Genetic medicines: treatment strategies for hereditary disorders

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Abstract | The treatment of the more than 1,800 known monogenic hereditary disorders will depend on the development of 'genetic medicines' — therapies that use the transfer of DNA and/or RNA to modify gene expression to correct or compensate for an abnormal phenotype. Strategies include the use of somatic stem cells, gene transfer, RNA modification and, in the future, embryonic stem cells. Despite the efficacy of these technologies in treating experimental models of hereditary disorders, applying them successfully in the clinic is a great challenge, which will only be overcome by expending considerable intellectual and economic resources, and by solving societal concerns about modifications of the human genetic repertoire.

Metabolic manipulation
The use of dietary modification or small molecule therapy to compensate for a deranged biological process.

Protein augmentation
A therapy in which a missing protein is replaced by the administration of a protein that has been purified from mammalian cells/tissues or synthesized as recombinant protein.

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With the sequencing of the human genome and the concomitant understanding of genotype–phenotype relationships, increasing attention has been paid to applying this knowledge to treating inherited diseases. Whereas strategies such as metabolic manipulation and protein augmentation have been remarkably successful in treating some genetic diseases (BOX 1), the real therapeutic breakthroughs for hereditary disorders will depend on the development of 'genetic medicines': therapies that are centred on transferring genetic material to correct or compensate for an abnormal phenotype associated with a particular genotype.

Of the approximately 25,000 genes that comprise the human genome, mutations in more than 1,800 have already been identified as causing hereditary disorders (Ensembl, OMIM). The focus of this review is the use of genetic medicines to treat monogenic hereditary disorders. Because so many single gene mutations are known, the logic is compelling that if sufficient correction or compensation can be achieved with genetic medicines, monogenic disorders could be prevented and/or treated. By contrast, current genetic medicines cannot correct the complex phenotype associated with the hundreds of genes that are typically affected in chromosomal disorders (such as trisomy 21) or the multiple genetic variations that underlie complex disorders. For these disorders, strategies are being developed to compensate for, or to modify, diseased organs. Examples include gene therapy to induce angiogenesis to bypass blocked coronary arteries, or stem cell therapy to regenerate cardiac myocytes to treat a failing myocardium.

Three broad categories of genetic medicines are being tested in the clinic: somatic stem cells (SSCs), gene transfer and RNA modification. In the future, the application of embryonic stem cells (ESCs) will be assessed. For each strategy, the fundamental approach is to modify the gene expression repertoire of a subset of somatic cells/organs of the affected individual. No current strategy targets the germ line. The different categories of genetic medicine are not mutually exclusive, and it is likely that genetic medicines of the future will include various combinations of these approaches.

Genetic medicines are simple in concept, but challenging to make a therapeutic reality. We first outline the general concepts that are applicable to genetic medicines. We then review the genetic medicine strategies being developed to treat monogenic disorders, including those that involve the use of SSCs (excluding combined SSC–gene-transfer strategies, which are discussed in the section on gene transfer), gene transfer, RNA modification, and ESCs. For each of these strategies we describe the current status of applying these therapies to treat hereditary human disorders and the biological challenges in making genetic medicine therapies a reality. Finally, we discuss the future of genetic medicines, including the regulatory, economic and social hurdles in developing genetic medicines. To provide a historical context, strategies for treating these disorders in the pre-genetic medicine era are summarized in the Supplementary information S1–S3 (tables).

Genetic medicines — general considerations

The concept underlying all genetic medicines is that transfer of genetic material (for example, coding for a

Box 1 | **Treating hereditary disorders in the pre-genetic medicine era**

Before the development of genetic medicines, strategies for treating hereditary disorders focused on metabolic manipulation and protein augmentation therapy. For some monogenic disorders, success with these approaches has been remarkable.

Metabolic manipulation

The basic concept of metabolic manipulation is to use dietary or small molecule therapy to compensate for a deranged biological process, or in some instances, to prevent the complications of therapies used to correct the abnormal phenotype (see Supplementary information S2 (table) for a summary and references). The simplest form of metabolic manipulation is diet modification (such as phenylalanine restriction to treat phenylketonuria). For some disorders, successful therapy depends on combining diet manipulation with drugs (for example, for familial hypercholesterolaemia this involves the combination of a low-cholesterol diet and statin inhibitors of hydroxymethylglutaryl co-enzyme A (HMG CoA) reductase. Another strategy involves stimulating the expression of a protein that will substitute for the abnormal protein — for example, hydroxyurea drug therapy is used to stimulate expression of fetal haemoglobin (HbF, $\alpha_2\gamma_2$) levels to compensate for the abnormal sickle cell haemoglobin (HbS, $\alpha_2\beta_2$), therefore reducing the sickle crises of sickle cell anaemia. Alternatively, if the mutant protein is functional it can be effective to upregulate the expression of the mutant protein; an example is 'impeded' androgen therapy for C1-inhibitor deficiency, which is an autosomal dominant disorder that causes hereditary angioedema. Metabolic therapies can also be used to treat the complications from the treatment of genetic disorders, such as treatment of thalassaemia with the iron-chelating agent desferrioxamine; this drug prevents the organ failure that would otherwise be caused by the iron overload from the frequent red blood cell transfusions that are required to treat the primary phenotype. High-throughput screening of chemical libraries is being used to identify small molecules that modify the conformation of misfolded mutant proteins, which enables mutant proteins to traffic and/or function normally, or otherwise trick chaperones and other organelle-specific quality-control systems to accept the misfolded protein.

Protein augmentation

The concept of protein augmentation therapy is simple — purify the missing protein and return it to the patient. This therapy is used in several hereditary disorders, including cystic fibrosis, coagulation disorders, α1-antitrypsin deficiency, immunoglobulin deficiencies, endocrine disorders and lysosomal storage diseases (see Supplementary information S3 (table)). Protein augmentation therapy is most applicable to treating hereditary disorders in which the deficient protein functions in the extracellular milieu. If the protein has to reach sites from which it was prevented from diffusing (such as the brain), systemic protein augmentation therapy is not effective[118]. When the phenotype involves an intracellular protein, protein augmentation therapy can be effective only if there is a mechanism to import the protein into a compartment of the cell relevant to the abnormal phenotype, such as protein augmentation therapy for the lysosomal storage disorders[118]. Other challenges for treating hereditary disorders with protein augmentation therapy include: maintaining venous access to administer the protein; infection; supply shortages of the therapeutic agent; cost; requirement of frequent, repeated administrations; and the potential allergic, inflammatory and immune responses to the infused proteins[118].

single gene, which is typical for gene therapy, or fragments of coding sequences, as in RNA modification therapy) or the entire genome (as in the case of SSC and ESC therapy) will result in a modified phenotype for therapeutic purposes. Conceptually, the simplest strategy is to correct or compensate for the abnormal gene expression caused by the altered genotype. Alternatively, the genetic medicine can be designed to regenerate a diseased organ, either by re-engineering tissues by expressing embryonic 'master' genes that induce organ development or, in the case of stem cell therapy, by using wild-type or genetically corrected stem cells to generate normal tissues. The successful application of genetic medicines depends on addressing several general challenges, including those posed by the delivery of the genetic information, host immune responses, the inheritance mode of the disorder, the organs that manifest the abnormal phenotype, and the various mutations that result in the disease (BOX 2).

Phenylketonuria
An autosomal recessive error of metabolism that is caused by lack of the enzyme that converts phenylalanine to tyrosine. It causes abnormally high phenylalanine levels and severe, progressive mental retardation if untreated, but can be prevented by neonatal screening and a low phenylalanine diet from an early age.

Somatic stem cell therapy

In a sense, organ transplantation for a monogenic hereditary disorder is the ultimate 'genetic medicine', in that it involves replacing, along with the relevant SSCs and differentiated cells, the organ that is malfunctioning secondary to the abnormal phenotype. Organ transplantation for hereditary disorders has included replacing the liver, kidney, lung and heart[1].

Stem cells are unspecialized cells that are defined by their capacity for self-renewal and the ability to differentiate into specialized cells along many lineages[2–4]. There are two broad categories of stem cell (FIG. 1). Embryonic stem cells, which are derived from the inner cell mass of embryos at the blastocyst stage, are pluripotent[3–6]. Somatic stem cells, which are derived from various fetal and post-natal organs, can, at a minimum, differentiate into the cell types found in the tissue in which they reside[7]. Typically, SSCs are named on the basis of the organ from which they are derived (such as haematopoietic stem cells)[8]. In this section we review the use of non-autologous SSCs to treat hereditary disorders; in the section on gene transfer, we discuss the use of genetically modified autologous stem cells.

Haematopoietic stem cell transplantation. Bone marrow stem cell transplantation has been used in the clinic for more than 40 years as a means to replenish the body with haematopoietic stem cells (HSCs) — cells that can differentiate into all myeloid and lymphoid blood lineages[9–11]. Although HSC transplantation carries a potential risk owing to the requirement for immunosuppression of the host, HSC transplantation of unmodified bone marrow from individuals that express the normal gene has been used to treat various inherited diseases, including the lysosomal storage disorders, immunodeficiencies, haemoglobinopathies and leukodystrophies. Details about these clinical trials are summarized in Supplementary information S1 (table).

Non-haematopoietic sources of SSCs. In addition to HSCs, SSCs have been identified in the brain, gut, heart, liver, pancreas, skeletal muscle and skin/hair[7,8,12,13]. Although there is convincing evidence that SSCs can differentiate into multiple lineages from the organ from which the SSC is derived, it is controversial as to whether SSCs can differentiate across lineages[7,8,12,14–16]. There are many reports of pluripotency of SSCs, but most do not prove that a single cell from a fetal or post-natal organ can differentiate into a cell type from other organs, nor that the differentiated cells have both morphological and functional characteristics of cells from other organs[8].

One relevant application has been to use SSCs that are recovered from the human fetal brain as a potential therapy for neuronal ceroid lipofuscinosis (S.B. Basu, personal communication). When neural stem cells

from a human fetus were transplanted to the CNS of mice that were deficient for palmitoyl-protein thioesterase (*PPT1* — the gene that, when mutated, causes the infantile form of the disease) backcrossed onto a NOD/SCID background, differentiated cells were seen engrafted throughout the brain and there was some reduction in the amount of pathogenic storage material in the CNS.

Gene transfer

Of all of the therapeutic options for treating hereditary disorders, gene transfer is the most obvious: if the disorder is caused by a mutation in a single gene, why not correct the genotype by transferring copies of the normal gene into the affected individual? Like many good ideas, execution in a fashion that is efficacious, persistent and safe has been challenging. Despite the many mouse (and larger animal) models of hereditary disorders that have been 'cured' with gene transfer, in practice, correcting human hereditary disorders has been difficult. Since the first attempt in humans to transfer a gene to correct the adenosine deaminase deficiency form of severe combined immunodeficiency in 1990, by Blaese and Anderson[17], there have been 95 approved gene-transfer trials worldwide that have been directed towards treating monogenic disorders (TABLE I).

***Strategies:* ex vivo *and* in vivo.** There are two basic strategies of gene transfer for an hereditary disorder, *ex vivo* gene transfer and *in vivo* gene transfer. The *ex vivo* approach is limited to disorders in which the relevant cell population can be removed from the affected individual, modified genetically, and then replaced[18–20]. *Ex vivo* strategies are best suited to applications in which the corrected cells can be easily obtained (for example, bone marrow), or even better when the corrected cells have a selective advantage when they are returned to the patient (for example, the correction of HSCs for severe combined immunodeficiency). Other *ex vivo* applications for hereditary disorders include the use of corrected cells as sources of a secreted protein (for example, transfer of the factor VIII (*F8*) gene to autologous fibroblasts to treat haemophilia A) or to treat a complication from other therapies (for example, transfer of a suicide gene to T lymphocytes to control

'Impeded' androgen therapy
A means to overcome a deficiency in the C1-inhibitor (C1-INH) — a protease inhibitor that is involved in the plasma proteolytic system. The administration of attenuated androgens increases C1-INH expression levels.

Chemical libraries
Collections of tens or hundreds of thousands of organic chemicals, which are commonly referred to as small molecules, that can be characterized for potential utility in specific conditions using high-throughput screening.

Non-autologous
Refers to transplant material that is derived from a genetically independent source. An example is bone marrow transplantation in which the donor and recipient are distinct individuals.

Neuronal ceroid lipofuscinosis
A group of hereditary, fatal neurodegenerative disorders in which the phenotype is limited to the destruction of the retinal epithelium and the CNS.

NOD/SCID
A mouse strain that is derived from the transfer of a severe combined immunodeficiency (SCID) mutation onto a non-obese diabetic (NOD) strain background. This strain is an excellent model for testing cell-based therapies with human cells.

Severe combined immunodeficiency
A family of genetic disorders that affect T-cell differentiation and B-cell immunity, resulting in the absence of a functional immune system.

Ex vivo gene transfer
A gene-transfer strategy in which the target cells are removed from the individual to be treated, genetically modified in the laboratory, and then administered to the patient.

In vivo gene transfer
A gene-transfer strategy in which the vector carrying the expression cassette is administered directly to the patient.

Box 2 | **General considerations for the application of genetic medicines**

Global challenges

The main biological barriers for all genetic medicines are the delivery and maintenance of new genetic information. For gene-transfer therapy, this requires circumventing immune defences that are raised against the vectors that carry the new gene, transferring the gene to sufficient numbers of cells to modify the phenotype, and controlling the expression of the gene[18,23]. For RNA-modification therapy, the main challenge is delivery, and to a lesser extent specificity[89,92]. For adult and embryonic stem cell therapy, the significant issues relate to immune surveillance against 'foreign' cells, providing a 'niche' and selective advantage for the transplanted cells, and controlling and coordinating the proliferation, differentiation and anatomic location of the stem cells and their progeny[108,119–121]. To overcome these challenges, it is crucial to understand the target, including the molecular basis of the disorder, its mode of inheritance, the range of mutations and genotype–phenotype relationships that result in the disease phenotype, how the phenotype is modulated by alternative genes, and how, where and when the disease manifests.

Inheritance mode and functional category of a gene

For a genetic medicine to compensate for an autosomal recessive disorder, typically only 5–10% of normal gene expression is required to correct the phenotype[18,122]. The same is true for many X-chromosome-linked protein deficiency disorders[122]. For autosomal dominant disorders, the required levels of suppression are variable[96,123].

It is also important to consider that different categories of function of the affected gene tend to manifest at different ages. For example, mutations in transcription factors, such as the glioblastoma 3 (*GLI3*) mutations that are associated with cephalopolysyndactyly, usually manifest *in utero*[124]. By contrast, the phenotype of some disorders, such as the emphysema caused by α1-antitrypsin deficiency, is not expressed until the fourth or fifth decade of life[125].

Organs manifesting the abnormal phenotype

In designing a genetic medicine it is crucial to plan how to target the cells or organ that will correct the abnormal phenotype. For example, effective genetic therapy for late infantile neuronal ceroid lipofuscinosis — which is associated with mutations in ceroid lipofuscinosis 2 (*CLN2*; also known as tripeptidyl peptidase 1) genes[126] — must be directed to the brain and/or to the eye; organs that have different routes of administration, neither of which are susceptible to systemic administration of the genetic medicine[48].

The issue of which organs manifest the phenotype is more complex when considering stem cell therapy for an hereditary disorder, as it is necessary to provide a niche and/or selective advantage for the transferred stem cells and their progeny[119,120]. The challenge is even more daunting when considering using stem cells to enhance the function of, or to replace lost, neurons and other brain cells in a neurodegenerative disorder, where there are the substantive issues of directing the therapeutic cells to different locations, functions and interactions[127,128].

Genetic heterogeneity

For most monogenic disorders, many mutations result in a similar disease phenotype. If the genetic medicine is designed to compensate for a monogenic disorder by stem cell therapy or gene transfer, then the specific mutation is not as relevant. However, for an RNA-based strategy, the genetic medicine is targeted to specific sequences; the site and type of the mutation is therefore important, and it is challenging to design the RNA-modification therapy to fit all mutations for some disorders.

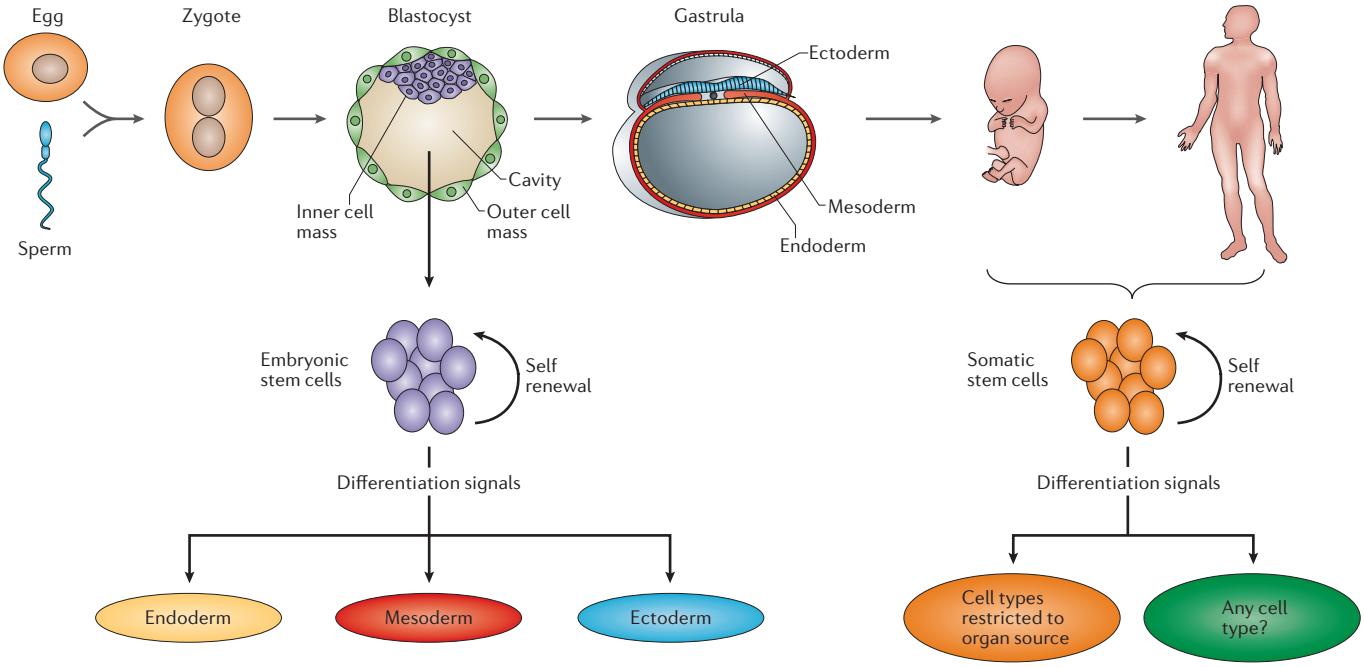


Figure 1 | **Embryonic and somatic stem cells as a source of genetic medicines.** The fusion of sperm and egg gametes during human fertilization establishes a diploid zygote and initiates a series of cell divisions that result in a multicellular embryo. The blastocyst stage is characterized by the presence of a blastocyst cavity, outer cell mass and inner cell mass. Embryonic stem cells are derived from the inner cell mass of the blastocyst. Embryonic stem cells in culture are capable of self-renewal without differentiation and are able to differentiate into all cell types of the endoderm, mesoderm and ectoderm lineages using appropriate signals. *In utero*, the blastocyst implants and all three embryonic germ layers are formed during gastrulation. Somatic stem cells are present in many fetal and post-natal tissues. Somatic stem cells are also capable of self-renewal and, with appropriate signals, differentiate into various cell types from the organ from which they are derived. The extent to which they are capable of differentiating into cell types from alternative lineages is controversial.

graft versus host adverse effects from non-autologous bone marrow transplants).

The *in vivo* approach is the most direct strategy for gene transfer and can theoretically be used to treat many hereditary disorders. Human *in vivo* studies for monogenic disorders have administered the vector containing the therapeutic DNA either directly to the organ of interest or into blood vessels that feed the organ (TABLE I). Although several studies in animal models have demonstrated that it is feasible to at least partially target viral gene-transfer vectors to different organs, targeting strategies have not been used clinically for hereditary disorders.

Effective gene-transfer strategies must involve the transfer of the gene to a cell population, where it should function *in vivo* to correct the abnormal phenotype. For disorders where the phenotype is intracellular, the gene has to be transferred to sufficient numbers of affected cells to correct the clinical phenotype. By contrast, if the phenotype results from a secreted protein, only protein levels that are sufficient to correct the phenotype need to be produced, with the caveat that the protein must be appropriately post-translationally modified and, if the levels need to be regulated, the gene-transfer strategy needs to take this into account (for example, by using a promoter that responds to the relevant signals). If these conditions are met, it does not matter to which cells or organ the gene is transferred. However, if the phenotype to be corrected is in the brain (or other organs that have barriers to the passage of proteins, such as the eye), the gene coding for the secreted protein needs to be directly delivered to the affected organ.

Expression cassettes. Typically, the gene is administered within an expression cassette that consists of a cDNA (or, if there is room, genomic DNA) flanked on the 5′ side by an active promoter and on the 3′ side by a transcription stop and polyadenylation site (BOX 3a). Although extensive studies that compare the efficiency of expressing cDNAs with genomic DNA forms of genes are lacking, there are examples where the genomic forms (or 'pseudo-genomic' forms that have artificial or truncated introns separating two or more exons) are more effective[21,22]. As gene transfer becomes more sophisticated, there will probably be a shift to using genomic forms of genes, or at least genes that have exons and artificial introns. In theory, this would preserve the ability of the spliceosome machinery of the cell to produce different mRNA transcripts of the gene that are based on intron sequences flanking each exon, thereby preserving the subtle complexity of functions of multiple splice forms of each gene.

Suicide gene
A gene that encodes a protein that can convert a non-toxic prodrug into a cytotoxic compound.

Cephalopolysyndactyly
A condition that is characterized by abnormal skull morphology and digital malformations.

Table 1 | **Gene-transfer trials for monogenic hereditary disorders***

Vector	Hereditary disorder (references[§])	Transgene	Target cells	Evidence for phenotype correction[‡]		
				In vivo GE[‖] (mRNA)	*In vivo* biochem., physiological or imaging methods	CI[¶]
Plasmid ±; liposome (*ex vivo*)	Haemophilia A (136)	Factor VIII	Fibroblasts	NA	±	±
Plasmid ±; liposome (*in vivo*)	Cystic fibrosis (137–144)	*CFTR*	Nasal and airway epithelium	±	±	No
	α1-antitrypsin deficiency (145)	α1-antitrypsin	Nasal and respiratory tract epithelium	±	No	No
	Canavan disease (146)	Aspartoacylase	CNS	?	?	?
	Muscular dystrophy (147)	Dystrophin	Muscle	±	No	No
Retrovirus (*ex vivo*)	Adenosine deaminase deficiency (17,75,77,78,148,149)	Adenosine deaminase	T cells, CD34 cells, cord blood, bone marrow	YES	YES	YES
	Familial hypercholesterolaemia (74,150)	Low-density lipoprotein receptor	Hepatocytes	Yes	Yes	No
	Gaucher disease (151)	Glucocerebrosidase	Blood CD34+, bone marrow CD34+	±	No	No
	Fanconi anaemia (152,153)	Complementation group C or A	Blood CD34 cells	±	No	No
	Chronic granulomatous disease (154)	p47 phagocyte NADPH oxidase	Blood CD34+	±	No	No
	X-linked severe combined immunodeficiency (80–82,155)	Common γ-chain of multiple cytokine receptor	Cord blood and bone marrow CD34+	YES	YES	YES
	Leukocyte adherence deficiency (156,157)	*CD18*	Blood CD34+	±	No	No
	Severe combined immunodeficiency secondary to JAK3 deficiency (158)	*JAK3*	Bone marrow CD34+	?	?	?
	Haemophilia B (159)	Factor IX	Skin fibroblasts	NA	±	No
Retrovirus (*in vivo*)	Haemophilia A (160)	Factor VIII	Intravenous[#]	±	±	No
Adenovirus serotypes 2 and 5** (*in vivo*)	Cystic fibrosis (32,37, 42–45,161,162)	*CFTR*	Nasal and airway epithelium	Yes	Yes	No
	Ornithine transcarbamylase deficiency (49,163)	Ornithine transcarbamylase	Liver	±	±	No
	Haemophilia A (50)	Factor VIII	Liver	±	±	No
Adeno-associated virus serotype 2 (*in vivo*)	Cystic fibrosis (67,164–166)	*CFTR*	Nasal, airway and maxillary sinus epithelium	No	No	No
	Haemophilia B (66,167; see the ASGT Stakeholder's Report in the Further information)	Factor IX	Muscle, liver	Yes	Yes	No
	Muscular dystrophy (66)	α,β,γ,Δ-sarcoglycan	Muscle	?	?	?
	Canavan disease (146,168)	Aspartoacylase	CNS	?	?	?
	Late infantile ceroid lipofuscinosis (169)	*CLN2* (tripeptidyl peptidase 1)	CNS	?	?	?

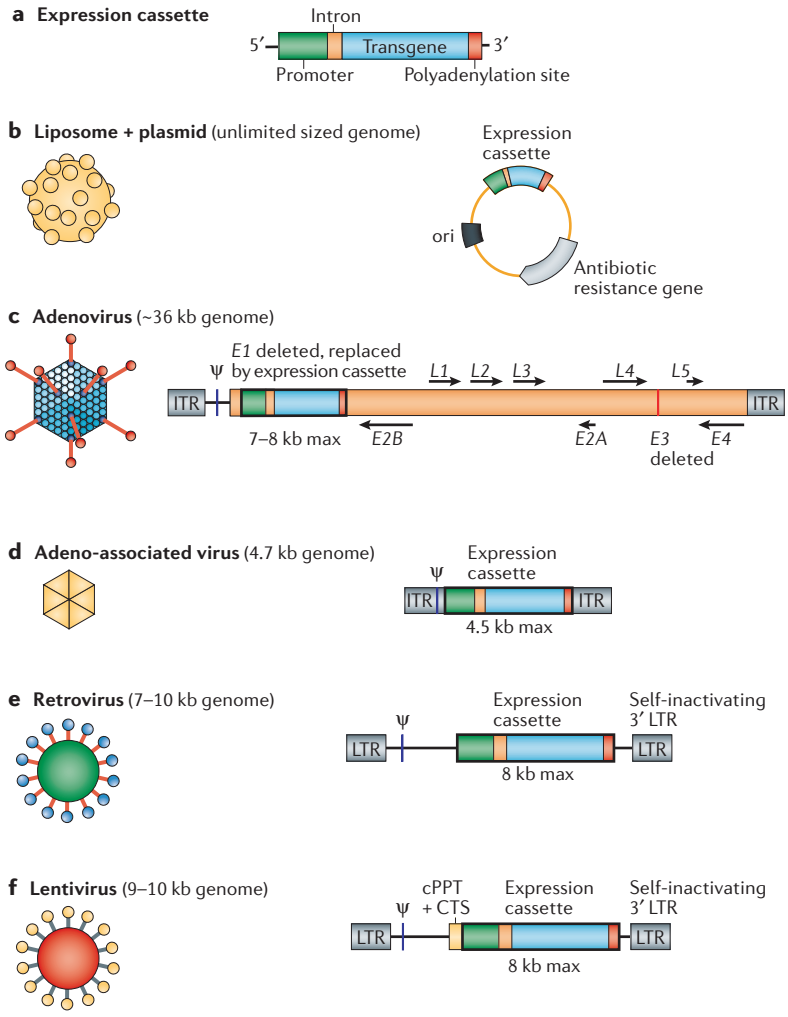
*The trials listed are compiled from lists of approved gene-transfer protocols and published details of the trials; the lists of approved trials worldwide can be found on the Gene Therapy Clinical Trials Worldwide web site (www.wiley.co.uk/genetherapy/clinical) and on the Clinical Trials in Human Gene Transfer web site (www4.od.nih.gov/oba/rac/clinicaltrial.htm). [‡]A summary of the clinical studies so far; where multiple studies have been carried out, the summary is that of the best results. [§]Outcomes of some of the trials are available in reviews[18,19,26,66,77]. [‖]GE, gene expression. [¶]CI, clinical improvement. [#]Probably liver, but not known. **Most studies have been with adenovirus serotype 5. Biochem., biochemical; *CFTR*, cystic fibrosis transmembrane conductance regulator; *CLN2*, ceroid lipofuscinosis 2; *JAK3*, janus kinase 3; NA, not assessed; NADPH, nicotinamide adenine dinucleotide phosphate; Yes, clear evidence of phenotype correction, although not persistent; YES, persistent phenotype correction sufficient to permanently reverse the abnormal phenotype; ±, some evidence of phenotype correction, but not sufficient to be curative; ?, not yet reported.

Gene-transfer vectors: general issues. The barriers to successful gene transfer are severe: not only does the expression cassette have to reach the relevant cells within the organ targeted by gene transfer, but once it reaches the cell, the gene has to successfully breach the plasma membrane, traffic through the cytoplasm, and enter the nucleus where it can use the endogenous transcriptional machinery to express the therapeutic gene[18,19,23–25]. To accomplish this, non-viral and viral gene-transfer vectors are used (BOX 3).

To avoid the problems associated with insertional mutagenesis, some gene-transfer strategies deliver the expression cassette to the nucleus at an extrachromosomal location[26,27]. This strategy is effective as long as the target cell is not proliferating, in which case the transgene gets progressively diluted, and expression wanes over

Box 3 | **Gene-transfer vectors that are used to treat hereditary disorders**

There are five classes of gene-transfer vectors and a prototypic expression cassette that are used to treat hereditary disorders (see figure)[18,26,31,129]. Panel **a** shows a typical expression cassette. The therapeutic transgene is flanked at the 5′ end by the promoter and at the 3′ end by a polyadenylation site. Plasmid DNA has an unlimited size capacity and is delivered either naked or formulated with liposomes (**b**). The expression cassette contains a bacterial origin of DNA replication and an antibiotic resistance gene for production in bacteria. Panel **c** shows a first generation adenovirus vector that is based on serotype 5. The 36-kb dsDNA genome of wild-type adenoviruses contains left- and right-inverted terminal repeats (ITR) that facilitate viral DNA replication, a packaging signal (Ψ), early (*E1–E4*) genes and late (*L1–L5*) genes. A typical adenovirus vector has the essential *E1* region deleted (to prevent replication) and lacks most *E3* genes (to increase the cargo space). Expression cassettes of 7–8 kb are inserted into the *E1* region. The 4.7-kb ssDNA genome of wild-type adeno-associated viruses (**d**) contains 5′ and 3′ ITRs and 2 genes, *rep* and *cap*. The 5′ ITR contains Ψ. Deletion of the viral *rep* and *cap* genes allows expression cassettes of up to 4.5 kb to be accommodated. The 7–10 kb retrovirus ssRNA genome (**e**) contains left and right long terminal repeats (LTRs) that flank *rev*, *gag*, *pol* and various regulatory genes that are required for viral function. The RNA genome of replication-defective retroviral vectors contain an expression cassette of up to 8 kb that replaces all viral protein-coding sequences. LTRs flank the expression cassette and allow transcription initiation by host cell factors. Vectors are rendered self-inactivating by deletion of the promoter and enhancer regions in the 3′ LTR to prevent LTR-driven transcription. Packaging of genomic RNA is controlled in *cis* by Ψ. The vectors are produced in packaging cells that provide the missing components in *trans*. The 9–10 kb lentivirus ssRNA genome (**f**), which comprises components of HIV1, is similar to that of retrovirus vectors. All viral protein-coding sequences are deleted and replaced with an expression cassette that is up to 8 kb in size, as packaging efficiency and titre production decline significantly beyond this limit. The central polypurine tract (cPPT) and central termination sequence (CTS) are *cis*-acting sequences, which are unique to lentiviruses, that improve nuclear import of proviral DNA, with consequent increases in transduction compared with retrovirus vectors. The cPPT and CTS coordinate the formation of a central DNA flap — this is a short triplex DNA element not found in retroviruses, which might have a role in nuclear entry. As for retroviral vectors, a *rev* responsive element can be incorporated into the vector to facilitate nuclear export of unspliced RNA and packaging of genomic RNA is controlled in *cis* by Ψ.



First generation adenovirus vector
A gene-transfer vector that is based on adenovirus serotype 5 and is characterized by the deletion of the *E1* gene, to prevent viral replication, and the *E3* gene, to increase cargo space.

time. Neither the non-viral vectors nor the adenovirus vectors mediate insertion of the transgene into the target cell genome, and both mediate only transient expression. Adeno-associated virus (AAV) vectors, retrovirus vectors and lentivirus vectors are associated with persistent expression; AAV vectors accomplish this by functioning in an extrachromosomal fashion (although there might be small amounts of genome integration), whereas retrovirus and lentivirus vectors mediate permanent insertion into the genome[18,26,27].

Non-viral vectors. The non-viral vectors consist of typical dsDNA plasmids, which are usually combined with cationic liposomes to help break the barrier of the plasma membrane[28] (BOX 3b). Although there have been many attempts to correct experimental animal models of hereditary disorders with plasmid gene transfer alone or with plasmids that are combined with liposomes (or other means to enhance the efficiency of transferring the plasmid to the nucleus), it is challenging to correct an hereditary disorder on a permanent basis with a plasmid-based gene-transfer system. Similarly, several human trials in hereditary disorders (for example, haemophilia A, cystic fibrosis, α1-antitrypsin deficiency and Canavan disease) that have been carried out using non-viral gene-transfer strategies have failed, with little evidence of expression of the plasmid-directed mRNA, let alone evidence of correcting the abnormal phenotype (TABLE I). In addition to involving transient vector systems, this failure of non-viral vectors rests on inefficiency. The non-viral-based vectors that have been used in the human studies conducted so far have no means to traffic the plasmid to the nucleus[29]. Despite the challenges in using non-viral vectors, some investigators are convinced that a non-viral-based gene-transfer system will prove successful for treating the respiratory manifestations of cystic fibrosis, and have developed programmes to move this therapy to the clinic[30].

Viral vectors. Viruses provide an efficient vehicle for transferring genes to target cells, *ex vivo* and *in vivo*. The viral vectors that have attracted the most attention of gene therapists are the DNA-based adenovirus and AAV vectors, and the RNA-based retrovirus and lentivirus vectors. Although lentiviruses belong to the family Retroviridae, they are characterized by their ability to infect non-dividing cells and are generally discussed separately. The following sections discuss the ways in which viral-based vectors have been used to treat monogenic disorders. For readers who are interested in details of the design, production, function and trafficking of gene-transfer vectors, several reviews are available[18,19,24–27,31].

Viral strategies: adenovirus vectors. Adenovirus vectors were the first gene-transfer vectors to be used *in vivo* to treat an hereditary disorder[32] (BOX 3c). All human gene-transfer studies in hereditary disorders have been carried out with human group C, serotype 5, and to a lesser extent, serotype 2 (TABLE I). The dsDNA adenovirus binds to target cells through epitopes in its fibre and penton bases[33]. By altering these sequences, the targeting of the vector can be modified and the efficiency of gene transfer improved[33–36].

In mouse experimental models and in humans, correction with adenovirus vectors has almost always been transient[33,37,38]; although there has been some more persistent expression with adenovirus vectors in which most of the adenovirus genes are deleted (for example, 'gutless vectors')[39]. Although the adenovirus vector is the 'gold standard' of gene-transfer vectors as it can achieve the highest levels of gene expression after *in vivo* administration, it is highly immunogenic, and innate and acquired host responses to the vector limit expression from the transgene that is carried by the adenovirus vector to only a few weeks at the most[23,40,41].

In humans, we and others have used adenovirus vectors to correct the abnormal phenotype in cystic fibrosis airway epithelium at the mRNA level, but the correction was short-lived, and could not be maintained by repeated administration[37,42–46]. Despite various strategies to delete more genes from adenovirus vectors[37], and the use of 'sero-switch' strategies to circumvent immunity against the viral capsid[47], adenoviruses are unlikely to be useful for treating monogenic hereditary disorders (unless successful gutless vectors are created[39]). An exception to this principle is the lysosomal storage disorders, for which it might be possible to 'set back the clock' by using an adenovirus gene-transfer vector to transiently correct the relevant cell populations and, in doing so, clear the lysosomes of the storage material that has taken months to years to accumulate[48].

The host responses to adenovirus vectors can pose a risk, as demonstrated in a vector-related death following intravascular administration of high doses (3.8×10^{13}) of adenovirus particles to a patient with ornithine transcarbamylase deficiency[49]. Likewise, a trial for haemophilia A using intravascular delivery of an adenovirus vector was stopped because of transient thrombocytopoenia and transaminase elevation[50]. It is now recognized that, if adenovirus vectors are administered directly to target organs (not directly into the vasculature), doses of up to 10^{12} particles can be used[51–53].

Viral strategies: adeno-associated virus vectors. The AAV virus is a small ssDNA virus with a simple protein coat[18,26,27,54] (BOX 3d). Like adenoviruses, many AAV serotypes have been assessed as gene-transfer vectors: 6 from humans and more than 20 from non-human primates[31,55]. The advantage of AAV vectors is that their genome persists in organs in which cells are not turning over rapidly, including the liver, brain, heart, pleura and retina[26,27,31,56,57]. Typically, a serotype 2 genome is used, and the vector is 'pseudotyped' with the capsid of the AAV serotype that provides the best properties for gene transfer for the specific application[55,56]. For example, the AAV8 capsid has high tropism for the liver, whereas AAV5 is efficient in the lung epithelium[58,59]. The AAV genome used in gene-transfer vectors contains no expressed genes, and therefore induction of host immunity against the vector is less problematic than for adenovirus vectors; except with induction of antibodies that are directed against the AAV capsid[40,41]. One

α1-Antitrypsin deficiency
An autosomal recessive disorder that is associated with emphysema and liver disease. It results from the deficiency of a serine protease inhibitor that is produced in the liver and secreted into the plasma, where it inhibits the activity of trypsin and elastase.

(Viral vector) serotypes
Viral vectors that belong to the same viral family, but that have sufficiently distinct capsids that they can be distinguished by differences in the antibodies that they evoke *in vivo*, for example, adenovirus serotypes 2 and 5 are group C Adenoviridae.

Sero-switch
A gene-transfer strategy that involves the repeated administration of alternating adenovirus vectors that are derived from different serotype subgroups, in order to circumvent anti-adenovirus humoral immunity.

Thrombocytopoenia
A persistent decrease in the number of blood platelets. It is often associated with haemorrhagic conditions.

limitation of using AAV vectors is their low packaging capacity (4.5 kb)[26,31,56,57].

AAV-vector-mediated gene transfer of the wild-type cDNA has corrected the abnormal phenotype of several experimental animal models of hereditary disorders on a persistent basis, including haemophilia A and haemophilia B, mucopolysaccharidosis I and mucopolysaccharidosis IIIb, Niemann–Pick A, and glycogen storage disease type II (REFS 60–64). One of the most striking examples has been the restoration of vision in a dog model of Leber congenital amaurosis, which is an autosomal recessive disorder that is associated with blindness, using AAV2-mediated delivery of the cDNA of the normal retinal pigment epithelium 65 to the retina[65]. AAV vectors have also been evaluated in humans with hereditary disorders, including cystic fibrosis, muscular dystrophy, α1-antitrypsin deficiency, haemophilia with factor IX (*F9*) deficiency (haemophilia B), and two paediatric neurodegeneration disorders — aspartoacylase deficiency (Canavan disease) and late infantile ceroid lipofuscinosis (a form of Batten disease)[66].

The first clinical use of AAV vectors to treat an hereditary disorder used an AAV2 vector to transfer the cystic fibrosis transmembrane-conductance regulator (*CFTR*) cDNA to the respiratory epithelium of individuals with cystic fibrosis[67]. This, as well as other attempts with AAV2-based vectors to transfer the *CFTR* cDNA to the nasal, sinus and airway epithelium of individuals with cystic fibrosis failed to convincingly demonstrate significant amounts of wild-type *CFTR* mRNA transfer in the target cells[66,67]. One problem was that the serotype 2 capsid does not effectively transfer genes to the human airway epithelium[59]. Furthermore, the 4.5-kb *CFTR* cDNA is the maximum size that can fit into AAV vectors, which necessitates the use of short, weak promoters[66]. Attempts have also been made to treat muscular dystrophy with AAV-mediated gene transfer. Although most cases of muscular dystrophy are caused by mutations in the dystrophin (*DMD*) gene, it is too large (11 kb) to fit into an AAV vector. Initial clinical gene-transfer studies have been carried out in the rare cases of muscular dystrophy that are caused by mutations in smaller genes[66].

Clinical studies attempting to correct serum protein deficiencies using AAV vectors have taught another lesson — humans are not just big mice, and to successfully use gene transfer to produce enough protein to correct a serum deficiency phenotype is a big hurdle. Whereas the liver is the obvious site for delivery of genes to treat hereditary disorders when the phenotype is a serum protein deficiency, using AAV vectors to deliver genes to the liver in sufficient quantities to correct the phenotype is a challenge. For example, hepatic artery administration of an AAV serotype 2 vector that codes for F9 to treat haemophilia B was associated with some initial success in mediating small, transient elevations in F9 (REF. 68; see the ASGT Stakeholder's Report in the Further information). However, as the doses were increased, host defences were incited, causing transient hepatitis and a decline in *F9* expression levels. Choosing a serotype that more efficiently transfers genes to the liver might solve this issue.

One solution to the small cargo space that is available in AAV vectors is to deliver portions of the expression cassette in separate vectors. For example, two vectors can be used to deliver partial DNA sequences that are designed to concatamerize in the nucleus[69]. Alternatively, gene segments that are delivered in two vectors can be individually expressed as separate mRNA molecules, which then hybridize in a unique (correct) orientation[70]. These and other strategies are discussed in the later section on RNA modification.

Viral strategies: retrovirus vectors. The Moloney murine leukaemia retrovirus (MMLV) was the first gene-transfer vector to gain widespread attention, and the first to be used in an *ex vivo* strategy to treat an hereditary disorder[17–19]. MMLV vectors have undergone several modifications and are now referred to collectively as the 'retrovirus' gene-transfer vectors[71,72] (BOX 3e). In recent years, considerable effort has been placed on pseudotyping retrovirus vectors with various coats, based on observations that different coats enhance binding/entry to target cells[71,72].

Because retrovirus vectors are sensitive to complement activation, and are difficult to concentrate in high titres, the principal use of retrovirus vectors to treat monogenic hereditary disorders has been in *ex vivo* strategies[17–19,71–73] (TABLE I). One early adoption of this strategy was to use a retrovirus vector to transfer the normal low-density lipoprotein receptor cDNA to cultured hepatocytes from an individual with familial hypercholesterolaemia and return the corrected cells to the liver of the individual[74]. There were no serious safety issues with this trial, and some phenotypic correction, although the numbers of corrected hepatocytes returned to the patients were insufficient to permanently correct the phenotype — probably, in part, because the corrected cells had no selective advantage over the endogenous hepatocytes that express the abnormal gene.

Most clinical studies with retrovirus vectors to treat hereditary disorders have used the vector to transfer the wild-type gene to T lymphocytes or to autologous HSCs to correct haematological diseases. The selective advantage of the transplanted, genetically modified cells might not be sufficient; indeed, the issue of providing a niche for the transplanted cells has been one reason for the failure of most *ex vivo* retrovirus gene-transfer studies[75,76]. ADA-SCID is a good target for gene therapy because the disease manifests in a well-defined localized target tissue (HSCs or mature T cells), individuals with as little as 5% of normal ADA activity are phenotypically normal, and genetically corrected cells should have a selective advantage[17,77]. The first gene-therapy clinical trial for an hereditary disorder was a treatment for ADA-SCID in which a retrovirus vector was used to transduce mature T cells *ex vivo*[17]. Subsequent clinical trials have targeted various sources of HSCs and have used improved retrovirus vectors[77]. Most children in the trials have remained on ADA-polyethylene glycol protein augmentation therapy, so the effects of gene therapy *per se* have been difficult to assess. However, sustained engraftment of transduced cells has been documented

Complement
Groups of plasma enzymes and regulatory proteins that function in innate immunity and that are activated in a cascading fashion to promote cell lysis.

Niche
A subset of tissue cells and extracellular substrates that can house one or more stem cells and control their self-renewal and progeny production *in vivo*.

ADA-SCID
An autosomal recessive disorder that presents in infants. The immunodeficiency results from the sensitivity of lymphocytes to the accumulation of adenosine degradation products.

10 years post-infusion and no severe adverse events have been reported[78]. A more recent trial involving two children for whom ADA protein augmentation therapy was not available used non-myeloablative conditioning immunosuppression to create an initial advantage for infused HSCs that had been corrected *ex vivo* using a retroviral vector[75]. There was sustained engraftment of the transduced HSCs, increased lymphocyte counts, improved immunity and lowered levels of toxic metabolites.

The great advantage of using retrovirus vectors for treatment of hereditary disorders is that they permanently integrate into the genome of the target cell[71,79]. However, this feature also carries the risk of insertional mutagenesis, and consequent development of a neoplasm[80]. Notably, these two features of retrovirus vectors — persistence in the genome and insertional mutagenesis — have been shown in an *ex vivo* strategy that used MMLV-based vectors to correct CD34+ HSCs with the γc-cytokine receptor cDNA to treat X-linked SCID[77,81,82]. The treatment resulted in sustained restoration of the immune system and the treated children no longer had to live in protected environments. However, severe adverse events in three of the patients have now been observed, with an uncontrolled clonal T lymphoproliferative syndrome, similar to acute lymphoblastic leukaemia. One of these children has now died[80]. In two of these three cases, there was retrovirus vector integration in proximity to the LIM domain only 2 (*LMO2*) promoter[82]. The third case with probable insertional mutagenesis is now under investigation[80]. These findings highlight the promise of genetic therapies using gene-transfer vectors that integrate, but also the critical need for strategies to reduce the risk of adverse events from insertional mutagenesis.

The lentivirus vectors are retrovirus vectors that are most commonly based on components from HIV1 (REFS 26,27,31,71,83) (BOX 3f). Although lentiviruses belong to the family Retroviridae, they have the advantage over MMLV-based vectors that they can transfer genes to non-dividing cells[31,83]. Despite the difficulties in producing high-titre stocks and the safety concerns that stem from the fact that the vectors are derived from HIV1 (REFS 26,83,84), production issues are slowly being solved[84]. The HIV1 derivation issue has been solved by deleting *rev*, *tat* and accessory gene sequences from the packaging construct[26,83]. To produce lentivirus vectors, the packaging construct is co-transfected with multiple separate plasmids that provide these functions, and the transfer vector is engineered to be self-inactivating by modifications to the 3′ long terminal repeat (LTR)[83,84]. Further enhancements to improve the efficiency of lentiviruses included the use of different coat proteins (for example, vesicular stomatitis virus G glycoprotein) as pseudotyping agents, which broadens target-cell tropism[26,83]. Like retroviruses, insertional mutagenesis is a safety concern for clinical applications of lentiviruses[80].

Owing to their ability to transduce non-dividing cells, lentivirus vectors can be used in strategies to deliver transgenes *in vivo* to tissues that would not be effectively transduced by retrovirus vectors, such as the CNS and liver[71,83]. Lentivirus vectors have been used to deliver transgenes to the CNS to correct mouse models of inherited disorders such as metachromatic leukodystrophy[85] and mucopolysaccharidosis type VII (REF. 83). Lentivirus vectors have also been used effectively in *ex vivo* applications, including transduction of HSCs with the β-globin gene to improve the phenotype of thalassaemia mouse models[86]. Although no clinical trials have used lentiviruses to transfer genes to correct an hereditary disorder, lentivirus vectors have been used in an *ex vivo* strategy in humans to treat HIV1 infection[87].

The future of gene transfer. The main thrust over the next several years will be to further develop AAV vectors for *in vivo* studies, retrovirus vectors for autologous HSC-related *ex vivo* studies, and probably lentivirus vectors for *ex vivo*, and possibly *in vivo*, applications. Together, the human trials have shown that it is feasible to transfer genes to humans, to achieve persistent expression, and to induce phenotypic modifications, at least at the gene-expression level. The biggest remaining challenges are how to achieve expression that is sufficient to correct the clinical phenotype, without inducing host defences that compromise safety and, for the integrating vectors, how to minimize the risk of insertional mutagenesis, particularly if the corrected cells have a subsequent selective advantage and are continuing to proliferate. As the vectors are further developed, particularly with identification of serotypes and modifications of coat proteins that enhance gene transfer, the doses that are required to gain adequate expression will be reduced, with consequent enhancement of safety and efficacy.

RNA-modification therapy

As the name implies, RNA-modification therapy targets mRNA, either to suppress mRNA levels, or by correcting or adding function to the mRNA. There are four basic approaches to modifying mRNA to treat monogenic disorders: antisense oligonucleotides, RNAi, *trans*-splicing and ribozymes (BOX 4). All have broad possible applications for treating genetic disorders, but also have major challenges of delivery, efficiency and specificity.

Antisense oligonucleotides. The most common antisense oligonucleotide (ASO) strategy uses ssDNA sequences, which are typically 18–30 bases in length, that target the degradation of mRNA with sequence complementarity[88] (BOX 4a). The outcome of this process is a knockdown of gene-expression levels of the target.

In theory, ASO strategies could treat genetic diseases in which decreasing the levels of a mutant protein would favourably alter the phenotype; for example, in the autosomal dominant disorders. One obstacle is the stability of ASOs in the cell[88], which could be improved by modifying the ASO backbone. However, whereas newer generation ASO agents show increased stability, other crucial properties, including cellular uptake efficiencies, target specificity and binding affinities can be compromised[89]. Furthermore, the mechanisms of cellular uptake of ASOs are not clearly understood, so all chemical modifications require trial-and-error

X-linked SCID
A fatal immunodeficiency disorder that results from mutations in the γc-cytokine receptor. These mutations cause an early block in T and NK lymphocyte differentiation.

Thalassaemia
A group of related genetic blood disorders that result from mutations in the genes encoding either the α or β-proteins of haemoglobin, which results in anaemia of varying severity.

Dicer
A highly conserved cytoplasmic enzyme that cleaves dsRNA into small interfering RNAs.

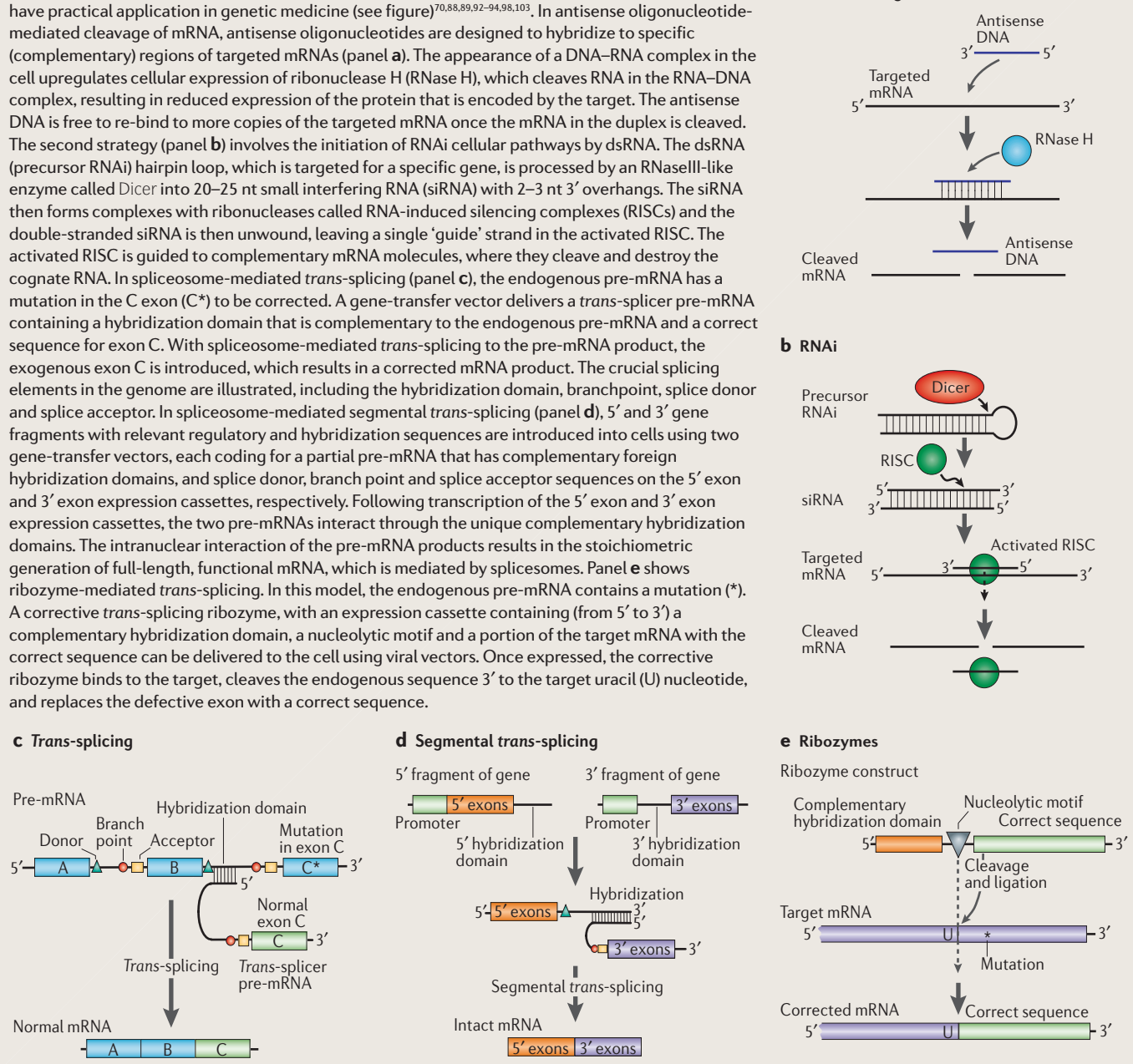
studies[88,89]. Finally, ASO therapies can be associated with unintended effects, such as the interactions of ASOs with DNA or cellular proteins, downregulation of expression levels of similar non-target mRNAs and immune activation[90].

There have been no clinical trials for genetic diseases using ASOs. One interesting pre-clinical strategy is to treat Duchenne muscular dystrophy (DMD) by affecting pre-mRNA processing[91]. ASOs can induce exon skipping patterns in the DMD pre-mRNA that restores the reading frame, resulting in a truncated, but still semi-functional, dystrophin protein.

RNAi. RNAi is a natural process in which small (typically 22 bp) dsRNAs with 2–3 nucleotide 3′ overhangs are processed intracellularly, forming an RNA-silencing complex that mediates degradation, or translation block, of targeted mRNA[92–94] (BOX 4b). The main challenges for realizing RNAi as a genetic medicine include improving efficiency, specificity and, most importantly, delivery. Assuming the RNAi can reach the cytoplasm in sufficient amounts, the efficiency of mRNA silencing depends on the abundance of the mRNA target and on the kinetics of hybridization of the guide strand to the mRNA target. This is part science (by choosing appropriate target

Box 4 | **RNA-modification strategies for genetic medicine**

There are five strategies for modifying gene expression at the pre-mRNA or mRNA levels that might have practical application in genetic medicine (see figure)[70,88,89,92–94,98,103]. In antisense oligonucleotide-mediated cleavage of mRNA, antisense oligonucleotides are designed to hybridize to specific (complementary) regions of targeted mRNAs (panel **a**). The appearance of a DNA–RNA complex in the cell upregulates cellular expression of ribonuclease H (RNase H), which cleaves RNA in the RNA–DNA complex, resulting in reduced expression of the protein that is encoded by the target. The antisense DNA is free to re-bind to more copies of the targeted mRNA once the mRNA in the duplex is cleaved. The second strategy (panel **b**) involves the initiation of RNAi cellular pathways by dsRNA. The dsRNA (precursor RNAi) hairpin loop, which is targeted for a specific gene, is processed by an RNaseIII-like enzyme called Dicer into 20–25 nt small interfering RNA (siRNA) with 2–3 nt 3′ overhangs. The siRNA then forms complexes with ribonucleases called RNA-induced silencing complexes (RISCs) and the double-stranded siRNA is then unwound, leaving a single 'guide' strand in the activated RISC. The activated RISC is guided to complementary mRNA molecules, where they cleave and destroy the cognate RNA. In spliceosome-mediated *trans*-splicing (panel **c**), the endogenous pre-mRNA has a mutation in the C exon (C*) to be corrected. A gene-transfer vector delivers a *trans*-splicer pre-mRNA containing a hybridization domain that is complementary to the endogenous pre-mRNA and a correct sequence for exon C. With spliceosome-mediated *trans*-splicing to the pre-mRNA product, the exogenous exon C is introduced, which results in a corrected mRNA product. The crucial splicing elements in the genome are illustrated, including the hybridization domain, branchpoint, splice donor and splice acceptor. In spliceosome-mediated segmental *trans*-splicing (panel **d**), 5′ and 3′ gene fragments with relevant regulatory and hybridization sequences are introduced into cells using two gene-transfer vectors, each coding for a partial pre-mRNA that has complementary foreign hybridization domains, and splice donor, branch point and splice acceptor sequences on the 5′ exon and 3′ exon expression cassettes, respectively. Following transcription of the 5′ exon and 3′ exon expression cassettes, the two pre-mRNAs interact through the unique complementary hybridization domains. The intranuclear interaction of the pre-mRNA products results in the stoichiometric generation of full-length, functional mRNA, which is mediated by splicesomes. Panel **e** shows ribozyme-mediated *trans*-splicing. In this model, the endogenous pre-mRNA contains a mutation (*). A corrective *trans*-splicing ribozyme, with an expression cassette containing (from 5′ to 3′) a complementary hybridization domain, a nucleolytic motif and a portion of the target mRNA with the correct sequence can be delivered to the cell using viral vectors. Once expressed, the corrective ribozyme binds to the target, cleaves the endogenous sequence 3′ to the target uracil (U) nucleotide, and replaces the defective exon with a correct sequence.



sequences) but, given the current technology, also a lot of art. Specificity is usually demonstrated by showing abrogation of mRNA silencing when a single nucleotide difference is introduced into the RNAi sequence, but microarrray analysis of cells that are treated with mRNA-specific RNAi show that other mRNAs might also be affected[92–95]. Another concern about RNAi as a therapeutic agent is that it involves dsRNAs, which can induce expression of interferon genes[92,93]. In practice, this induction is both concentration and sequence dependent, and probably can be overcome by engineering the RNAi molecule.

For hereditary disorders and their requirement for persistent effectiveness, it is challenging to deliver enough RNAi to the target cells on a persistent basis. RNAi molecules do not pass across cell membranes with ease (in humans) and have short half-lives in blood[92,93]. Finally, if given systemically, the dilution problem in the human body is daunting. In the present technology, strategies to deliver RNAi molecules include combining them with lipids or various 'delivery' proteins that pass membranes effectively. One obvious strategy for RNAi delivery for hereditary disorders is gene transfer with viral vectors that mediate persistent expression[94,96,97]. Because RNAi can only correct the phenotype of the cell to which it is delivered, the concomitant challenge is to deliver the viral vector to sufficient numbers of relevant cells to alter the phenotype. Combined gene-transfer–RNAi strategies to treat hereditary disorders have been used to correct the abnormal phenotypes in two mouse models of neurodegenerative disorders that are caused by the dominant-negative expansion of polyglutamine tracts: spinal cerebellar ataxia type 1 and Huntington disease[96,97]. In both models, CNS delivery of AAV2 vectors that code for RNAi-targeting sequences specific to the mutant alleles was associated with phenotypic improvement.

Trans-*splicing*. Therapeutic *trans*-splicing differs from ASOs and RNAi in that, in addition to reducing the expression levels of targeted genes, *trans*-splicing can be used to modify the genetic repertoire at the pre-mRNA level to correct the phenotype. In therapeutic *trans*-splicing, the sequence of the target pre-mRNA is altered by being *trans*-spliced to an independent pre-mRNA that is delivered exogenously by a gene-transfer vector[70,98] (BOX 4c). *Trans*-splicing strategies have not been evaluated in clinical trials, but they can correct animal models of haemophilia A[99], X-linked immunodeficiency with hyper IgM[100] and cystic fibrosis[101]. One hurdle to this approach is that efficiency is limited by the dependency of hybridization on the initial concentrations of the molecular participants.

Our laboratory recently developed a new *trans*-splicing model, called 'segmental *trans*-splicing' (STS), which addressed the challenge of gene transfer for genes — such as the von Willbrand factor gene (8.6 kb) or the muscular dystrophy gene (dystrophin; 11.0 kb) — that are too large to fit into conventional viral vectors[70,102] (BOX 3d). STS involves the delivery of two separate gene-transfer vectors, each encoding independent pre-mRNAs that are then joined through spliceosome-mediated *trans*-splicing to create an intact pre-mRNA.

Ribozymes. Ribozymes are RNA molecules with enzymatic activity that recognize specific RNA sequences and catalyse a site-specific phosphodiester bond cleavage within the target molecule[70,88,103]. As applied to hereditary disorders, if effective, ribozymes could be used to replace mutant sequences or to reduce mutant mRNA levels in loss-of-function dominant disorders. The structure of ribozymes consists of two regions of antisense RNA (referred to as the flanking complementarity regions) that flank the nucleolytic motif and provide the target specificity. The type of ribozymes most relevant for therapeutic applications are hammerhead ribozymes[103].

The main hurdles to applying ribozymes to the clinic are obtaining efficient delivery, stability and efficiency. Pre-synthesized ribozymes can be delivered exogenously, but this approach suffers from low levels of cellular uptake and, once taken up, rapid degradation of the ribozyme[88,103]. Ribozyme stability and efficiency are also issues, as with ASOs. Improvements can be made by engineering structural modifications to the bases and sugars of synthetic ribozymes, but can result in significant inhibition of the catalytic activity of the ribozyme. An alternative strategy for delivery is to use gene transfer to generate endogenous expression of a ribozyme, including strategies for ribozyme-mediated *trans*-splicing (BOX 4e).

In laboratory studies, ribozyme constructs have been used to correct mutations *in vitro* for hereditary disorders such as familial amyloidotic polyneuropathy[104]. There have been no clinical trials of ribozyme therapies for inherited disorders, although one strategy is focused on the autosomal dominant form of retinitis pigmentosa. The approach is to have the ribozyme selectively target the dominant version of the gene transcript, a strategy that has shown some efficacy in rodent and large mammalian models[105].

The future of RNA-modification therapy. Delivery is the big hurdle to effectively using RNA-modification therapies to treat hereditary disorders. Although mouse hereditary disease models have been corrected by RNAi and *trans*-splicing strategies combined with gene-transfer delivery, the low efficiencies and requirement to effectively treat most affected cells make effective human application a significant challenge. Because effective delivery is crucial, the breakthroughs for RNA-modification therapies for treating monogenic disorders will depend on advances in the development of gene-transfer vectors.

Embryonic stem cells

Stem cells from mouse embryos were first isolated and cultured 25 years ago, and although ESCs have now been derived from humans and several other mammalian species, most of what is known about ESC biology has been gleaned from studies of mouse cells[2,5,6]. The combination

Interferon
A family of glycoproteins that are produced and secreted by cells of the immune system to boost immune responses to viral infection.

Hammerhead ribozymes
One of the smallest ribozymes (only 30–40 nt), they are characterized by a structure consisting of three base-paired helices that are connected by two invariant single-stranded regions, which form the catalytic core.

Familial amyloidotic polyneuropathy
An autosomal dominant disorder that is characterized by deposition of amyloid fibrils in the peripheral nerves and various organs.

Retinitis pigmentosa
A retinal degeneration disease that results from one of hundreds of mutations in the rhodopsin gene. There are several varieties of this disorder, including both autosomal dominant and autosomal recessive types.

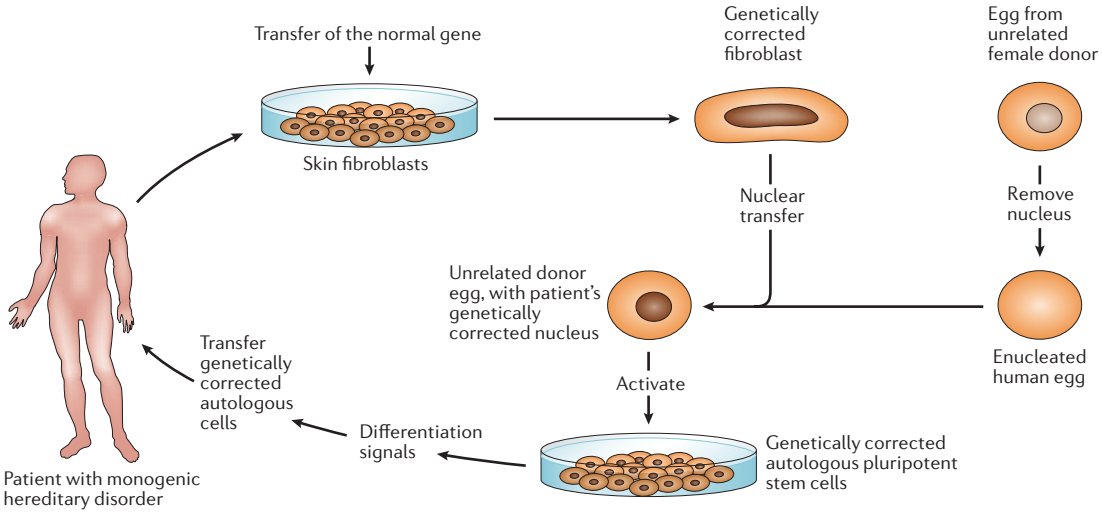


Figure 2 | **A model for genetic medicine using gene transfer, somatic cell nuclear transfer and stem cell technologies.** The strategy depicted starts with culturing skin fibroblasts from a patient with a monogenic disorder and then compensating for the abnormal gene using gene-transfer methods, such as retrovirus or lentivirus vectors, that mediate integration of the correct gene with appropriate regulatory sequences into the skin fibroblasts. The nucleus from a corrected cell is then transferred to an enucleated egg obtained from an unrelated donor using the technology of somatic cell nuclear transfer. The egg, now containing the genetically corrected genome of the patient, is activated to develop into a blastocyst *in vitro* and corrected autologous pluripotent stem cells are derived from the inner cell mass. Depending on the phenotype of the patient's disorder, the stem cells are then directed to differentiate into a specific cell type and administered into the patient, thereby correcting the disorder. The feasibility of this strategy has been demonstrated by Rideout *et al.*[135] in a mouse model of immunodeficiency that is caused by inactivation of *Rag2* (recombinase activating gene 2), which results in the complete absence of mature T and B cells in lymphoid organs.

of pluripotency and indefinite self-renewal in culture without spontaneous differentiation have made ESCs an attractive system for developing genetic medicines. However, no trials for human hereditary disorders with ESCs have been initiated, in part because of the immaturity of the science so far and in part because of societal concerns, and no trials are being seriously contemplated at this time.

One potential strategy for using ESC therapeutically for hereditary disorders is a regenerative approach in which ESCs are differentiated *in vitro* to specialized cell types (or progenitors of the target specialized cells), and then transplanted *in vivo* to replace diseased cells or tissues. The crucial first step in developing this application is to determine ways to control ESC differentiation *in vitro*. This has been the focus of considerable work, and both mouse and human ESCs have been successfully differentiated into specific cell types from all three primary germ layers[2,4–6]. Fair *et al.*[106] corrected F9 deficiency in mice by deriving 'putative endodermal precursors' from mouse ESCs, and transplanting the derived cells into the liver of an F9 deficient mouse. An alternate strategy is to use ESC-derived cells as delivery vehicles for genes that mediate phenotype correction through gene-transfer technology.

Another potentially important application of human ESCs for the treatment of inherited diseases is the derivation of human ESCs from individuals with genetic disorders to use as *in vitro* models. Verlinsky *et al.*[107] have established 18 human ESC lines with genetic disorders, such as adrenoleukodystrophy, Duchenne and Becker muscular dystrophy, Fanconi anaemia, and Huntington disease. These lines were established from embryos at the 8-cell stage with specific mutations that were identified in the course of pre-implantation genetic diagnosis. Provided this approach is verified by other laboratories, the lines will be an important resource for gaining insight into the pathogenesis and pathophysiology of these diseases and also provides an *in vitro* system for drug screening.

One potential barrier to using human ESCs to treat genetic disorders is immunorejection of the transplanted cells by the host[108,109]. This obstacle could be circumvented by using gene transfer with the relevant wild-type gene to autologous cells (such as cultured skin fibroblasts), transfer of the corrected nucleus to an enucleated egg from an unrelated donor, development of 'corrected' ESCs and, finally, differentiation and transplantation of the corrected relevant cells to the same patient (FIG. 2). A crucial component of future clinical applications of this strategy is the ability to derive 'personalized' human ESC lines using nuclear transfer with some degree of efficiency. Although research on this technology has been controversial[110,111] the efficient transfer of somatic cell nuclei to enucleated oocytes from unrelated donors, and the subsequent derivation of human ESC lines from the resulting blastocysts is a technical hurdle that should be overcome in the next few years.

Finally, but even further in the future, human ESCs could be used to develop *in vitro* three-dimensional biological structures for the purpose of replacing,

Box 5 | **Challenges for developing genetic medicines**

Regulatory

Genetic medicines are 'drugs', and so their development must be regulated with the same rigour as other therapeutics. Therapy for hereditary disorders must meet the high standard of being chronic, and so it is mandatory to prove both safety and efficacy. This standard is balanced by the fact that most hereditary disorders are untreatable, and many cause severe disability and often premature death. Furthermore, most are rare, which makes it difficult to apply the usual method of drug testing, including blinded, controlled studies[1].

Because genetic medicines aim to modify the genetic repertoire and/or gene expression, concerns have been voiced as to the appropriateness of applying such therapies to humans[130–132]. In response, governmental regulatory and advisory bodies (see the links to the GTAC and RAC web sites in the Further information) have been implemented that, in addition to carrying out routine drug review, have a specific function in the public review of genetic medicines. Whereas clinical investigators, biotechnology companies and large pharmaceutical companies often voice frustration about these further regulatory hurdles, the general consensus is that public review and further regulatory scrutiny are a good idea and help to allay public fears about genetic medicines.

Economic

The economics of drug development have an important role in attracting resources to developing genetic medicines for hereditary disorders. Academic centres do not have the financial resources nor the drug production and regulatory infrastructure to take drug development through to regulatory approval. Therefore, the development of genetic medicines that can be widely used will depend on the biotechnology or pharmaceutical industries diverting their interests to disorders that, for the most part, offer few financial incentives to compensate for the risk and costs of developing drugs. From the commercial viewpoint, most of the low-hanging fruit has been harvested (for example, protein augmentation therapies for common hereditary deficiency disorders), and therefore there will have to be a significant shift in practices to bring the biotechnology and pharmaceutical industries to focus on genetic disorders.

Socio-political

Because of the social implications of modifying the genome (in the case of gene transfer) and the sources of genetic medicine (for stem cell therapy), genetic medicines are topics of national debate in many countries. The socio-political hurdles to the use of human ESCs for therapeutic purposes have attracted significant attention in the United States, where there is a ban on using federal funds to develop new ESC lines or to do research on non-approved lines[3]. For those that oppose the development of human ESCs as therapeutics, using the blastocyst as the source of ESCs is equivalent to the destruction of human life. The consequence of the present restrictions is that most ongoing work on human ESCs in the United States is carried out by companies or in the academic world, supported by state initiatives (for example, the California referendum; see the CIRM web site in the Further information) or private funds. Although innovative work continues, including strategies to bypass the derivation of ESCs from the intact blastocyst[133,134], without access to federal funds to energize the US biomedical academic infrastructure, the promise of using human ESCs as effective genetic medicines is in the distant future.

repairing or regenerating tissues and organs[112]. Along with the challenge of circumventing immune rejection and the development of effective matrix and scaffold materials for establishing such structures, the ESC must be induced to correctly differentiate into multiple lineages in the correct organization and function, including the ability to interact appropriately with neighbouring cells and/or tissues.

The future of embryonic stem cell therapy. Given that human ESCs were first isolated and successfully cultured in only 1998, and the limitation in some countries in the use of government funds for research with deriving new ESC lines[3], human ESCs are not nearly as well understood as mouse ESCs. Much progress has been made in identifying methods for *in vitro* directed differentiation of human ESCs, and human ESCs can differentiate *in vitro* into neural precursors that can be transplanted into rodent brains and develop into neurons, astrocytes and oligodendrocytes[113–115]. Challenges for the use of ESC strategies for treating inherited disorders include improving the efficiency by which differentiated cells with appropriate functional characteristics are derived, successful avoidance of immune rejection, robust engraftment and avoidance of tumour formation.

Future prospects

The most elegant genetic medicine for a monogenic genetic disorder would be to correct the mutation and thereby allow the production of a normal protein under the endogenous regulatory signals of the cell. An advance in this direction was the recent report of efficient targeted gene correction using engineered zinc-finger nucleases (ZFNs). Urnov *et al.*[116] designed ZFNs that targeted the interleukin 2 receptor-γc mutation that causes X-linked severe combined immunodeficiency. In human cells lines in culture, ZFNs corrected 18% of the primary blood cells and 5% of the T cells without any selection. The proposed strategy for using ZFN strategies in the clinic would be for applications, such as sickle cell anaemia, in which bone marrow stem cells could be harvested from a patient, corrected *ex vivo*, and then returned to the patient. Important challenges for this technology include how to develop the ZFNs, managing the immune responses to a foreign protein and achieving target specificity[117]. Because direct 'correction' of mutations is not ready for application in humans, almost all genetic medicine strategies that have reached the clinic have focused on compensating for the abnormal phenotype.

With all the human and financial resources that have focused on using genetic medicines to treat monogenic disorders, an obvious question is: why does none of these therapies alter the abnormal phenotype in a reproducible, efficacious manner, without significant toxicity? The simplistic answer is that drug development takes years, averaging 12–15 years from concept to governmental approval (see the New Drug Development timeline in the Further information). There are also large societal hurdles, which result in regulatory hurdles that can be overcome, but slow the development process. But it might also be that genetic medicines have not yet developed to the extent that the technology is sufficiently robust to 'cure' a human monogenic disorder.

In addition to developing the technology of genetic medicines and applying them to treating experimental models and humans with hereditary disorders, some regulatory, economic and socio-political issues must also be overcome before genetic medicines can become a reality (BOX 5). Despite these societal issues, the fact that no genetic medicine has been approved for use in the treatment of any hereditary human disorder, and the daunting challenges to making genetic medicines a

Zinc-finger nucleases (ZFNs). Synthetic proteins that are composed of a highly specific DNA-binding domain, which comprises a string of zinc-finger motifs, and a nonspecific DNA-cleaving domain. The combination of ZFNs and DNA repair by homologous recombination represents a strategy of gene correction.

reality, significant intellectual and economic resources are focused on genetic medicines for one major reason: everyone agrees that, as a general class of drugs, genetic medicines are a good idea. The paths for the development of ground-breaking therapies that we accept as standard today, such as bone marrow transplantation, monoclonal antibodies, *in vitro* fertilization and transplantation of internal organs such as the heart, lung and liver, were littered by disappointments and nay-sayers that predicted inevitable failure. In a similar vein, the biological, regulatory, economic and socio-political barriers to success in the development of genetic medicines will be overcome, and we predict that, within 10 to 20 years, doctors of genetic medicine will take their place in the front lines of treating human disease.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

The following terms in this article are linked online to:
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American Society for Gene Therapy (ASGT) Stakeholder's meeting summary: http://www.asgt.org/member_resources/recent_course_materials/stakeholders/index.shtml
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