *RANKL* mRNA levels were significantly higher in the three MPS I BMSC samples *versus* three HD BMSC samples (p=0.03) (Figure 5, panel D), whereas *OPG* mRNA levels were similar. *RANKL* and *OPG* mRNA levels were also measured in osteoblast progeny from MPS-I and HD BMSC. *RANKL* mRNA levels were higher in day 7 and 14 progeny of MPS I BMSC compared with HD BMSC, reaching significance by day 21 (P=0.04) (Figure 5, panel E). Again, *OPG* expression levels were similar in osteoblasts derived from MPS I and HD BMSC. Accordingly, the RANKL / OPG index, as an index of osteoclastogenic stimulation capacity, in supernatants of day 21 osteogenic progeny of MPS I was higher than in supernatants of osteoblasts generated from HD BMSC (Figure 5, panel F), even though not statistically significant (p=0.2).

Discussion

Among the widespread clinical features of MPS, the toxic effect of GAGs in bones and joints leads to the complicated skeletal disease known as *dysostosis multiplex*. To date, few studies have described the characteristics of the skeletal defects in Hurler patients [1,3,34-36],

and a limited number of them has investigated possible mechanisms involved in the bone pathophysiology of this disease [9,10,37]. One of the limitations of all studies, including this study, is the restricted availability of patient samples, which hampers investigation of rare disorders like MPS IH and often does not permit adequate statistical analysis. As a result, the pathogenesis of skeletal changes in MPS, and how the disease genotype correlates with a skeletal phenotype, remain unclear. In particular, which cells in the bone environment are critical mediators of skeletal abnormalities remain elusive. Skeletal cells (osteoblasts, chondrocytes, adipocytes and marrow stroma) primarily involved in development, morphogenesis and growth of skeletal tissues, are of mesenchymal lineage. Osteoclasts, (which resorb bone and mineralized cartilage throughout development, modelling, remodelling and growth of the skeleton) in contrast, are of hematopoietic origin. Dissecting the relative role of skeletal cells of mesenchymal origin on the one hand, and of osteoclasts on the other, in generating skeletal abnormalities has obvious relevance to the design of cell-targeted therapies.

In several bone disorders, changes in bony structures are associated with distinct functional changes in BMSC [13]. The latter are known to include postnatal progenitors of skeletal cells (skeletal stem cells, aka mesenchymal stem cells). In general, the notion of skeletal stem cells provides a novel angle for elucidating key pathogenetic mechanisms of skeletal disorders, an approach that has been productive in other genetic diseases of the skeleton such as fibrous dysplasia [38]. To begin elucidating pathogenic mechanisms implicated in the musculoskeletal defects of Hurler disease, we

isolated and characterized BMSC from pediatric patients diagnosed with MPS IH and compared their biological and functional characteristics with BMSC from age-matched HD. The DNA mutation analysis of the three MPS I BMSC primary lines confirmed the pattern found in the parental cells at diagnosis. As expected, an extremely low level of iduronidase activity was detected in these cells. However, similar to HD BMMNC, MPS I BMMNC cultured in the appropriate culture conditions gave rise to expandable BMSC colonies. Colony-Forming efficiency (CFE) estimates the frequency of stromal clonogenic progenitors among marrow cells, giving important information about the biological activity of stromal cells [39]. As reported by Kuznetsov *et al*. [40], CFE can change in pathological conditions and may be reflective of bone tissue or hematopoietic disorders, even in the absence of other evidence of histological alteration of bone or stroma. The frequency of CFU-F in BMMNC of three MPS IH patients was significantly lower than in BMMNC of four HD, which may suggest an altered status of the bone marrow stromal compartment in MPS I.

MPS I BMSC were otherwise very similar to HD BMSC. BMSC from three independent MPS IH patients or HD donors exhibited typical BMSC characteristics, including fibroblastoid morphology, expression of the surface proteins CD90, CD73, CD105 and CD146, but not CD14, CD34 and CD45. MPS I and HD BMSC had also similar proliferation capacity and cell cycle distribution. The telomerase activity of MPS I and HD BMSC was similar and a similar cellular senescence profile was observed (data not shown).

As one possible explanation for the defects in skeletal tissues in MPS IH could be abnormal differentiation of BMSC to chondrocytes and / or osteoblasts, we compared the differentiation capacity of MPS I and HD BMSC to these lineages. Similar to HD BMSC, MPS I BMSC differentiated to the chondrogenic lineage *in vitro*, as demonstrated by their capacity to form pellets of cartilage with typical morphology and chondrocyte-specific gene expression. Thus, the *in vivo* observed cartilage anomalies may not result from an impaired differentiation of cartilage tissue, but might be due to abnormalities in, and dysfunction of, mature chondrocytes. This hypothesis is supported by previous reports demonstrating that GAGs accumulation leads to chondrocyte apoptosis in the articular cartilage and growth plates of MPS animals, with a consequent triggering of inflammatory events and further destruction [41]. It is also possible that, because MPS I BMSC lines used in our work were isolated from pediatric patients, at this early stage of the disease the capacity of BMSC to differentiate into cartilage is still not altered. In addition, no differences in MPS I BMSC compared with HD-BMSC were found as related to smooth muscle, osteoblast and adipocyte differentiation *in vitro*. Furthermore, MPS I BMSC were able to generate genuine, histology-proven bone *in vivo*, similar to HD BMSC, which suggests that the osteogenic potential of BMSCs *per se* is not altered in MPS I.

The generation of a murine model for MPS-I was first described by Clarke *et al.* in which the main features of Hurler disease in humans were recreated in the mouse [42]. At the skeletal level, the most severe difference between MPS-I and wild-type mice is an increase in the amount of cartilage in the subepiphyseal growth plate, despite a

simultaneous increase of osteoclasts, as recently described by Wilson *et al.* [9]. Similarly, Simonaro *et al.* have described an increase in osteoclast-precursors and osteoclasts in BM of MPS VI rats, with an up-regulation of RANKL in fibroblast-like synoviocytes, an important molecule involved in osteoclastogenesis [10]. RANKL is produced by osteoblasts and stromal cells and functions as a key regulator for osteoclast differentiation and activation [43]. The involvement of RANKL in several inflammatory and bone diseases, such as osteoporosis and arthritis, has also been well documented [44,45]. Based on these findings, we hypothesized that a possible increase of the osteoclast number in MPS I growth plates could result from abnormalities in the levels of RANKL/OPG locally produced by BMSC. To test our hypothesis we evaluated the capacity of MPS I *versus* HD BMSC to support the generation of osteoclasts from normal CD34 positive cells *in vitro* [33]. The number of osteoclasts differentiated in the presence of MPS I BMSC was significantly higher. We further demonstrated that RANKL expression is higher in MPS I BMSC and osteoblasts derived from these BMSC, compared to HD BMSC and osteoblasts. We could not detect any significant difference in the expression of other inflammatory or pro-osteolytic cytokines, as such as TGF-β, DKK-1, IL-6 (data not shown). These data might explain the increased number of osteoclasts during *in vitro* culture of CD34+ cells with MPS I BMSC. It is thus possible that a cell-autonomous dysfunction in MPS I BMSC results in increased osteoclastogenesis via overproduction of RANKL, thus contributing to explain the excess osteoclasts noted in MPS I mice [9]. Wilson *et al.* [9] hypothesized that an osteoclast cell-autonomous decrease in the

collagenolytic activity of cathepsin K could impair osteoclast function, leading to pathological effects on the bones. On the other hand, our data suggest an independent, cell-autonomous functional change in stromal cells that drive osteoclastogenesis. Taken together, our data and those from previous studies focusing on the function of differentiated osteoclasts seem to reveal a more complex derangement of skeletal remodelling than currently appreciated. A vicious cycle of enhanced osteoclastogenesis and ineffective osteoclastic degradation of bone matrix would contribute to the skeletal phenotype in MPS I, explaining the enhanced numbers of osteoclasts noted *in vivo*.

In conclusion, we report that BMSC derived from MPS IH patients can generate normal cartilage, bone and smooth muscle cells *in vitro*, and bone tissue *in vivo*. However, MPS I BMSC and osteoblasts derived from these BMSC produce increased amounts of RANKL and display an increased capacity to stimulate the formation of osteoclasts *in vitro*. Our results support the hypothesis that aberrant osteoclastogenesis may be one of the aspects involved in Hurler bone disease and that the abnormal ability of MPS I BMSC to support osteoclastogenesis may contribute to explain aspects of the skeletal phenotype seen in this disease.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

All authors state that they have no conflicts of interest.

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mutation analysis of the IDUA gene

Genomic DNA was extracted from BMSC derived from the three MPS IH patients using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Mutation analysis of the IDUA gene was limited to confirm the presence of mutations identified at diagnosis: p.W402X (exon9) for patient 1, p.Q70X (exon2) and c.398_403del6 (exon4) for patient 2, p.Q70X (exon2) and p.P496R (exon10) for patient 3. The exons 2, 4, 9 and 10 were amplified and sequenced using intronic primers designed for both amplification and sequencing. Exons 2 and 4 were amplified in 25µl containing 100ng of genomic DNA, Buffer 1X with MgCl2 1.5mM, dNTP mix 200mM, 15pmol of each primer, GC-RICH solution 1X and 0.5U of the proofreading PWO SuperYeld DNA Polymerase (ROCHE, Monza, Italy). Exons 9-10 co-amplification was carried out in 25µl volume containing 100ng of genomic DNA, Buffer 1X with MgCl2 1.5mM, dNTP mix 200mM, 15pmol of each primer and 0.5U of the GoTaq DNA Polymerase (Promega). Cycling conditions were: initial denaturation at 96°C for 5 min, 30 cycles at the following conditions:

denaturation at 96°C for 1 min, annealing at 67°C for exon 2, 65°C for exon 4 and 63°C for exons 9-10 for 1 min, extension at 72°C for 1 min, followed by final extension at 72°C for 7 min. PCR products were purified using an enzymatic reaction containing 5U of Exonuclease I (Celbio, Pero, Italy) and 1U of Alkaline Phosphatase (Promega) using the following conditions: 15 min at 37°C followed by 15 min at 80°C. Purified fragments were sequenced in both forward and reverse directions using BigDye v3.1 terminator technology and then purified with the BigDye XTerminator Purification Kit. Sequence reactions were carried out and purified according to the manufacturer's instructions and were analyzed on an ABI Prism 3130 Avant Automatic Sequencer (Applied Biosystems, Foster City, CA).

Supplemental Table 1.

Assays used for Q-RT-PCR.

Name	Code
GAPDH	4352934E
COL 2	Hs01060345_m1
COL 10	Hs00166657_m1
SOX 9	Hs00165814_m1
AGGRECAN	Hs00202971_m1
OPN	Hs00959010_m1
RUNX 2	Hs00231692_m1
OTC	Hs00609452_g1
OTN	Hs00234160_m1
ALP	Hs01029144_m1
COL 1 A 2	Hs01028970_m1
RANKL	Hs00243519_m1
OPG	Hs00171068_m1

Chapter 3

Patient-specific induced pluripotent stem cells for modeling Hurler Syndrome

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Abstract

Mucopolysaccharidosis type I (MPS I) is a rare genetic disease belonging to lysosomal storage disorders. It is caused by mutations in α-L-iduronidase (*idua*) gene, leading to an intra-cellular accumulation of undegraded glycosaminoglycans in all body tissues. Hematopoietic cell transplantation (HCT) is a life-saving treatment for the most severe form of MPS I, the Hurler syndrome (MPS IH). However, HCT is associated with morbidity and mortality and does not prevent the disease progression in organs. Human induced pluripotent stem cells (iPSCs) represent a great promise for advancements in cell replacement therapy, developmental biology, screening candidate drugs, and in the study of modeling disease phenotypes of human pathologies. Here, we showed the use of human iPSCs for modeling the MPS IH. Skin fibroblasts from MPS IH patients were used to generate patient-specific iPS cell lines. We found that iPSCs-MPS IH showed lysosomal storage characteristics of MPS IH cells (*idua* mutations, reduced α-L-iduronidase activity) and could be differentiated into committed cell types such as hepatocyte-like cells and neurons as well as derivative cells of the three germ layers. Targeted gene transfer into iPSCs holds promise for correction of monogenic diseases without the insertional mutagenesis caused by multisite integration of viral vectors. Zinc finger nuclease–mediated gene targeting of *idua* gene into the "safe harbor" *AAVS1* locus in iPSCs-MPS IH could result in sustained expression of IDUA activity. We aim to evaluate the potential of iPSCs-MPS IH cells to generate, and eventually correct, differentiated cell types belonging to those

anatomical sites not fully corrected with HCT in order to study disease mechanisms in those compartments.

Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive metabolic disorder caused by mutations in the *idua* gene, leading to inactivity of the lysosomal enzyme α-L-iduronidase. The deficiency of IDUA causes a progressive intracellular accumulation of undegraded glycosaminoglycans (GAGs), dermatan and heparan-sulfate [1] [2]. This disrupts cell functions and gives rise to multiorgan dysfunction. MPS I is a highly heterogeneous disorder with a wide spectrum of clinical manifestations. Hurler syndrome (MPS IH) is the most severe form of the disease, affecting approximately 50% of all patients with MPS. The clinical condition is marked by hepatosplenomegaly, obstructive airway disease complicated by respiratory infections, cardiac failure, and progressive mental retardation. The array of musculoskeletal abnormalities observed in Hurler syndrome is known as *dysostosis multiplex*, and underlies the typical dwarfism and gargoyle-like features.

The sole agent needed for MPS I correction is the missing α-L-iduronidase, which after secretion and intercellular transfer is taken up by IDUA-deficient cells through receptor-mediated endocytosis. Nowadays, two treatments are mainly available for the therapy of MPS I: enzyme replacement therapy (ERT) with human recombinant iduronidase and hematopoietic cell transplantation (HCT). Weekly doses of intravenous iduronidase have been used for milder forms of MPS I. However, because iduronidase does not cross the blood-brain

barrier efficiently, ERT alone is not indicated for the severe form of IDUA deficiency [3]. Allogeneic HCT, in contrast, leads not only to donor hematopoietic engraftment and systemic expression of IDUA but also to donor myeloid cells crossing the blood-brain barrier and correcting IDUA deficiency in the brain [4] [5] [6]. Although HCT is a life-saving measure in MPS IH, a suitable HCT donor is difficult to find for everyone. To achieve a cure, children with MPS IH must survive both the disease and its therapy because allogeneic HCT is associated with significant morbidity and mortality from physical and immune injury by both the myeloablative conditioning regimen and the transplantation of an immunologically matched allogeneic cellular graft [7] [8] [9]. Furthermore, some of the tissue-specific manifestations of MPS IH in cardiovascular, nervous, and skeletal systems persist even after full hematopoietic donor engraftment after allogeneic HCT. Thus, HCT is not able to prevent the disease progression in some anatomical sites.

Patient-specific stem cells, such as the recently identified induced Pluripotent Stem cells (iPSCs) [10] [11] [12], represent a useful tool to study still unknown disease mechanisms in MPS IH, providing a means of better understanding the sequence of downstream events initiated by IDUA deficiency in the most immature human cell type available. Demonstration that iPSCs can recapitulate disease-specific phenotypes in vitro has been accomplished only with a few monogenic diseases [13] [14] [15] [16] [17] [18] [19] [20].

Zinc finger nuclease (ZFN)–facilitated homologous recombination (HR)–mediated gene targeting of autologous cells

could allow precise genomic modification. However, HR rates with some cell types are modest [21]. By contrast, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have unlimited proliferative self-renewal potential in vitro [22] [23] [11]. Specific gene targeting can be performed in human ESCs and iPSCs using ZFNs to induce a sequence-specific double-strand DNA break that enhances site-specific HR [24] [25]. Studies in human ESCs and iPSCs have shown that the *AAVS1* locus, which lies within the first intron of the *PPP1R12C* gene on chromosome 19 and is the common integration site of adeno-associated virus 2, can be used as a nonpathogenic "safe harbor" to target a function correcting minigene with persistent and strong transgene expression [26] [25] [27]. The *AAVS1* locus has an open chromatin structure that is flanked by insulator elements that shield the integrated cassette from *trans*-activation or repression [28]. Although gene insertion at the *AAVS1* locus may affect *PPP1R12C* gene expression, no haploid insufficiency of *PPP1R12C* has been reported, and disruption of one *AAVS1* allele in transgenic mice and in human ESCs and iPSCs cells appears to have no adverse effects [27] [29].

In our present study, we show that iPSCs can be isolated from patients with MPS IH (iPSCs-MPS IH) and that they can be gene-corrected with an alternative and safer method than viral vectors. We report that iPSCs-MPS IH can be derived from MPS IH fibroblasts and they can be differentiated to both three germ layers tissues and more committed cell types such as hepatocyte-like cells and neurons. As a proof of principle, we then demonstrate the functional correction of the α-L-iduronidase defect in the iPSCs following ZFN-mediated

site-specific HR targeting of the *idua* gene to the *AAVS1* safe harbor locus. These data suggest the potential of iPSCs in modeling MPS IH providing a useful tool for studying disease mechanisms in those cell types derived from the anatomical sites not fully corrected after successful HCT.

Materials and Methods

Patients and fibroblast isolation

After obtaining appropriate ethical approval and patient consent, 3-mm skin punch biopsies were collected from 3 MPS IH pediatric patients. Fibroblasts were isolated as follow. Briefly, the biopsy was washed in PBS (Invitrogen, San Giuliano Milanese, IT), minced in thin fragments with surgical scissors and transferred in a T25 cm^2 flask (Nunc, Rochester, NY) with standard fibroblast culture (SFC) medium. SFC medium contains RPMI (Gibco, Grand Island, NY), 15% of FBS (Biosera, East Sussex, UK), 1% L-glutamine (Gibco) and 1% Penicillin/Streptomycin (Gibco). After 5 days the medium was replaced and the fragments were removed from the culture. The fibroblasts were expanded in SFC medium until they reached 90% confluence. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Human iPSCs isolation and culture

Moloney murine leukemia virus–derived vectors, each containing the coding sequences of 1 of the 4 human genes OCT4, SOX2, c-Myc, and KLF4, and the corresponding viral particles were generated by Vectalys (Toulouse, France) and used to infect the fibroblasts as

previously described [10] [30]. On day 1, 10^5 fibroblasts were plated in each well of 6-well plates in SFC medium. The following day the cells were transduced with the four viruses at a multiplicity of infection of 10 (for each virus) for 24 hours. On day 3, the cells were washed 3 times in PBS (Gibco) and then grown in medium containing FBS for 3 additional days. On day 7, the cells were passaged on plastic plates containing irradiated mouse embryonic fibroblasts (iMEF, GlobalStem, Rockville, MD) and then grown for 2 additional days in medium containing FBS. After day 9, the cells were grown in standard hESC culture conditions (knockout [KSR](Gibco) + FGF2 (4 ng/ml; R&D Systems Inc., Minneapolis, MN). The first iPS colonies appeared 15-18 days later and they could be picked after 5-8 additional days of culture. Individual colonies were picked and either transferred into a single well of 12-well plates containing iMEF feeders in KSR + FGF2. The resulting colonies were then expanded using enzymatic dissociation: cells were harvested using a solution of 1 mg/ml dispase:collagenase IV (ratio 1:1) (Gibco).

In vitro differentiation of human iPSCs into three germ layers

For in vitro differentiation, human iPSCs were switched to a feeder-free condition of culture with Chemically Defined Medium (CDM or CDM-PVA) (see ref.30). Initially, iPSCs were grown for the first 2 days in CDM supplemented with recombinant Activin (10 ng/ml; R&D Systems Inc.) and FGF2 (12 ng/ml). To obtain extraembryonic tissue, iPSCs were grown for 7 days in CDM in the presence of bone morphogenic protein 4 (BMP4, 10 ng/ml, R&D Systems Inc.). To obtain neuroectoderm progenitors, iPSCs were grown in CDM-PVA in the presence of SB431542 (10 µM, Tocris Bioscience, Bristol, UK)

and FGF2 (12 ng/ml) for 7 additional days. To obtain mesendoderm precursors, iPSCs were grown for the 3 following days in CDM-PVA in the presence of BMP4 (10 ng/ml), FGF2 (20 ng/ml), Activin (100 ng/ml) and LY294002 (10 μM, Promega, Madison, WI).

In vivo differentiation

Human iPSCs were harvested with the enzymatic treatment immediately prior to implantation, and approximately $2x10^6$ cells were resuspended in mixture of PBS and Matrigel (BD Biosciences, Buccinasco, IT) (ratio 1:1) in a final volume of 400 μL. The cell suspensions were inoculated subcutaneously into the dorsal flank of 8-week-old Balb/c-$Rag2^{-/-}$ $\gamma C^{-/-}$ male mice. Within eight weeks after the injection, tumors were surgically dissected from the mice. Samples were fixed in PBS containing 4% formaldehyde and embedded in paraffin. Serially sections of 4 μm were stained with hematoxylin-eosin and characterized.

RNA extraction and real-time PCR

Total RNAs were extracted from iPSCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Each sample was treated with RNase-Free DNase (Qiagen) to avoid DNA contamination. For each sample 0.5 μg of total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR mixtures were prepared as described (SensiMiX protocol; Quantace, London) then denatured at 94°C for 5 minutes and cycled at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes after completion of 40 cycles. Primer sequences are described elsewhere [31]. Real-time PCR reactions

were performed using a Stratagene Mx3005P (La Jolla, CA, http:// www.stratagene.com) in triplicate and normalized to porphobilinogen deaminase (PBGD) in the same run.

Immunofluorescence

Human iPSCs or their differentiated progenitors were fixed for 20 minutes at 4°C in 4% paraformaldehyde and then washed three times in PBS. Cells were incubated for 20 minutes at room temperature in PBS containing 10% donkey serum (Serotec Ltd.) and subsequently incubated overnight at 4°C with primary antibody diluted in 1% donkey serum in PBS. For the dilutions of primary antibodies see the following reference [30]. Cells were then washed three times in PBS and incubated with Texas Red or fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma-Aldrich; 1:200 in 1% donkey serum in PBS) or rabbit IgG (Jackson Laboratory, Bar Harbor, ME, http://www.jax.org; 1:400 in donkey serum in PBS) or goat IgG (Jackson Laboratory; 1:400 in donkey serum in PBS) for 2 hours at room temperature. Unbound secondary antibody was removed by three washes in PBS. Hoechst 33258 was added to the first wash (Sigma-Aldrich; 1:10,000).

Differentiation of iPSCs in hepatocyte-like cells

For the differentiation of human iPSCs into hepatocyte-like cells an adapted protocol published by Roelandt et al [32] was followed. Briefly, iPSCs were plated on Matrigel (BD Bioscience) coated 12 well plates with mTeSR1 medium (STEMCELL Technologies, Vancouver, CA) until they reached 70% of confluence. Afterwards, liver differentiation protocol was started: iPSCs were grown for 28

days in liver basal medium in addition with different citokynes sequentially added during the differentiation steps. To monitor and evaluate the liver differentiation efficiency, gene expression analysis and immunocytochemistry of specific hepatic markers were performed at fixed time points (day 0, day 10 and day 28). As functional assays of hepatocyte-like cells albumin secretion and detoxification capacity of cytochrome P450 were analyzed.

Differentiation of iPSCs in neurons

Human iPSCs were differentiated into neurons following an adapted protocol described by Gaspard N [33]. Briefly, iPSCs were grown on Matrigel coated 12 well plates with mTeSR1 medium for 2 days. Then, neural differentiation protocol was started: iPSCs were grown for 16 days in a chemically defined default medium (DDM) in addition with Noggin (100 ng/ml, R&D Systems Inc.) and until the day 24 only with DDM medium, changing it every 2 days. At day 24 neural precursor cells were splitted on polylisin/laminin coated 12 well plates and grown until day 40 in DDM medium. To monitor and evaluate the neuronal differentiation efficiency, gene expression analysis and immunocytochemistry of specific neuronal markers were performed at fixed time points (day 5, day 11, day 19, day 24 and day 40).

Mutation analysis of the *idua* gene

Genomic DNA was extracted from human iPSCs using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Mutation analysis of the *idua* gene was limited to confirm the presence of mutations identified at diagnosis: p.Q70X (exon2) and p.P496R

(exon10) for patient 1 and p.W402X (exon9) for patient 2. The exons 2, 9 and 10 were amplified and sequenced using intronic primers designed for both amplification and sequencing. Exons 2 was amplified in 25µl containing 100 ng of genomic DNA, Buffer 1X with MgCl2 1.5mM, dNTP mix 200mM, 15pmol of each primer, GC-RICH solution 1X and 0.5U of the proofreading PWO SuperYeld DNA Polymerase (ROCHE, Monza, Italy). Exons 9-10 co-amplification was carried out in 25µl volume containing 100 ng of genomic DNA, Buffer 1X with MgCl2 1.5mM, dNTP mix 200mM, 15pmol of each primer and 0.5U of the GoTaq DNA Polymerase (Promega). Cycling conditions were: initial denaturation at 96°C for 5 min, 30 cycles at the following conditions: denaturation at 96°C for 1 min, annealing at 67°C for exon 2, 65°C for exon 4 and 63°C for exons 9-10 for 1 min, extension at 72°C for 1 min, followed by final extension at 72°C for 7 min. PCR products were purified using an enzymatic reaction containing 5U of Exonuclease I (Celbio, Pero, Italy) and 1U of Alkaline Phosphatase (Promega) using the following conditions: 15 min at 37°C followed by 15 min at 80°C. Purified fragments were sequenced in both forward and reverse directions using BigDye v3.1 terminator technology and then purified with the BigDye XTerminator Purification Kit. Sequence reactions were carried out and purified according to the manufacturer's instructions and were analyzed on an ABI Prism 3130 Avant Automatic Sequencer (Applied Biosystems, Foster City, CA).

Specific α-L-iduronidase enzymatic activity

IDUA activity was determined according to Clements et al. [34]. Briefly, cells were resuspended in 150 mM NaCl and freeze-thawed 6

times. Protein extracts were assayed for total protein content using the BCA assay. 5 µg of protein was added, in triplicate, to a solution containing 8 mM D-Saccharic acid 1,4-lactone and 2 mM 4-methylumbelliferyl-α-L-iduronide in 0.1 M sodium formate buffer, pH 3.2. Samples were incubated for 1 hr at 37°C before stopping the reaction by adding 1 ml of 0.5 M carbonate buffer, pH 10.7. The cleaved substrate was quantified on a Perkin Elmer fluorometer. The enzyme activity was calculated from a reference curve obtained by using 4-methylumbelliferone and was expressed as nmoles/hr/mg.

Construction of a pAAVS1-Puro-EF1α-eGFP-p2A-IDUA-pA donor plasmid for ZFN-mediated targeted expression of GFP/IDUA gene in iPSCs

The human IDUA cDNA (kindly provided from Dr. Alessandra Biffi, TIGET, San Raffaele Institute, Milan, Italy) was amplified by PCR with Phusion® High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) under the following conditions with the use of primers that incorporated a 5' Spe I and 3' Pme I restriction endonuclease site: 94°C for 2 minutes, 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes. The PCR product was gel-purified, digested with Spe I and Pme I (New England BioLabs, Ipswich, MA), and ligated into the pEF1α-eGFP-P2A-SV40pA plasmid obtained from Prof. Catherine Verfaillie, Catholic University of Leuven (Belgium) with the use of T4 DNA ligase (Roche, Monza, IT). The optimized sequence from the pEF1α-eGFP-P2A-IDUA-SV40pA was enzimatically inserted into the commercially available AAVS1 locus-targeting donor plasmid pAAv backbone (pZDonor-AAVS1 Puromycin Vector Kit, Sigma Aldrich). This backbone is flanked at

the 5' and 3' ends by sequence upstream and downstream of the AAVS1 locus targeting site of the ZFNs. The resultant donor plasmid, pAAVS1-Puro-EF1α-eGFP-p2A-IDUA-pA contains a splice acceptor element and 2A linker placed in front of a promoterless puromycin-polyA cassette, which will express the puromycin resistance element only if it is inserted downstream of a constitutively active promoter such as the PPP1R12C promoter. The eGFP/IDUA cassette is placed downstream of the puromycin resistance element using the EF1α promoter to express a codon-optimized eGFP/IDUA cDNA-polyA.

Gene targeting in human iPSCs using AAVS1 ZFN mRNAs

A qualified ZFN pair optimized for targeting the AAVS1 locus is commercially available as mRNAs from Sigma-Aldrich (CompoZrAAVS1 targeted integration kit CTI1). One million human iPSCs were digested for 2-3 minutes using 0.05% trypsin (Invitrogen). The cells were centrifuged at 100g for 5 minutes and resuspended in 100 μL of Amaxa P3 Primary cell nucleofection medium (Amaxa Biosystems) with 10 μg of the eGFP/IDUA donor plasmid targeting vector and 5 μL of mRNA of the AAVS1 ZFNs. The cells were transfected using an Amaxa Nucleofector device with program H9, human ES cells, and then plated immediately onto puromycin-resistant DR4 MEF feeders (GlobalStem) with 10 μM ROCK inhibitor Y27632 (Stemgent, Cambridge, MA) and KSR+FGF2 medium. Puromycin selection (250 ng/mL) was started 2 days after nucleofection. Individual clones were picked 10 days later and then expanded and maintained without puromycin.

Karyotype analysis

Human iPSCs were grown to confluence on 10-cm dishes, then harvested and metaphase spreads obtained by the San Gerardo Hospital Cytogenetics diagnostics laboratory.

Results

Generation of iPS cell lines from fibroblasts of MPS IH patients

Dermal fibroblasts were obtained from skin biopsies of 3 MPS IH pediatric patients before their treatment with HCT. All patients had biochemical and molecular confirmation of the diagnosis and presented with coarse facies, multiple dysostosis including dorsolumbar spine kyphosis and hip dysplasia and other typical clinical signs. The age of the patients was respectively of 11 months (female), 13 months (female) and 20 months (female). Human fibroblast cell lines were established from skin biopsies and used for reprogramming experiments at early passages (< P3). As several groups have reported, human fibroblasts can be reprogrammed into iPSCs by overexpressing the four transcription factors c-Myc, Oct-4, Klf-4 and Sox2 [23] [11]. Using such an approach, we generated human iPS cell lines from fibroblasts of MPS IH patients. 2 out of 3 sources of fibroblasts were successfully reprogrammed and we isolated approximately 10 different cell lines from each patient. The success rate of iPS cells derivation was respectively of 0.024% and 0.11%, confirming the existent variability in capacity to reprogram fibroblasts from patients. The resulting iPS cell lines (iPS-MPS IH)

were grown in human ES system of culture for several passages, in the presence of a murine feeder and with serum-free medium. 3 human iPS-MPS IH cell lines per individual were selected for a fully characterization. All the selected cell lines showed the morphology of typical iPS clones with round shape, cells with scant cytoplasm and large nucleoli (Fig. 1A, inset). The iPS-MPS IH clones revealed the expression of pluripotency markers such as Nanog, Oct-4, Sox2, TRA-1-60 (Fig. 1A) and alkaline phosphatase (Fig. 1B). We further characterized the iPS-MPS IH cell lines generated by defining the expression profiles of the 4 exogenous transgenes and endogenous pluripotency genes using real-time PCR. These analyses showed that the exogenous transgenes were detected at low levels in human iPSCs-MPS IH, demonstrating the efficiency of the retroviral vector to be silenced in pluripotent stem cells (Fig. 1C). Finally, we determined the number of transgene copies inserted in the genome of the isolated iPS-MPS IH cell lines, showing that each line contains an average of 3 copies for each transgene (Fig. 1D), according to previous studies [23] [11] [12]. Furthermore, abnormal karyotype was observed in human iPSCs grown for long periods of time (i.e. passage 30). Thus, only early-passage human iPSCs-MPS IH (i.e. before passage 15) were used for the present study. Taken together, these results demonstrate that the human iPS cell lines generated from somatic cells of MPS IH patients were successfully reprogrammed and can be used as a source for further investigations in the disease modeling of Hurler syndrome.

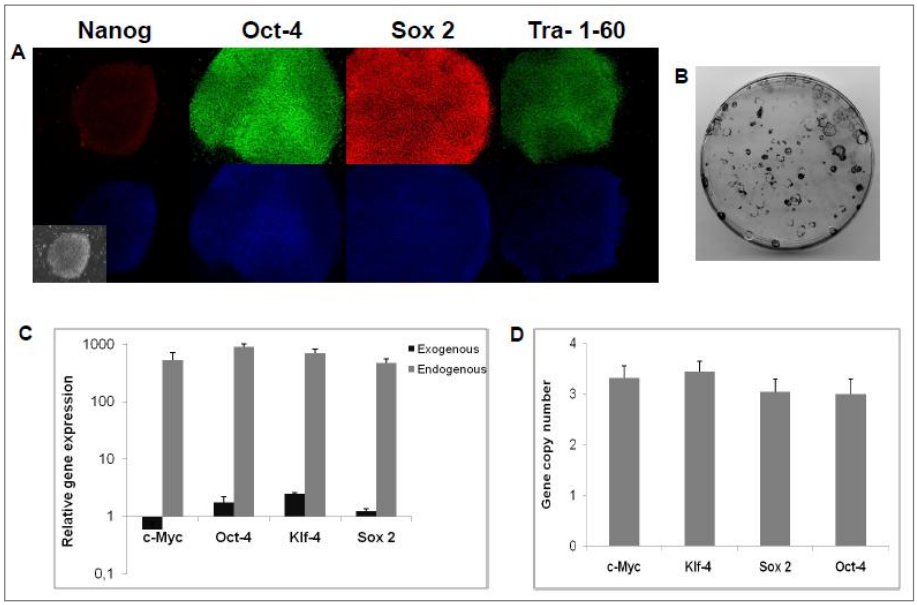


Figure 1: Human iPSCs are isolated from fibroblasts of MPS IH patients. (**A**) iPSCs grown in human ES system of culture show pluripotency markers. The picture (inset, magnification 10X) represents the morphology of an iPS clone at passage 3. The clone shows round shape, cells with scant cytoplasm and large nucleoli. The immunostaining was performed at passage 5 to detect the expression of the pluripotency markers Nanog, Oct4, Sox2, and Tra-1-60. DAPI stained nuclei blue fluorescence. Magnification 10X. (**B**) Alkaline Phosphatase assay. This colorimetric assay was performed to detect alkaline phosphatase activity after fibroblast reprogramming. With this assay is also possible to calculate the reprogramming efficiency, counting the alkaline phosphatase positive colonies. (**C**) Relative gene expression of exogenous and endogenous c-Myc, Oct-4, Klf-4 and Sox2 in human iPSCs generated from fibroblasts of MPS IH patients. Human iPSCs-MPS IH were grown in hES system of culture and the expression of endogenous and exogenous c-Myc, Oct-4, Klf-4 and Sox2 was determined using real-time PCR and specific primers. The graph shows the mean of three different iPS cell lines derived from the same patient. hESCs (H9 line) were used as reference control. Bars indicate ± SD. (**D**) Number of transgene copies in human iPSCs generated from MPS IH fibroblasts. Copy number for each gene used to reprogram MPS IH fibroblasts was determined using real-time PCR. The graph shows the mean of three different iPS cell lines derived from the same patient. Bars indicate ± SD.

Characterization of iPS-MPS IH cell lines

After reprogramming of Hurler fibroblasts, we wanted to evaluate whether the isolated iPSCs maintained the original characteristics of the disease-affected cell types. We analyzed the disease-specific genotype of the derived iPSCs-MPS IH. In both patients, the sequence analysis of the *idua* gene confirmed the mutations previously identified in the somatic counterparts used for the diagnosis, specifically fibroblasts and peripheral blood mononuclear cells (Fig. 2A). In addition, specific IDUA enzymatic activity, a hallmark of the biochemical defect in MPS IH patients, was dramatically low in iPS-MPS IH cell lines compared to iPS-HD cell line (3.6 *vs* 54.1)(Fig. 2B).

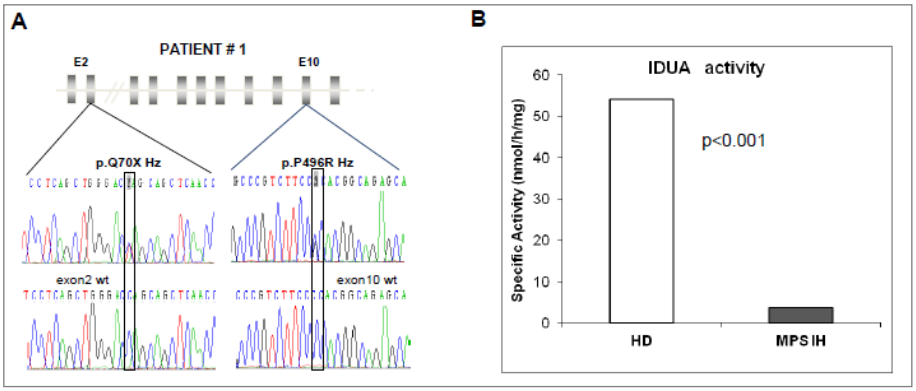


Figure 2: Molecular analysis of *idua* mutations. (**A**) The automated sequencing of the *idua* coding region has identified the mutations in the genomic DNA of iPSCs-MPS IH generated from patient #1. The panel shows the positions of mutations (electroferograms higher) compared with the wild-type sequence (electroferograms lower). This patient reveals a compound heterozygosity for p.Q70X and p.496R mutations; the p.Q70X introduce a premature STOP codon at position 70 of protein chain and the p.496R, a missense mutation in exon 10, results in a non conserved aminoacid change at position 496 of protein chain. IDUA activity evaluation (**B**)

Specific IDUA enzymatic activity was measured in protein extracts of iPS-MPS IH and iPS-HD cell lines. Activity was expressed as nmoles of enzyme normalized against protein content (mg) and incubation time (hr). Each bar represents the mean of 2 cell lines.

In vitro and *in vivo* differentiation of human iPSCs

In order to further characterize human iPS cell lines, we tested the developmental potential of the iPSCs-MPS IH to differentiate into derivatives of the embryonic germ layers both *in vitro* and *in vivo*. For the *in vitro* differentiation, we performed three different assays in a feeder-free system of culture as reported by Vallier L. *et al.* [30]. Therefore, we analyzed the expression of specific markers for the three germ layers and extra-embryonic tissues to evaluate the pluripotency potential of the cells previously generated. The immunostaining revealed that iPSCs-MPS IH could be differentiated *in vitro* into derivative cells of the three germ layers and into extra-embryonic tissues, as neuroectoderm (PAX6, SOX-1, SOX-2,), mesendoderm (SOX-17, Brachyury, FOXA2) and extra-embryonic (CDX-2, GATA-4, SOX-7) (Fig. 3A). These differentiations assays were performed in fully chemically defined medium, devoid of factors that could obscure the analysis of developmental mechanisms or render the resulting tissues incompatible with future clinical applications. Recently, several studies have reported that teratoma formation is the hallmark to test the pluripotency of the isolated iPSCs *in vivo.* Thus, we injected subcutaneously our iPSCs-MPS IH in the dorsal flanks of immunodeficient mice. Within 8 weeks after injection, well-differentiated cystic teratomas were observed,

confirming the phenotype-defining ability of iPSCs to differentiate *in vivo* into cells of ectodermal, endodermal and mesodermal origins (Fig. 3B).

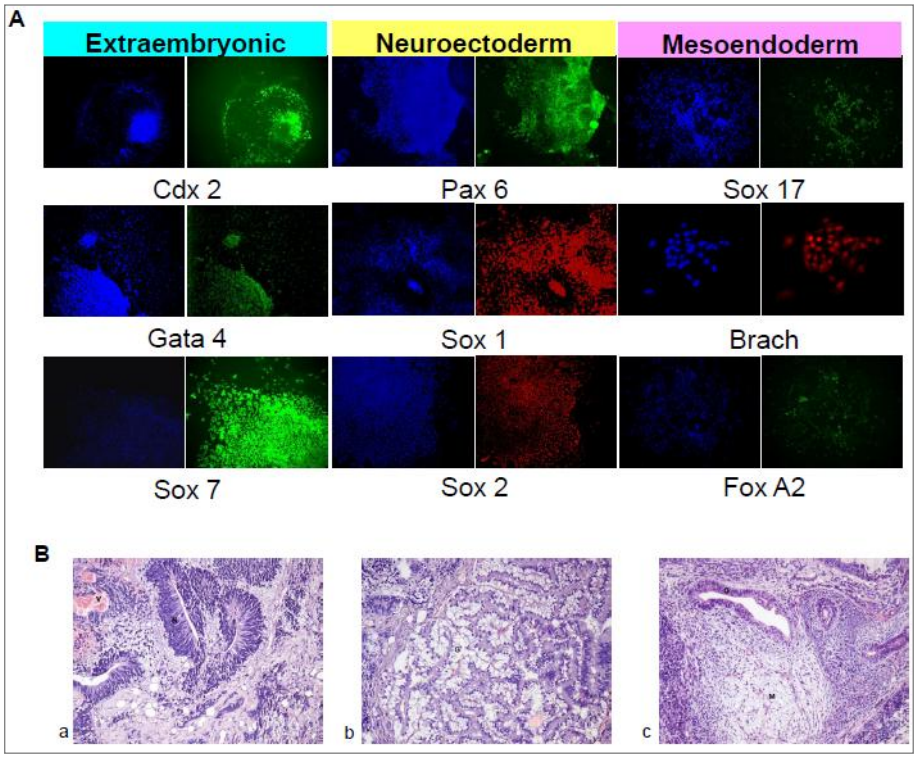
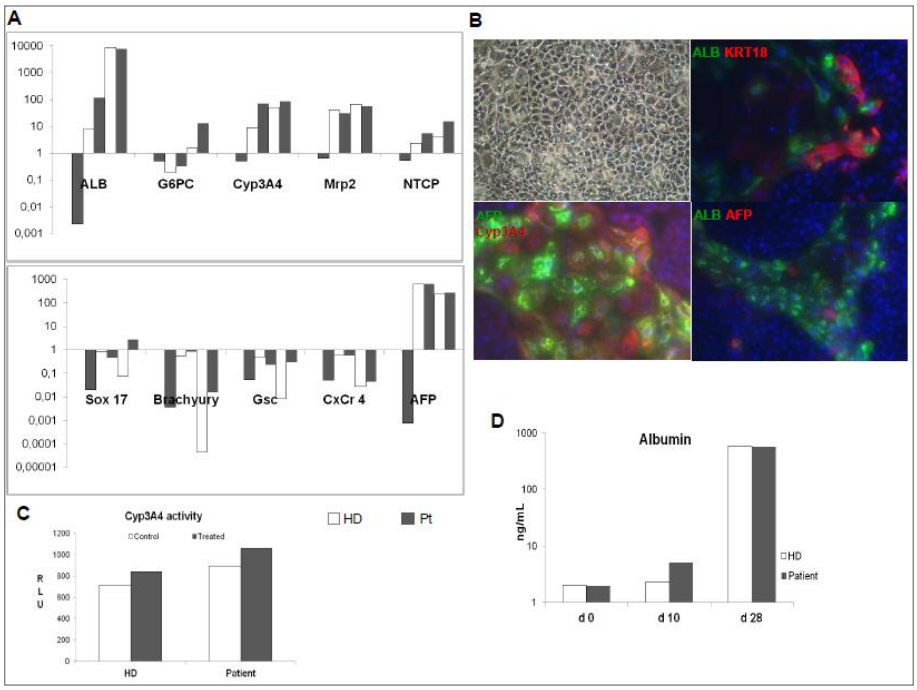


Figure 3*: In vitro* differentiation of human iPSCs-MPS IH (**A**): The immunostaining demonstrated that patient-derived iPS cell lines could be differentiated *in vitro* in a chemically defined medium into derivatives of the three germ layers and into extra-embryonic tissues: extraembryonic (Cdx-2, Gata-4, Sox-7), neuroectoderm (Pax6, Sox-1, Sox-2) and mesendoderm (Sox17, Brachyury, FoxA2). *In vivo* differentiation of iPS-MPS IH cell lines. (**B**) Teratomas from human iPSCs-MPS IH. $2x10^6$ cells grown for 10 passages in hES system of culture were injected into the dorsal flank of Balb/c-$Rag2^{-/-}$ $\gamma C^{-/-}$ mice. The resulting tumors were harvested approximately 8 weeks after injections. Hematoxylin and eosin stained sections of teratomas formed showing the formation of cell types characteristic of all three germ layers including neural epithelium (neuroectoderm) (a), glandular epithelium (endoderm) (b) and mesenchyme (mesoderm) (c).

Subsequently, we wanted to investigate the differentiation of the iPS-MPS IH cell lines into two of the most affected cell types in Hurler patients, hepatocytes and neurons. Following the protocol described by Roelandt P. *et al.* [32], we derived a heterogeneous population of hepatocyte-like cells in feeder-free system of culture from iPS-MPS IH cell line. The gene expression profile of the hepatic markers, obtained through real time PCR, showed that there are no significant differences between the hepatocyte-like cells derived from iPS-MPS IH and iPS-HD cell lines (an iPS cell line isolated from a healthy donor, used as control) (Fig. 4A).



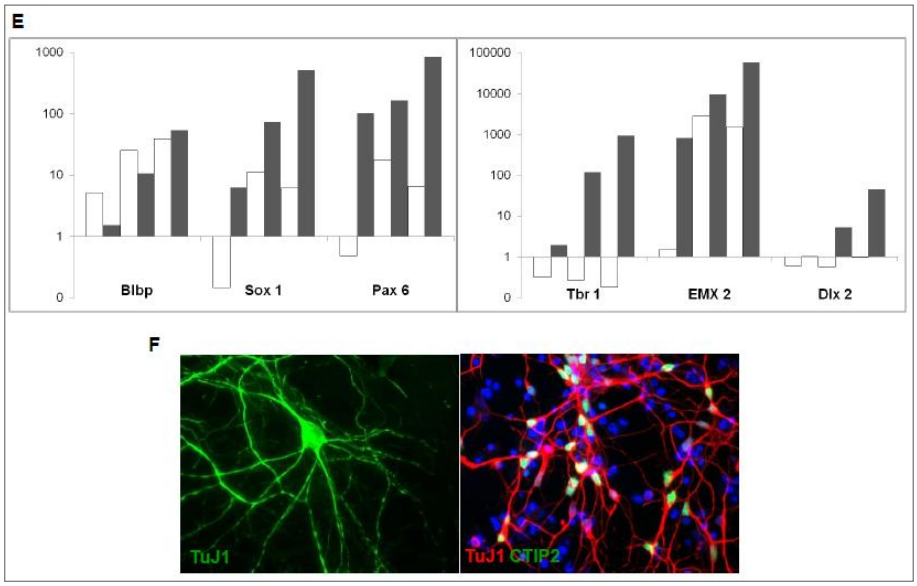


Figure 4: Differentiation of iPSCs-MPS IH in hepatocyte-like cells. (**A**) The panel represents the gene expression profile of hepatic markers (Sox 17, Brachyury, Gsc, CxCr4, Afp, Alb, G6PC, Cyp3A4, Mrp2, NTCP) obtained through real-time PCR at different time points (day 0, day 10 and day 28). Bars are the means DDCt. (**B**) After 28 days of hepatic differentiation medium, the differentiated iPSCs show the morphology of hepatocyte-like cell, rectangular shape with clear nuclei (magnification 20X). The immunocytochemistry analysis at day 28 reveals the expression of Albumin, Krt18, α-fetoprotein and Cyp3A4 in the differentiated iPS-MPS IH cell lines. (**C**) The panel represents the Cyp3A4 activity induced by phenobarbital in the differentiated progeny of iPSCs after 28 days of differentiation. (**D**) In the graph is depicted the albumin secretion analyzed at different time points during hepatic differentiation of iPSCs. Differentiation of iPSCs-MPS IH in neurons. (**E**) The panel represents the gene expression profile of neuronal markers (Tbr 1, EMX 2, Dlx 2, Blbp, Sox 1, Pax 6) in the progeny of differentiated iPSCs, obtained through real-time PCR at different time points (day 5, day 11, day 19 and day 24). Bars are the means DDCt. (**F**) The differentiated iPSCs-MPS IH express neuronal markers TuJ1 and CTIP2 by immunocytochemistry.

The expression of the hepatic markers were evaluated at different time points, following the embryonic liver differentiation from primitive endoderm to hepatocytes. The immunostaining also confirmed that iPSCs-MPS IH derived hepatocyte-like cells revealed the expression

of such hepatic markers at their final step of differentiation (Fig. 4B). To provide further evidence of the differentiated iPSCs on a fuctional level, we analyzed albumin secretion and detoxification capacity (cytochrome P450) of the hepatocyte-like cells derived from iPS-MPS IH and iPS-HD cell lines. In both assays, no differences among MPS IH and HD derived hepatocyte-like cells have been detected (Fig. 4C and 4D). Similarly, we performed the differentiation of the iPS-MPS IH cell line into neurons. No significant differences in the expression profile of neural markers at different time points between MPS IH and HD derived neurons (Fig. 4E). The neurons derived from iPSCs-MPS IH expressed the typical neuronal markers TuJ 1 and CTIP2 (cortical neurons) (Fig. 4F). Taken together our results demonstrated that iPSCs-MPS IH are able to differentiate into committed cell types such as hepatocyte-like cells and neurons with the same ability of an iPS cell line derived from a healthy donor.

Gene editing of iPS-MPS IH cell lines ZFNs-mediated

To achieve a level of IDUA expression sufficient for the phenotypic rescue of different cell types in Hurler patients, we proposed a Zinc Finger Nuclease-mediated approach for gene correction of the mutated *idua* gene in the iPSCs-MPS IH, safer than viral vectors. For this purpose, we developed a 7Kb plasmid carrying the homologous arms of the *AAVS1* locus, the puromycin resistant gene, EF1α as ubiquitous promoter, the fusion protein eGFP/IDUA and a rtTA sequence (Fig. 5A). In order to demonstrate the activity of the created plasmid, we tested the construct in the permissive Huh7.5 cell line. The immunofluorescence analysis and the specific IDUA enzymatic activity showed the expression of both eGFP and IDUA proteins in

the transduced cell line (Fig. 5B and 5C). Moreover, we used a nucleofection method for the delivery of the exogenous DNA in the iPSCs-MPS IH. To setted up the protocol, we used an "easy-access" donor plasmid (the pMax, available in the nucleofection kit) which reached a trasfection efficiency of ~ 30% viable transfected iPSCs-MPS IH (Fig. 5D).

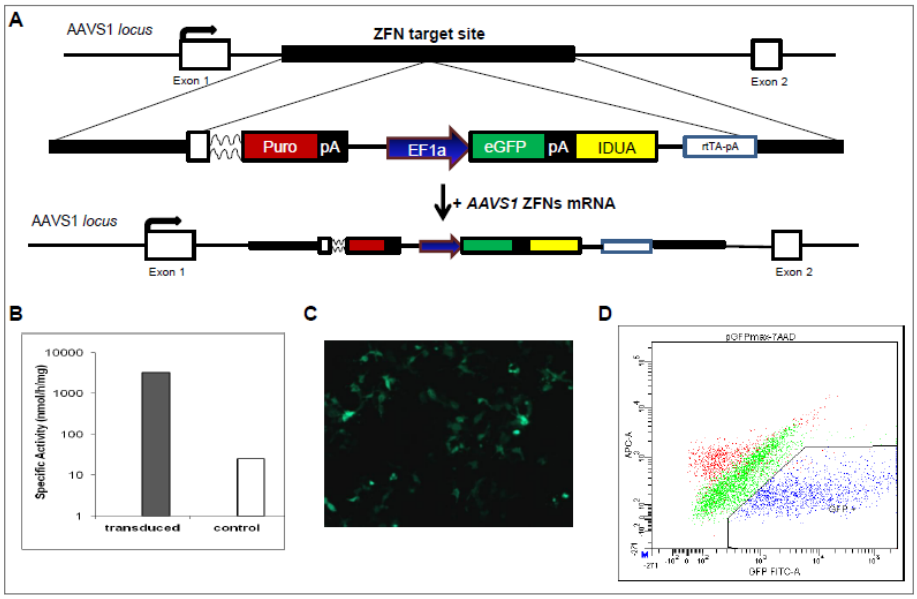


Figure 5: Gene targeting of iPSCs-MPS IH. (**A**) Schematic representation of the eGFP/IDUA minigene donor plasmid (upper part) and schematic *PPP1R12C* gene structure that includes exons 1 and 2 flanking the *AAVS1* locus (lower part). (**B**) Specific IDUA enzymatic activity evaluated in the Huh7.5 transduced cell line. (**C**) eGFP protein expression detected by immunofluorescence in the transduced Huh7.5 cell line. (**D**) Flow cytometry analysis of iPSCs transfected with the donor plasmid pMax via nucleofection method. The percentage of viable eGFP positive cells is ~30%.

Discussion

Lysosomal storage disorders (LSDs) are rare genetic diseases due to an intracellular accumulation of undegraded glycosaminoglycans. To date, only few studies have been accomplished on studying the characteristics of iPS from LSDs. Two works published by the group of Eto Y and collaborators described the generation of iPSCs from mouse models of Fabry disease, globoid cell leukodystrophy (GLD), mucopolysaccharidosis VII (MPSVII) and Pompe disease, demonstrating defects in disease-specific enzymes and the consequent accumulation of relative substrates. In addition, Fabry-iPSCs, GLD-iPSCs, MPSVII-iPSCs and Pompe-iPSCs were efficiently differentiated into disease-relevant cell types in vitro, such as cardiomyocytes and neural stem cells, without showing any significant difference in comparison with normal iPSCs [35] [36]. Interestingly, MPSVII-iPSCs exhibited a decreased ability to form embryoid bodies in vitro, suggesting possible abnormal embryogenesis in MPSVII. This observation demonstrated how disease-specific iPSCs could be an extraordinary source to study the disease at a very early stage of embryogenesis, especially in humans. In the report proposed by Kawagoe et al., skeletal muscle cells were successfully generated from murine Pompe-iPSCs cells and showed typical morphological features, spontaneous contraction and positive staining with the myosin heavy chain antibody [36]. Similarly, two studies described the isolation of iPSCs from LSDs in a human setting. Tolar and co-workers generated iPSCs from patients with Hurler syndrome. They isolated iPSCs both from keratinocytes and mesenchymal stem cells of MPS IH patients and then differentiated

them into hematopoietic cells in vitro, showing their ability to give rise to erythroid and myeloid colonies when plated in a semisolid medium. Interestingly, they gene-corrected the autologous hematopoietic progeny via lentiviral transduction, suggesting their future application for graft in HCT [19]. More recently, iPSCs have been isolated from fibroblasts of children with mucopolysaccharidosis type IIIB (Sanfilippo syndrome type B). The NAGLU enzyme deficiency affected the proliferation of emerging iPS clones, necessitating compensation of the genetic defect through enzyme replacement for efficient isolation. Notably, when MPS IIIB-iPSCs were differentiated into neuronal progeny, they expressed cell disorders consisting of storage vescicles and severe disorganization of Golgi ribbons, associated with modified expression of the Golgi matrix protein GM130 [37].

In the present study, we generated iPSCs from fibroblasts of two MPS IH patients, showing typical morphological features and expression for the pluripotency markers Oct-4, Nanog, Sox 2 and TRA-1-60. IPSCs-MPS IH demonstrated enzymatic IDUA activity reduction compared with normal iPSCs. This is consistent with the current view that the pathogenic cascade resulting from the *idua* gene lesion is established early in prenatal development, according to other LSDs [19, 35-37]. When injected into immunodeficient mice, iPSCs-MPS IH gave rise to the teratoma formation. This is an hallmark to test the pluripotency of iPSCs to differentiate *in vivo* into different tissue types belonging to all the germ layers. Interestingly, iPSCs-MPS IH and teratoma sections will be used for our future experiments

to explore the cellular compartment of lysosomes and the intracellular GAGs content in the newly formed tissues from iPSCs-MPS IH.

Similarly to wild type iPSCs, iPSCs-MPS IH could be induced to generate in vitro cell types of three germ layers, showing a normal capacity to differentiate into early neuroectoderm, mesoendoderm and extraembryonic tissues cells, as demonstrated by peculiar markers. In order to investigate the differentiation potential of iPSCs-MPS IH into disease-relevant cell types, we set up the differentiation of iPSCs-MPS IH into two committed cell types, such as hepatocyte-like cells and neurons. About hepatocytes differentiation, iPSCs-MPS IH have been able to form hepatocytes with typical cuboidal morphology, expression of characteristic genes (albumin, α-fetoprotein, Cyp3a4, glucose-6-phosphatase, Mrp2 and Ntcp) and markers (albumin, α-fetoprotein, keratin 18 and Cyp3a4). To address the hepatocytes functional activity of differentiated cells, two functional assays have been performed, evaluating the ability to secrete albumin in the medium and the detoxification capacity after drug administration. Similarly, iPSCs-MPS IH differentiated into neurons expressing their typical genes (Tbr1, Sox 1; Pax 6, Blbp and Emx 2) and markers (Tuj1, Nestin, Pax 6, CTIP2). Nevertheless, further investigations are needed to analyze the GAGs (heparan and dermatan sulphate) content and the specific enzymatic IDUA activity of hepatocyte-like cells and neurons derived from MPS-IH patients. Importantly, all the in vitro differentiation assays showed in our work, were performed in chemically defined medium, which enables the resulting differentiated cells compatible with possible future clinical applications. Indeed, most of the current methods available for inducing differentiation of

human iPSCs are based on culture system containing factors (such as serum, stroma cells, and complex matrices) that obscure analysis of developmental mechanisms or render the differentiated tissues incompatible with future clinical applications [38] [39].

As second objective of the present study, we considered the possibility to gene-correct the iPSCs-MPS IH using an alternative and safer method than viral vectors. We adopted the alternative approach of targeting a corrective *idua* gene specifically to a predesigned safe harbor site such as the *AAVS1* locus, which has previously been shown to permit stable expression of transgenes, and which is also a location that has been shown to have minimal effects on nearby genes [27] [29]. At the moment, we have been able to create a construct carrying the fusion protein eGFP/IDUA into the *AAVS1* locus and show its activity in the permissive cell line Huh7.5. Furthermore, we set up a nucleofection system to deliver the exogenous DNA into iPSCs; to do that, we used, as a preliminary approach, a smaller-size plasmid which is of "easy-access" for iPSCs. Previous studies demonstrated that ZFN targeting of a disease-corrective donor plasmid gene to the *AAVS1* safe harbor site is a broadly applicable approach to correction of monogenic disorders in iPSCs [20] [27]. The correction of iPSCs-MPS IH with the *idua* gene using the adopted system will explore the opportunity of targeting Hurler disease and will demonstrate, as a proof of principle, the realistic possibility to modify pluripotent stem cells derived from this disease.

In conclusion, human iPSCs could be generated from Hurler patients, showing similar morphological and functional features with normal iPSCs. Thus, our data demonstrates the peculiar promise of

iPSCs for studying the pathogenesis of MPS IH disease and exploring new therapeutic approaches. In particular, gene modification of Hurler disease with the proposed ZFN-based system is of interest and future efforts will aim to verify the feasibility of such innovative approach.

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Chapter 4

Summary, Conclusions and Future perspectives

Summary, Conclusions and Future perspectives

Mucopolysaccharidosis type I (MPS I) is a rare genetic disease belonging to the family of Lysosomal Storage Disorders (LSDs). It is caused by mutations in the *idua* gene, leading to a partial or total deficiency of the lysosomal enzyme α-L-iduronidase. This enzyme is involved in the physiological degradation of glycosaminoglycans (GAGs), particularly heparan and dermatan sulfate. In MPS I, GAGs progressively accumulate in the lysosomes, ultimately causing cell, tissue, and organ dysfunction by largely unknown pathophysiological mechanisms. Because of the ubiquitous presence of α-L-iduronidase, all body tissues are more or less severely affected. Depending on the severity of the disease, the MPS I patients share many clinical features, although in variable degrees. These include a chronic and progressive course, multisystem involvement, organomegaly, dysostosis multiplex, and abnormal facies. Hearing, vision, airway, cardiovascular function, and joint mobility are also affected; profound mental retardation is characteristic of the severe form. Based on clinical factors, the Hurler syndrome (MPS IH) is the most severe form of MPS I [1-4]. Nowadays, two treatments are mainly available for the therapy of MPS I: enzyme replacement therapy (ERT) with human recombinant iduronidase and hematopoietic cell transplantation (HCT). Weekly doses of intravenous iduronidase have been used for milder forms of MPS I. However, because iduronidase does not cross the blood-brain barrier efficiently, ERT alone is not indicated for the severe form of IDUA deficiency [5]. Allogeneic HCT, in contrast, leads not only to donor hematopoietic engraftment and systemic expression of IDUA, but also to donor myeloid cells

crossing the blood-brain barrier and correcting IDUA deficiency in the brain [6-8]. However, HCT is associated with significant morbidity and mortality from physical and immune injury [9-11] and some of the tissue-specific manifestations of MPS IH in cardiovascular, nervous, and skeletal systems persist even after full hematopoietic donor engraftment after allogeneic HCT. Thus, HCT is not able to prevent the disease progression in some anatomical sites.

Mesenchymal Stem Cells (MSCs) are multipotent stem cells from mesodermal origin able to differentiate *in vitro* into a variety of cells of the mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts [12-17]. Importantly, MSCs have an essential role to support the development and differentiation of hematopoietic cells [18]. Over the years, it has also become progressively clear that MSCs could be the basis for an extremely powerful "natural system of tissue repair": MSCs have been demonstrated, upon exogenous administration, to serve as effective therapeutic agents in a variety of experimental models of tissue injuries [19-23]. Some evidences in literature support the theory that MSCs can home to tissues, particularly when injured or inflamed, involving migration across endothelial cell layers.

The recent discovery by Yamanaka's group made a breakthrough in the stem cell field [24]. Indeed, adult somatic cells can be reprogrammed to a pluripotent state via the over expression of 4 defined transcription factors (Klf 4, Sox 2, Oct 4 and cMyc) into the so called induced Pluripotent Stem Cells (iPSCs). Once reprogrammed, iPSCs show pluripotent differentiation potential, independently of the tissue of origin, differentiating in nearly all cell

types of the three germ layers. iPSCs are morphologically, epigenetically and trascriptionally similar to embryonic stem cells (ESCs). In addition, they pass the hallmark test of pluripotency: when injected into immunocompromised mice, they form teratomas, showing their potential to differentiate *in vivo* into the three embryonic germ layers.

In our first study, we isolated MSCs from bone marrow of Hurler patients in order to better understand disease mechanisms involved in the skeletal defects. We characterized MSCs for their expansion rate, phenotype, IDUA activity, telomerase activity, and differentiation capacity towards adipocytes, osteoblasts, chondrocytes and smooth muscle cells *in vitro,* showing similar characteristics compared to controls. However, patient-derived MSCs displayed an increased capacity to support osteoclastogenesis *in vitro* which may correlate with the upregulation of the RANKL/RANK/OPG molecular pathway in MPS IH-MSCs compared to normal MSCs. Our results support the hypothesis that aberrant osteoclastogenesis may be one of the aspects involved in Hurler bone disease and that the abnormal ability of MPS IH-MSCs to support osteoclastogenesis may contribute to explain aspects of the skeletal phenotype seen in this disease. To date, only few studies have described the characteristics of the skeletal defects in Hurler patients [1-3, 25, 26] and a limited number of them has investigated possible mechanisms involved in the bone pathophysiology of this disease. A recent study accomplished in a MPS I mouse model showed that, at the skeletal level, the most severe difference between MPS-I and wild-type mice is an increase in the

amount of cartilage in the subepiphyseal growth plate, despite a simultaneous increase of osteoclasts. Moreover, the high concentrations of GAGs in MPS I may inhibit the collagenolytic activity of cathepsin K, a lysosomal cysteine protease highly expressed by osteoclasts and responsible for a significant part of total bone resorption. This study suggest that an impairment of cathepsin K activity may contribute to the deranged bone remodelling in MPS I [27]. Similarly, Simonaro *et al.* have described an increase in osteoclast-precursors and osteoclasts in bone marrow of MPS VI rats, with an up-regulation of RANKL in fibroblast-like synoviocytes [28]. Taken together, our data and those from previous studies focusing on the function of differentiated osteoclasts seem to reveal a more complex derangement of skeletal remodelling than currently appreciated. A vicious cycle of enhanced osteoclastogenesis and ineffective osteoclastic degradation of bone matrix would contribute to the skeletal phenotype in MPS I, explaining the enhanced numbers of osteoclasts. Our results support the hypothesis that aberrant osteoclastogenesis may be one of the aspects involved in Hurler bone disease and that the abnormal ability of MPS IH-MSCs to support osteoclastogenesis may contribute to explain aspects of the skeletal phenotype seen in this disease. Further studies are needed to elucidate bone remodeling mechanisms in Hurler syndrome. In the future experiments it will be intriguing to investigate generated osteoclasts function and a possible correlation between the abnormal osteoclasts production and the expression of cathepsin K as well as other molecules involved in the osteoclastogenesis process.

The second part of this PhD project was focused on the isolation of iPSCs from fibroblasts of Hurler patients in order to derive, in a first instance, different patient-specific cell types. The generated iPSCs-MPS IH cell lines were fully characterized for their pluripotency markers, gene expression profile, viral copy number integration and their potential to differentiate into the three germ layers both *in vitro* and *in vivo*. Interestingly, the iPSCs-MPS IH were able to differentiate into two disease-affected cell types, hepatocyte-like cells and neurons. No differences in morphology, gene expression profile, immunocytochemistry and functional activity of differentiated populations have been reported. As second objective, we are attempting to gene correct the iPSCs-MPS IH with an alternative and safer method than viral vectors, using a Zinc Finger Nucleases-mediated approach for gene targeting of pluripotent stem cells.

Further studies will be focused on testing the ability of iPSCs-MPS IH to differentiate in other affected cell types in Hurler syndrome, such as hematopoietic stem cells and chondrocytes. Particularly, it will be interesting to analyze the chondrocyte-derived cells in order to find altered disease mechanisms also in the cartilage compartment. This might recapitulate defects in the endochondral ossification process already detected studying MSCs (manuscript in preparation).

Introducing a set of transcription factors linked to pluripotency it is possible to directly reprogram human somatic cells to produce iPSCs, a method that has been achieved by several groups worldwide [29-32]. For cases where murine and human physiology differ, disease-specific

pluripotent cells capable of differentiation into the various tissues affected in each condition could undoubtedly provide new insights into disease pathophysiology by permitting analysis in a human system, under controlled conditions *in vitro*, and in a manner specific to the genetic lesions. Furthermore, creation of iPS lines from patients with single-gene disorders allows experiments on disease phenotypes *in vitro*, and an opportunity to repair gene defects *ex vivo*. The resulting cells, by virtue of their immortal growth in culture, can be extensively characterized to ensure that gene repair is precise and specific, thereby reducing the safety concerns of random, viral-mediated gene therapy. Repair of gene defects in pluripotent cells provides a common platform for combined gene repair and cell replacement therapy for a variety of genetic disorders, as long as the pluripotent cells can be differentiated into relevant somatic stem cell or tissue populations. Given the robustness of the approach, direct reprogramming promises to be an easy source of patient-derived cell lines. Such lines would be immediately valuable for medical research, but current methods for reprogramming require infecting the somatic cells with multiple viral vectors, thereby precluding consideration of their use in transplantation medicine at this time. Such patient-specific stem cells offer an unprecedented opportunity to recapitulate both normal and pathologic human tissue formation *in vitro*, thereby enabling disease investigation. Another useful application of iPS cell technology is the study of drug development. By comparison of normal and pathologic tissue formation, and by assessment of the reparative effects of drug treatment *in vitro*, cell lines generated from patients offer an unprecedented opportunity to recapitulate pathologic

human tissue formation *in vitro*, and a new technology platform for drug screening.

Most of the lysosomal storage diseases are life-threatening genetic diseases. Unfortunately, the pathogenesis of these diseases is poorly understood. Induced pluripotent stem cell technology offers new opportunities for both mechanistic studies and development of future stem cell–based therapies. The paucity of mechanistic studies of LSDs is due largely to the absence of faithful in vitro disease model systems. iPSCs offer the opportunity to establish such in vitro models of LSDs, because they can be generated from skin fibroblasts of patients and theoretically can differentiate into multiple cell types in vitro. In addition, the embryonic stem cell–like properties of iPSCs permit to study the disease in a culture system at a very early stage of embryogenesis that is difficult or nearly impossible to study *in vivo*, especially in humans. This unique capability provides an invaluable tool for investigating the abnormal early embryonic development that may occur in these genetic disorders. Notably, one of the limitations of rare disease studies, including this study, is the restricted availability of patient samples, which hampers investigation of rare disorders like MPS IH. Exploiting iPS cell technology, it is possible to derive different cell types from a patient specimen just reprogramming fibroblasts (or other adult cell sources).

The complexity of MPS IH, as evidenced by several biochemical events downstream of the *idua* lesion and by different phenotypes of human disease, is perhaps matched by the versatility and promise of iPS cell technology. Thus, even at present, iPSCs-MPS IH offer a new model of MPS IH disease and may help to understand pathogenic

cascades underlying the inherent complexity of MPS IH, including the effect of normal and aberrant GAGs, which have fundamental roles in cell growth, differentiation, and tissue pattern formation [33-35] as well as to study the disease mechanisms in those anatomical sites not fully corrected after HCT.

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Gatto F., Vallier L., Biondi A., Serafini M. *iPS cells: a modelling to study Mucopolysaccharidosis type I* . 18th ESGLD (European Study Group Lysosomal Disease) Workshop. Langvik, Finland, 3-6 September 2011

Gatto F., Vallier L., Biondi A., Serafini M. *iPS cells: a novel cellular source for Mucopolysaccharidosis type I* . 9th Annual Meeting ISSCR. Toronto (Ontario), Canada, 15-18 June 2011

Gatto F. *iPS: una nuova fonte cellulare per lo studio della MPS I".* Invited speaker at the XXIV International Congress AIMPS. Senago (MI), Italy, 14-17 April 2011

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Gatto F., Biffi A., Biondi A., Serafini M. *Isolation and characterization of Mesenchymal Stem Cells derived from MPS-I mouse model.* VI DTI Telethon Retreat. Bardolino (VR), Italy, 21-23 October 2008

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