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Genetic Manipulation of Stem Cells

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Abstract

Stem cells have the remarkable potential for self-renewal and differentiation into many cell types in the body during early life and development. In addition, in many tissues they constitute a source of internal repair system, dividing essentially without limit to replenish damaged or dead cells. After division, each new cell has the potential either to retain the stem cell status or to differentiate to a more specialized cell type, such as a red blood cell, a brain cell or a heart cell.

Until recently, three types of stem cells from animals and humans have been characterized, i.e. embryonic stem cells, fetal stem cells and somatic adult stem cells. However, in late 2007, researchers accomplished another breakthrough by identifying conditions that allow some specialized adult cells to be "reprogrammed" genetically to assume a stem cell-like state. These cells, called induced pluripotent stem cells (iPSCs), express genes and factors important for maintaining the unique properties and features of embryonic stem cells.

This review analyzes the mechanisms of genetic manipulation of stem cells, including the transfer of therapeutic genes into patients' cells via recombinant viral vectors for gene therapy purposes and discusses the mechanisms of generation and the resulting properties of induced pluripotent stem cells.

Keywords: Gene therapy; Lentiviral vector; Oncoretroviral vector; Somatic stem cells (HSCs); Induced pluripotent stem cells (iPSCs)

Abbreviations: SSC: Somatic Stem Cell; ESC: Embryonic Stem Cell; NSC: Neuronal Stem Cell; HSC: Hematopoietic Stem Cell; iPSC: Induced Pluripotent Stem Cell; MLV: Murine Leukemia Virus; HIV: Human Immunodeficiency Virus; TU/ml: Transduction Unit/millilitre; SIN: Self Inactivating; LTR: Long Terminal Repeat; PIC: Pre-Integration Complex

Gene Therapy Technologies and Stem Cell Therapeutic Approaches

Stem cells constitute a very important subset of tissue cells, as they can generate all the different cell types included in the body. All stem cells, regardless of their originating source, have three major general properties:

- They are capable of dividing and self-renewing for long periods.
- They are maintained in an undifferentiated state.
- They can give rise to multiple types of specialized cells.

Given their unique regenerative abilities, stem cells offer a new exciting potential for treating debilitating diseases such as diabetes, heart disease and degenerative neurological disorders. However, much work remains to be done in the laboratory and in the clinic to understand how to efficiently use these cells for cell-based therapies in order to treat a growing number of diseases in the context of the emerging field referred as regenerative medicine. In parallel, genetic manipulation of stem cells can also be used to treat important diseases in a gene therapy context. According to the American Society of Gene and Cell Therapy, gene therapy is defined as the introduction or alteration of genetic material within a cell or organism with the intention of curing or treating a disease [1]. Therapeutic genes, also referred to as transgenes, are transferred into the cells of patients either through a recombinant virus, a non-viral vector, or naked DNA.

Three main types of gene therapy approaches are currently being employed:

In situ

Gene therapy in which the vector carrying the therapeutic genetic material is directly administered to the affected tissue, such as by an injection into a tumor nodule or organ [1].

Ex vivo

Gene therapy during which the patients' cells are harvested and co-cultured in the laboratory in the presence of the therapeutic vector. The corrected cells with the new genetic material are then transplanted back to the patient from whom they were originally derived [1].

In vivo

Gene therapy is defined as the administration of the vector carrying the therapeutic genetic material directly to a live animal. The vector can be delivered by a variety of methods, such as intravenous injection or by other physical means of administration such as hypodermic injection, aerosol, or employing other routes [1].

In all cases, the most important issue to resolve in a gene therapy context is the adequate correction of the phenotype of the disease, by constructing the most efficient vector in terms of a) gene delivery, b) stability, c) appropriate and tissue-specific transgene expression and d) safety. Furthermore, the type of cell to be selected for correction, is of critical importance, and will depend on the type of organ(s) and/or tissues that manifest the abnormal phenotype. Gene therapy in

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general, is not suitable for the treatment of complex disease phenotypes associated with multiple affected genes or for the multiple genetic variations that underlie complex disorders. Rather, gene therapy, with the exception of cancer and several other multifactorial disorders, is mostly applicable for monogenic disorders, such as the hereditary disorders caused by mutations in more than 1,800 gene loci [2]. Therefore, it is conceivable that if sufficient correction or compensation can be achieved with gene transfer, monogenic disorders could be prevented and/or treated [2].

In vivo and *in situ* gene therapy targets a whole organ or tissue, including both differentiated and undifferentiated types of cells. On the other hand, e*x vivo* gene therapy is especially attractive in correcting somatic stem cells (SSCs) located in the organ that is malfunctioning, secondary to the abnormal phenotype. Somatic stem cells derive from various fetal and postnatal organs and are able to differentiate into the cell types located in the tissue in which they reside [3,4]. Typically, SSCs are designated on the basis of the organ from which they derive from, such as hematopoietic stem cells or HSCs [5]. Target monogenic diseases for this type of therapy, among others, include the X-linked severe combined immunodeficiency, chronic granulomatous disease, Fanconi anemia, β-thalassemia and sickle cell disease.

For the aforementioned hematopoietic disorders the stem cell of choice is the $CD34^+$ positive cell fraction derived mainly from the bone marrow [6].

Mesenchymal stem cells constitute another type of somatic stem cells, mainly characterized by their ability to differentiate into numerous tissues, including bone, cartilage and adipose tissue [7]. Specifically, it is this multipotent differentiation potential that is being used as a functional criterion for the definition of MSCs, since there are no specific markers assigned for the identification of this cell population. *Ex vivo*-expanded MSC express a number of non-specific surface molecules such as CD105, CD73, CD90, CD166, CD44 and CD29. MSCs are also involved in the regulation of the immune system, since they exert suppressive effects on T and B cells, natural killer (NK) cells, as well as on dendritic cells. Finally, MSCs have also been shown to recruit and/or induce regulatory T cells, leading to enhanced immunosuppressive effect. So far, mesenchymal stem cells have not been involved in gene therapy clinical trials but are rather used as carriers in a cell therapy context. In that sense, MSCs are not genetically engineered in order to improve the phenotype of the respective tissue they derive from, but are used to carry a therapeutic gene to a damaged tissue. Recently, Zagoura and colleagues [7] have documented the effects of genetically engineered MSCs for the treatment of acute liver failure in an animal model, utilizing human amniotic fluid MSCs. Another major advantage of MSCs comprises their innate tropism to solid tumors *in vivo* that readily qualifies these cells as the perfect carriers for anti-tumour agents [8]. In the aforementioned settings, MSCs are transduced in the laboratory with a vector carrying the desired molecule to be transferred and are then transplanted back to the animals.

Neurological diseases considered amenable to gene therapy include neurodegenerative disorders, brain tumours and autoimmune defects that lead to the destruction of nerve tissues. One approach to coping with neuronal loss is the activation of endogenous neural stem cells (NSCs) in the adult brain. Neural stem cells can be defined as cells that can continuously self-renew and have the potential to generate intermediate and mature cells of both glial and neuronal lineages. There are several subpopulations of neural stem cells that could be restricted to particular developmental stages or regions of the mature brain, and each of these populations is expected to have specific biological features [9]. One site of NSCs source throughout life was identified within the subventricular zone [10]. Neural stem cells have been considered for use in cell replacement therapies in various neurodegenerative diseases because of their high migration rate to areas of brain pathology, such as ischemic and neoplastic lesions. However, the general rationale for gene therapy is the treatment of diseases that are caused by the lack of some crucial factor(s) by restoring the factor(s) using appropriate gene expression vectors. For example, proposed gene therapies for Alzheimer's disease, include targeted expression of choline acetyltransferase to compensate for deficits in acetylcholine [10]. The lack of dopamine in the putamen, caused by the degeneration of innervating neurons in the substantia nigra, has a central role in the pathogenesis of Parkinson's disease (PD). Systemic L-DOPA (L-3,4-dihydroxyphenylalanine) therapy is an effective treatment for the symptoms of PD and because the degeneration lesion is relatively localized, PD was one of the first targets for cell therapy [10]. Finally, ectopic expression of neurotrophic factors mediated either by viral vectors or the transplantation of genetically modified cells have shown some promise in the treatment of mouse models of Huntington's disease [11].

Gene Therapy Vectors

Vectors constitute the vehicles or carriers that contain the therapeutic transgene for delivery to the cells [1]. The vectors derive either from genetically engineered viruses or from non-viral formulations such as those manufactured by the use of nanotechnology (i.e. liposomes) and naked DNA [12]. Vectors deliver the therapeutic gene either by transducing the right type of stem cell *in vitro*, or by direct injection in the animal or the tissue. In all cases, the designed vector should not induce cytotoxic effects and should be rescuable after transplantation and/or injection in order to maximize the safety of the whole gene/cell therapy approach.

Several types of recombinant viruses are used as vehicles of the therapeutic genes in the patients' stem cells. These include:

- Retroviruses belonging in the subfamily of *Oncoretroviridae* (e.g. MLV) or *Lentiviridae* (e.g. HIV)
- Adenoviruses
- Adeno-Associated viruses
- Herpes viruses
- Vacinnia viruses and many others.

Of the aforementioned viruses, retroviruses are widely used as efficient tools for genetic manipulation of stem cells, mainly because of their ability to integrate into the host cell's genome through the reverse transcription process during which the viral RNA is converted to DNA. Integration of the retroviral DNA genome into the host cell DNA is an essential step in the retrovirus replication cycle, permitting viral genomes to become permanently fixed as proviruses into the DNA of the host. During this process, the retroviral DNA is associated within a large complex with a subset of retroviral proteins known as the pre-integration complex (PIC). For oncoretroviruses (gamma-retroviruses) such as the Murine Leukemia Virus (MLV), uncoating, DNA synthesis, and formation of the PIC occur at the same rate, both in non-dividing cells as well as in dividing cells, but integration fails to occur. However, during mitosis, the nuclear membrane disassembles, rendering the chromosomes accessible to the virus, suggesting that infection by oncoretroviruses such as MLV requires cell division [13].

Oncoretroviruses consist of an enveloped capsid that contains a plus (+) strand RNA genome ranging from 7 to 10 kb. Their tropism often includes hematopoietic stem cells. The advantages and disadvantages of oncoretroviral vectors are depicted in Table 1.

On the other hand, lentiviruses, display one major difference from oncoretroviruses, i.e. they do not require dissociation of the nuclear envelope in order to integrate their genome into the host's genome, as it has been extensively documented that they can efficiently infect both dividing and non-dividing cells. HIV in particular, has the capacity to cross the nuclear membrane of interphasic cells. This represents a crucial aspect for genetically modifying tissues, especially those considered as the main potential cell targets of gene therapy, such as the brain, muscle, liver and the hematopoietic system [14], as they can transduce even non-dividing cells residing in the G_o phase of the cell cycle.

Another virus used as gene therapy vector is the adeno-associated virus (AAV). AAV is a non-pathogenic, non-enveloped virus containing a 4.7 kb single-stranded DNA genome that encodes the structural proteins of the viral capsid, encoded by the cap gene and the non-structural proteins necessary for viral replication and assembly, encoded by the rep gene, flanked by short inverted terminal repeats. Its life cycle involves two phases, the replicative and the latent phase. In the productive phase, it co-infects the host cell only when a helper virus is present [15]. In the absence of a helper virus, AAV usually enters the latent phase, integrating into the human genome, commonly within a specific region of chromosome 19, although this has been observed only in cell lines. Its principal advantage for gene therapy is the poor inflammatory response to infected cells. As a therapeutic vector, AAV consists only of the inverted terminal repeats which are necessary for replication, packaging, and integration, while the viral coding

Oncoretroviral vectors	
Advantages	**Disadvantages**
Efficient and stable gene transfer • Transduction rates of up to 40% of HSCs in non-human primates	Low rates of expression • One or fewer copies of provirus per cell • Sensitive to chromosomal position effects • Sensitive to DNA repeats, introns
High fidelity gene transfer due to intact integration and absence of chromosomal rearrangements	Limits on the size of the therapeutic gene (< 7 kb) insert
Extensive clinical experience suggests that they are generally safe	Difficult to deliver *in vivo* due to low titers (<10^7 TU/ml)
Safety concerns. Potential problems with insertional mutagenesis due to integration inside or near a proto-oncogene .	
Lentiviral vectors	
Advantages	**Disadvantages**
Efficient and stable gene transfer • Transduction rates of up to 90% of HSCs • High levels of transgene's expression • High titers (>10^8 TU/ml) • Extensive clinical experience due to AIDS	Sensitive to chromosomal position effects
High fidelity gene transfer due to intact integration and absence of chromosomal rearrangements	Limits on the size of the therapeutic gene (< 10 kb) insert
Safety concerns. Potential problems with insertional mutagenesis due to integration inside or near a proto-oncogene .	
Adenoviral vectors	
Advantages	**Disadvantages**
High efficiency of gene transfer • Delivery of many genome copies per target cell, that typically translates into very high expression • Relatively large therapeutic genes (25-30 kb with the latest generation vectors) High fidelity of gene transfer • Vector genomes are genetically stable	Transfer and expression are transient • Since adenoviruses are non-integrating viruses, the transgene expression typically lasts 1-2 months in non-dividing cells, while is much shorter in dividing cells
In vivo administration	The pre-existing immunity against adenoviruses in individuals may result in low levels of transgene delivery and expression
Extensive clinical experience	Vectors are immunogenic since the virus capsid and remaining viral proteins cause inflammation
No issues with insertional mutagenesis	Safety issues: Vectors are lethal at high doses and are highly infectious
Adeno Associated Vectors (AAV)	
Advantages	**Disadvantages**
Highly efficient gene transfer • Several genome copies per target cell	Small transgenes (4.7 kb)
High fidelity gene transfer • Vector genomes are genetically stable • Stable expression in some settings • Able to integrate into target cell genome	• Transfer and expression are not always stable as the virus does not always integrate • Questionable tropism for HSC
In vivo administration	Frequently immunogenic
Generally safe at doses tested	The pre-existing immunity in individuals may result in low levels of transgene delivery and expression
Safety concerns: Potential problems with insertional mutagenesis and small chromosomal rearrangements	

Table 1: Advantages and disadvantages of gene therapy vectors.

sequences are entirely removed, rendering AAV vectors replication-deficient. The resulting recombinant vectors can efficiently deliver a transgene and safely mediate long-term gene expression in dividing and non-dividing cells of numerous tissues. AAV has the potential to facilitate long-term transgene expression in the absence of destructive T cell responses and such vectors have been generally proven safe. However, naturally occurring AAV variants are typically inefficient in infecting a number of stem cell types, particularly human embryonic stem cells [15].

Human adenoviruses constitute another viral family used in gene therapy approaches, and classified into 51 immunologically distinct serotypes, divided into 6 subgroups, namely subgroups A–F. Adenoviruses are non-enveloped, double-stranded DNA viruses. Their genome size ranges from approximately 30 to 35 kilobases and contains five segments that encode early gene products (E1a, E1b, E2a, E2b, E3, and E4), and five segments that encode late gene products (L1–L5). E1, E2, and E4 gene products regulate transcription and translation of the late genes and therefore, are indispensable for viral replication [16]. Adenoviral vectors are among the most promising gene transfer vehicles for the *in vivo* treatment of a range of human diseases, e.g. cystic fibrosis and hemophilia because of their ability to infect a wide spectrum of cell types, including quiescent cells. Although replication-deficient vectors based on adenoviruses can be produced easily and at high titres, two main disadvantages occur: a) the transgene's expression is transient and often lasts less than one month and b) the stimulation of the host immune response, both cellular and humoral. First-generation adenoviral vectors may be applied where short-term expression and single dosing is adequate, such as cancer vaccine therapies. Enthusiasm toward the use of first-generation adenoviral vectors in gene replacement therapy diminished because not only they did fail to achieve sustained gene transfer, but also resulted in significant toxicity and in the death of an individual [16]. The advantages and disadvantages of adeno-associated and adenoviral vectors are also depicted in Table 1.

The Side Effects of Insertional Mutagenesis

While the possibility of insertional mutagenesis using replication-defective vectors has been considered as theoretically possible [17], such risks had been originally estimated to be extremely low [18], based on the assumption that proviral integration into the genome was random [19]. With the readily accessible human genome sequence data, mapping studies of retroviral integration sites in cell lines have uncovered non-random integration patterns, using wild-type HIV, HIV-derived, or murine leukemia virus (MLV)-derived vectors [20]. Moreover, these integration patterns have only been recently investigated in the most relevant primary cells for hematopoietic gene therapy, namely $CD34^+$ hematopoietic stem cells or HSCs [21,22]. These studies have disclosed that while MLV integrants were located predominantly around transcription start sites, HIV integrants strongly favoured transcription units and gene-dense regions of the genome. The clinical trial for adrenoleukodystrophy confirmed the typical distribution of the respective lentiviral vector into gene coding regions, without a particular preference for transcriptional start sites, and frequent occurrence in chromosomes harboring gene-dense regions [23].

Most importantly, the report of the clonal malignant lymphoproliferation due to insertional activation of the LMO2 gene following gene therapy for X-linked severe combined immunodeficiency [24], as well as the detection of retroviral insertion into the MDS1-EVI1 region, in a total of 14 times in 9 animals [25], has led to a re-evaluation of the mechanisms of insertional mutagenesis. These integration patterns suggest different mechanisms for integration as well as distinct safety implications for oncoretroviral versus lentiviral vectors. The basis for these preferences is unknown, but it is assumed that they may reflect interactions of the pre-integration complex with specific proteins or with specific DNA sequences or structures that are associated with transcription. Nevertheless, the use of specific DNA elements called insulators, exhibiting the capacity to block enhancer activity and maintain the chromatin status of distinct chromosomal regions, was eventually introduced in the construction of lentiviral vectors in order to a) diminish variable expression and silencing of the transgene and b) reduce the risk of insertional mutagenesis [26,27]. This strategy combined with the deletion of the U3 region of the LTR in the self-inactivating (SIN) configuration of lentiviruses, has rendered lentiviral vectors as powerful tools in the fields of neuroscience, hematology, developmental biology, stem cell biology and transgenesis over the past decade.

To this end, the SIN lentiviral vector used in the current β-thalassemia clinical trial organized by the group of P. Leboulch, upon request of the FDA, contains a fragment of the cHS4 insulator incorporated in the U3 region of the LTR. Despite the fact that this trial is considered successful, since the second patient who underwent gene transfer, reports good wellbeing and is free of transfusions for almost 2 years, still the results have revealed that the patient exhibits a relative clonal dominance [28]. Specifically, of the 10% of lentiviral-modified cells, a single clone, identified as having an integration site in the third intron of the HMGA2 gene, was represented in excessive proportion relative to the contribution of the other clones. There is some clinical relevance of the alteration of HMGA2 expression by the vector integration, due to the fact that this gene has been implicated as a potential oncogene in a variety of settings [29]. However, it remains unclear whether the vector insertion into this gene has actually resulted to the relative high contribution of this clone to hematopoiesis, since it is conceivable that the above observation may simply reflect the consequences of engraftment from a small number of transduced HSCs [28].

Current Clinical Trials

Chronic granulomatous disease (CGD)

Chronic granulomatous disease (CGD) is a primary immunodeficiency disorder, characterized by deficient antimicrobial activity in phagocytic cells due to defects in the nicotinamide dinucleotide phosphate (NADPH) oxidase complex that results in deficient antimicrobial activity of phagocytes. The NADPH oxidase plays a pivotal role in microbial killing by reducing molecular oxygen to superoxide, which subsequently reacts to form reactive oxygen species (ROS) like hydrogen peroxide, hypochlorous acid, and hydroxyl radicals [30]. Clinical trials for CGD with gene-modified cells were first initiated in the mid-1990s, as shown in Table 2, with vectors carrying genes encoding for the subunits of the NADPH oxidase such as the $gp91^{phox}$. The vectors delivered the transgene *ex vivo* to $CD34^+$ hematopoietic stem/progenitor cells deriving from the bone marrow, and the cells were reinfused back to the patients. Twelve patients have been so far treated with genetically modified $CD34^+$ cells throughout the world and they all showed initial clinical benefit [30]. Unfortunately, three of them developed clonal myeloproliferation due to insertional mutagenesis.

Adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID)

Adenosine deaminase-deficient severe combined

Disease	Affected tissue	Target cell	Type of vector	Reference
Chronic Granulomatous Disease (CGD)	Hematopoietic System, Immunodeficiency	$CD34^+$ hematopoietic stem/ progenitor cells	Retroviral (oncoretroviral)	30
Adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID)	Hematopoietic System, Immunodeficiency	CD34+ hematopoietic stem/ progenitor cells	Retroviral (oncoretroviral)	31
X-linked severe combined immunodeficiency (X-SCID)	Hematopoietic System, Immunodeficiency	CD34+ hematopoietic stem/ progenitor cells	Retroviral (oncoretroviral)	24
β-thalassemia	Hematopoietic System, Ineffective erythropoiesis	CD34+ hematopoietic stem/ progenitor cells	Retroviral (lentiviral)	28
X-linked adrenoleukodystrophy (ALD)	Brain	CD34+ hematopoietic stem/ progenitor cells	Retroviral (lentiviral)	23
Metachromatic leukodystrophy (MLD)	Brain	CD34+ hematopoietic stem/ progenitor cells	Retroviral (lentiviral)	33

Table 2: Current gene therapy clinical trials in humans.

immunodeficiency (ADA-SCID) represents an inherited disorder characterised by profound depletion of T, B, and natural killer cell lineages due to genetic defects in the purine salvage enzyme adenosine deaminase (ADA). Adenosine deaminase deficiency is treated with enzyme replacement and the results suggest that simple gene expression systems may be efficacious for this condition. In a current gene therapy trial, six children have undergone treatment with autologous $CD34^+$ hematopoietic bone marrow stem cells transduced by an oncoretroviral vector encoding the human ADA gene. Four of the six patients recovered immune function as a result of engraftment of gene-corrected cells. All patients remained free of infection and there were no adverse leukemic side effects suggesting that gene therapy is promising for ADA-SCID [31].

X-linked severe combined immunodeficiency (X-SCID)

X-linked severe combined immunodeficiency (X-SCID) is a primary immunodeficiency which is caused by naturally occurring mutations in the IL2RG gene that encodes the interleukin-2 receptor subunit gamma (IL2Rγ), rendering the IL-2 receptor absent or dysfunctional. This condition is characterized by the complete lack of T cells and natural killer cells, whereas B cells are present. In the so-called French clinical trial, nine patients, who lacked an HLA-identical donor, underwent *ex vivo* oncoretrovirus-mediated transfer of IL-2γ chain to autologous $CD34^+$ bone marrow stem cells between 1999 and 2002. Eight patients were alive after a median follow-up period of 9 years. Gene therapy was successful at correcting immune dysfunction in eight of the nine patients. Unfortunately, acute leukemia developed in four patients, one of whom died, due to overexpression of the LMO2 proto-oncogene [24] that was attributed to the integration of the oncoretroviral vector within this specific gene. After a 10 year-period of follow-up, gene therapy was proven to have corrected this immunodeficiency and may be an option for patients who do not have an HLA-identical donor for hematopoietic stem cell transplantation. However, this genetic treatment is associated with a risk of acute leukemia [32].

Thalassemia and hemoglobinopathies

The β-thalassemias are inherited anemias caused by mutations that reduce or suppress production of the β-globin chain of the hemoglobin molecule, being most prevalent in the Mediterranean region, the Middle East, the Indian subcontinent and South East Asia, representing a serious world health problem. On the other hand, sickle cell anemia or sickle cell disease (SCA or SCD, respectively) is caused by a point mutation in the β-globin gene (β^S), resulting in sickle hemoglobin (HbS). HbS polymerizes upon deoxygenation, thus creating sickle-shaped red blood cells that occlude microvasculature [6]. Current therapies for these diseases include chronic transfusions in combination with life-long iron chelation, hydroxyurea for the induction of fetal hemoglobin (HbF) and supportive care for episodic sickling. However, the complications of iron overload, together with the sequelae of the anemia and ineffective erythropoiesis, represent the major causes of morbidity and mortality. Patients with thalassemia, were actually the early targets for gene therapy because the β globin gene is small and well characterised, the target hematopoietic stem cell is easily obtainable and mouse models had been cured by transfer of the human globin gene. Therefore, gene therapy of autologous hematopoietic $CD34^+$ stem cells (HSCs) followed by transplantation could avoid adverse immunological consequences and will not be restricted by the availability of donors. It may also not require myeloablative-conditioning regimens, and thereby have lower toxicity. Following early attempts which were proven unsuccessful, detailed basic studies were undertaken to improve the efficiency of gene transfer and expression [28,29]. Thus, the major current drawbacks of the field affecting therapeutic efficacy, include 1) insufficient transduction efficiency of the target hematopoietic stem cells, 2) inconsistent expression of the transgene, 3) putative aberrant expression near integration sites raising safety issues and 4) lack of long term expression of the transgene, exhibiting eventual silencing [6]. There is one currently active gene therapy-based clinical trial for β-thalassemia conducted in France by the group of Dr Leboulch [28]. Details of this trial have been discussed in the insertional mutagenesis paragraph.

X-linked adrenoleukodystrophy (ALD)

X-linked adrenoleukodystrophy (ALD) is a severe brain demyelinating disease in boys that is caused by a deficiency in ALD protein, an adenosine triphosphate–binding cassette transporter encoded by the ABCD1 gene. The ALD protein participates in the peroxisomal degradation of very-long-chain fatty acids (VLCFAs) in oligodendrocytes and microglia, and deficiency of this protein disrupts myelin maintenance by these cells [23]. Allogeneic hematopoietic cell transplantation (HCT) is the only effective therapy to date and is mediated by the replacement of brain microglial cells derived from donor bone marrow myelo-monocytic cells. In the gene therapy-based clinical trial [23], autologous $CD34^+$ cells were initially harvested from the patients, were further genetically corrected *ex vivo* with a lentiviral vector encoding wild-type ABCD1, and then were re-infused into the patients. Beginning 14 to 16 months after infusion of the genetically corrected cells, progressive cerebral demyelination in the two patients stopped, providing evidence that lentiviral-mediated gene therapy of hematopoietic stem cells can provide clinical benefits in ALD.

Metachromatic leukodystrophy (MLD)

Metachromatic leukodystrophy (MLD) is a lysosomal storage disease caused by deficiency of the lysosomal enzyme arylsulfatase

A (ARSA) that results in intralysosomal storage of sphingolipid cerebroside 3-sulfates (sulfatides), which are abundant in myelin and neurons. These effects cause demyelination and neurodegeneration, creating various and ultimately lethal neurological symptoms. Restoration of only 1-5% of the normal enzyme level confers clinical benefit. Enzyme replacement and bone marrow transplantation represent current treatment options. The ongoing gene therapy phase I/II clinical trial started recruiting in late March 2010 in Italy. The protocol uses the patients' own genetically modified hematopoietic stem cells to increase ARSA production to 10-15-fold the normal rate. This super-production in the blood aims to offset the reduced number of cells that typically cross the blood-brain barrier [33]. The results of this trial remain to be announced.

The Use of Induced Pluripotent Stem Cells

Until recently, scientists primarily worked with three types of stem cells from animals and humans, i.e. embryonic stem cells, fetal stem cells and somatic adult stem cells [4]. However, in 2007, researchers identified conditions that would allow some specialized adult cells to be "reprogrammed" genetically to a stem cell-like state. These reprogrammed cells are called induced pluripotent stem cells (iPSCs) and express genes and factors important for maintaining the definitive properties of embryonic stem cells. iPS cells are supposed to be equivalent or comparable to embryonic stem cells in morphology, gene expression, and epigenetic status and can give rise to three-germ layers. Epigenetics is defined as the heritable changes of gene expression caused by mechanisms other than changes in the underlying DNA sequence. Epigenetic modification can happen at the DNA level by DNA methylation of promoter regions and/or by methylation or acetylation of histones that wrap any given genomic region. It should be noted, that these and other additional changes and modifications, can lead to specific transcriptional activity of iPS cells, reflecting primarily their cells of origin, thus unraveling the important issue of residual epigenetic memory, which should be taken in consideration when dealing with reprogramming attempts [34]. The necessary conditions initially identified to reprogram a somatic cell to a stem cell include the expression of the following genes, i.e. Oct3/4, Sox2, Klf4, c-Myc or Nanog and Lin28. The aforementioned proteins can be expressed in the originating cells either after retroviral transduction, or plasmid-based transfection or electroporation. These were first published by the Yamanaka [35] and Jaenisch group [36] and were later corroborated by the group of Kan [37]. This technology further offers the possibility of transforming a somatic cell to a stem-like cell and the additional ability to perform homologous recombination at that stage, in order to replace the mutated gene causing the disease, with the normal homologue. Thus, somatic cells from a patient may be isolated, genetically manipulated to form iPSCs, corrected by homologous recombination, differentiated to the desired cell lineage or tissue type, and then infused back to the patient. The proof of principle for the feasibility of such feature studies has been recently provided by the efficient correction of sickle cell anemia in a mouse model [36]. Several different techniques to induce pluripotency to a somatic cell and have been developed and are extensively reviewed by Patel and Yang [38]. Briefly, these techniques include somatic cell transfer, cell fusion, and reprogramming either through cell extracts or directly by using lentiviral vectors in order to increase the expression of the aforementioned genes. Thus, the major advantages of iPS cells include the following: a) they are easily accessible from adults, b) no embryonic material is needed, and therefore there are no ethical considerations involved and c) they can expand relatively well in culture compared to the respective adult stem cells that are generally rare and difficult to grow *in vitro*. Moreover, they provide a great potential for analyzing the mechanisms of human diseases *in vitro* and designing corrective strategies [39]. However, despite their great potential, the current bottlenecks of iPSCs include inadequate cell number, immune rejection, and teratoma formation upon transplantation [40,41]. Finally, other safety issues inherent to the iPSC technology that remain to be addressed before proceeding to the clinical use, are the genetic stability caused during the reprogramming phase, as well as the epigenetic changes of iPSCs [34,41].

Conclusions

Clinical trails involving gene transfer to somatic stem cells, are becoming increasingly prevalent for a wide range of disease targets, employing numerous types of therapeutic genes and gene delivery vectors. Most trials involve the use of recombinant viruses, such as retroviruses. Retroviruses, however, cause insertional mutagenesis due to integration in the genome of transplanted cells, an issue which currently represents the major challenge in treatments involving gene transfer. As a field, gene therapy has been marked by both well-publicized failures and a growing potential for profound successes. Finally, the recent development of induced pluripotent stem cells from differentiated cells has provided new insights but has also introduced new challenges such as the formation of teratomas. Continued success depends on careful and informed choices in study design, optimal vector development and regulatory oversight.

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