

## Candidate Diseases for Prenatal Gene Therapy

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### Abstract

Prenatal gene therapy aims to deliver genes to cells and tissues early in prenatal life, allowing correction of a genetic defect, before irreparable tissue damage has occurred. In contrast to postnatal gene therapy, prenatal application may target genes to a large population of stem cells, and the smaller fetal size allows a higher vector to target cell ratio to be achieved. Early gestation delivery may allow the development of immune tolerance to the transgenic protein, which would facilitate postnatal repeat vector administration if needed. Moreover, early delivery would avoid anti-vector immune responses which are often acquired in postnatal life.

The NIH Recombinant DNA Advisory Committee considered that a candidate disease for prenatal gene therapy should pose serious morbidity and mortality risks to the fetus or neonate, and not have any effective postnatal treatment. Prenatal gene therapy would therefore be appropriate for life-threatening disorders, in which prenatal gene delivery maintains a clear advantage over cell transplantation or postnatal gene therapy. If deemed safer and more efficacious, prenatal gene therapy may be applicable for nonlethal conditions if adult gene transfer is unlikely to be of benefit. Many candidate diseases will be inherited congenital disorders such as thalassaemia or lysosomal storage disorders. However, obstetric conditions such as fetal growth restriction may also be treated using a targeted gene therapy approach. In each disease, the condition must be diagnosed prenatally, either via antenatal screening and prenatal diagnosis, for example, in the case of hemophilias, or by ultrasound assessment of the fetus, for example, congenital diaphragmatic hernia.

In this chapter, we describe some examples of the candidate diseases and discuss how a prenatal gene therapy approach might work.

**Key words:** Prenatal gene therapy, Congenital disease, Pre-eclampsia, Fetal growth restriction

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### 1. Introduction

Gene therapy came onto the therapeutic scene in the 1980s and the first human gene therapy trials began over 15 years ago (1). But in spite of continuous technological progress most clinical results have been disappointing. The reasons for this are many and include difficulties in targeting the appropriate organ, a robust immune response to the therapy in adults and low level expression

of the therapeutic gene product. Many of these difficulties may be avoidable by applying the therapy prenatally, and recent preclinical work has indeed shown proof of principle for phenotypic cure of congenital disease in animal models using this approach. Selecting the right diseases for therapeutic intervention will be critical for clinical translation. Prenatal gene therapy has been proposed to be appropriate for life-threatening disorders, in which prenatal gene delivery maintains a clear advantage over cell transplantation or postnatal gene therapy and for which there are currently no satisfactory treatments available (2). In this chapter we consider the various congenital diseases and obstetric disorders that might be suitable for this therapeutic approach.

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## **2. Is There a Need for Prenatal Gene Therapy?**

Congenital disease places a huge burden on the community and the health service. A study of pediatric inpatient admissions in 1996 in a US children's hospital found that wholly genetic conditions accounted for one-third of hospital admissions and for 50% of the total hospital charges for that year (3). Thus a preventative strategy such as prenatal gene therapy could have an important social and economic impact.

A criticism leveled at a prenatal approach is that gene transfer to an individual after birth may be as effective, and probably safer, than prenatal treatment. Indeed current conventional treatment of some genetic diseases is highly effective. For example, hereditary hemochromatosis is caused mainly by mutations in the human hemochromatosis protein but can also be caused by mutations in hemojuvelin, hepcidin antimicrobial peptide, and the transferrin receptor 2, amongst others. The disease, which is characterized by iron overload, results in a variety of pathological changes including liver cirrhosis, cardiomyopathy, diabetes, and arthritis. Highly effective treatment consists of regular bloodletting which reduces iron concentrations to normal. For those who cannot tolerate phlebotomy iron chelation agents such as Deferoxamine are available. For many genetic diseases however, treatment is palliative rather than curative, resulting in patients living longer but with a reduced quality of life. This has been particularly seen in cystic fibrosis, in which life expectancy has risen from school age in 1955 to the mid 30s today (Cystic Fibrosis Foundation). To achieve this however, patients require daily chest physiotherapy, antibiotic treatment, dietary supplementation, insulin for diabetes mellitus, and in many cases, lung transplants, which require immunosuppressive therapy. Effective treatment in utero could cure genetic disease, or at least provide partial correction that may have a huge impact on disease progression.

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### 3. The Advantages of a Prenatal Approach for Some Diseases

1. Prenatal gene therapy may offer particular benefits in particular early onset genetic disorders in which irreversible pathological damage occurs to organs before or shortly after birth (4). For many such diseases, the organ may be difficult to target after birth, for example the lung in cystic fibrosis, the brain in urea cycle disorders, or the skin in epidermolysis bullosa, and prenatal treatment may take advantage of developmental changes to access organs that are inaccessible after birth.
2. Certain obstetric disorders may also be suitable for a prenatal gene therapy approach. Problems of deficient uteroplacental circulation underlie the common obstetric condition, fetal growth restriction (FGR) in which the fetus fails to achieve its genetic growth potential and which is currently untreatable. A targeted local gene transfer to improve the circulation is one approach that is being studied with some success.
3. The fetus has a size advantage in a number of ways for some congenital diseases, especially where transgenic protein expression levels may be important. Production of clinical grade vector is time-consuming and expensive and the small size of the fetus could lead to increased vector biodistribution at the same vector dose as an adult.
4. The fetus has a functionally immature immune system compared to an adult, which may be to its advantage for treatment of some diseases. Worldwide up to 50% of adults have preexisting humoral immunity to adenovirus and adeno-associated virus serotypes from which commonly used gene therapy vectors are derived (5). Even in the absence of a preexisting immune sensitivity, vector administration to adults often results in the development of an immune response that reduces the duration and the level of transgene expression. For example, after intramuscular injection of adenovirus vector containing the dystrophin gene into adult Duchenne muscular dystrophy transgenic mice, antibodies to the dystrophin protein were detected (6). This complication is particularly important when gene therapy is aiming to correct a genetic disease in which complete absence of a gene product is observed.

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### 4. Candidate Diseases

As with any potential therapeutic modality, the risks of prenatal gene therapy are not well characterized and the efficacy is still undetermined. In this regard, the report of the NIH Recombinant

DNA Advisory Committee (7) proposed that initial application of prenatal gene therapy should be limited only to those diseases that:

- Are associated with serious morbidity and mortality risks for the fetus either in utero or postnatally.
- Do not have an effective postnatal therapy, or have a poor outcome using available postnatal therapies.
- Are not associated with serious abnormalities that are not corrected by the transferred gene.
- Can be definitively diagnosed in utero and have a well defined genotype/phenotype relationship.
- Have an animal model for in utero gene transfer that recapitulates the human disease or disorder.

Some of the diseases that may be suitable for fetal treatment are listed in Table 1.

Preclinical studies of prenatal gene transfer are encouraging. Fetal application of gene therapy in mouse models of congenital disease such as hemophilia A (8) and B (9), congenital blindness (10), Crigler–Najjar type I syndrome (11) and Pompe disease (glycogen storage disease type II) (12) have shown phenotypic correction of the condition. In the following sections we consider groups of candidate diseases and discuss the factors that will influence the success of a prenatal gene therapy approach.

#### **4.1. The Hemophilias**

Inherited blood disorders would be a relatively simple target for prenatal gene therapy as the fetal circulation can be reached during its circulation through the umbilical vein (UV) at the placental cord insertion or the intrahepatic UV, or even via the peritoneal cavity, a route used successfully to transfuse anemic fetuses. Congenital blood disorders are relatively common in some populations, and prenatal screening and diagnostic services are available. Translation of prenatal gene therapy into man is probably most advanced when considering congenital blood disorders such as the hemophilias. Research has progressed from demonstrating proof of principle in mouse models, into larger animal models such as the sheep and non-human primate, where delivery techniques, long-term transgene expression and safety can be better addressed (see Table 2).

Deficiency in factor VIII (FVIII) and FIX proteins of the blood coagulation cascade, result in hemophilias A and B, respectively, and have a combined incidence of around 1 in 8,000 people (13). Current treatment uses replacement therapy with human FVIII or FIX which is expensive but effective (14). Beneficial effects occur after achieving only 1% of the normal levels of clotting factor. Unfortunately, a proportion of patients develop antibodies to therapy leading to ineffective treatment and occasional anaphylaxis (15). The complications of hemophilia treatment which include the major risk of HIV and hepatitis B infections, although less of an

**Table 1**  
**Candidate diseases for prenatal gene therapy**

Disease	Therapeutic gene product	Target cells/organ	Age at onset	Incidence	Life expectancy
Cystic fibrosis	CF transmembrane conductance regulator	Airway and intestinal epithelial cells	In utero	1:2,000–4,000	Mid-thirties
Duchenne muscular dystrophy	Dystrophin	Myocytes	2 years	1:4,500	25 years
Lysosomal storage disease	Glucocerebrosidase in Gaucher disease	Hepatocytes	9–11 years	1:9,000 overall	<2 years
Spinal muscular atrophy	Survival motor neuron protein	Motor neurons	In utero in type 0, 6 months in type 1	1:10,000	2 years
Urea cycle defects	Ornithine transcarbamylase in ornithine transcarbamylase deficiency	Hepatocytes	2 days	1:30,000 overall	2 days (severe neonatal onset)
Hemophilia	Human factor VIII or IX clotting factors	Hepatocytes	1 year	1:6,000	Adulthood with treatment
Homozygous $\alpha$ -thalassaemia	Globin	Erythrocyte precursors	In utero	1:2,700	Lethal
Severe combined immunodeficiency (SCID)	$\gamma$ c cytokine receptor (X-linked SCID); adenosine toxicity	Haematopoietic precursor cells	Birth	1:1,000,000	<6 months if no bone marrow transplant
Epidermolysis bullosa	Type VII collagen	Keratinocytes	Birth	1:40,000	Milder forms to adulthood
Severe fetal growth restriction	Vascular endothelial growth factor	Uterine arteries	In utero	1:100	Adulthood if individual survives the neonatal period
Congenital diaphragmatic hernia	Lung growth factors	Alveoli	In utero	1:2,200	Adulthood if individual survives neonatal surgery

**Table 2**  
**Translating fetal gene therapy into man, using hemophilia treatment as an example**

Animal model	Vector and transgene	Vector dose	Delivery route	Outcome
Mouse	Adenovirus encoding luciferase reporter gene	$1 \times 10^7$ pfu/fetus	Intrahepatic	High-level luciferase expression in neonatal liver ( <a href="#">193</a> )
Hemophilia A mouse*	Adenovirus encoding murine FVIII	$3.3 \times 10^5$ pfu/fetus	IP	Short-term correction of factor VIII deficiency in neonatal period ( <a href="#">8</a> )
Mouse	Adenovirus encoding luciferase reporter gene	$1 \times 10^7$ pfu/fetus	Intrahepatic to fetus and reinjection of 3 month-old neonate	Liver expression of luciferase up to 1 month of neonatal life. Antibodies to vector and protein developed after vector reinjection at 3 months postnatally ( <a href="#">194</a> )
Mouse	Adenovirus encoding hFIX	$2 \times 10^{11}$ pfu/fetus	Vitelline vessel	Induced immune tolerance to exogenous hFIX protein but only short-term transgenic hFIX protein expression in neonatal mouse plasma ( <a href="#">195</a> )
Hemophilia B mouse*	HIV Lentivirus encoding hFIX	$2.0 \times 10^{10}$ p/kg fetus	Vitelline vessel	Permanent cure of hemophilia with immune tolerance to exogenous hFIX ( <a href="#">9</a> )
Hemophilia B mouse*	AAV-1 encoding hFIX	$5 \times 10^9$ vg/fetus	IM (hindlimb) to fetus and reinjection postnatally	Induction of tolerance and long-term therapeutic hFIX expression ( <a href="#">23</a> )

Early gestation sheep	Adenovirus	$1.5 \times 10^{12}$ pfu/kg fetus	USS-guided IP or hepatic injection	Short-term high-level transduction of the liver after IP compared with intrahepatic injection with therapeutic levels of hFIX expression in the plasma (26)
Late-gestation macaque	AAV2 encoding eGFP	$8.6 \times 10^{10}$ p/fetus	USS-guided hepatic injection	Vector genomes in peripheral blood at birth (136)
Late-gestation macaque	Lentivirus encoding eGFP	$1 \times 10^7$ p/fetus	USS-guided IP injection	High-level liver transduction for at least 9 months postnatally (136–138, 196)
Early and late-gestation sheep	scAAV8 encoding hFIX	$1 \times 10^{12}$ vg/kg fetus	IP injection by USS	hFIX expression in blood up to 6 months but no immune tolerance (28)
Late-gestation macaque	scAAV5 and 8 encoding hFIX	$1.0\text{--}1.95 \times 10^{13}$ vg/kg fetus	USS-guided UV injection	hFIX expression in blood and liver for at least 1 year, non-neutralizing immune response (197)

Studies have moved from using short-term expressing vectors such as adenovirus to long-term expressing vectors such as AAV and lentivirus, in more clinically relevant animals such as the sheep and non-human primate

*hFIX* human factor IX clotting factor, *AAV* adeno-associated virus vector, *eGFP* enhanced green fluorescent protein, *USS* ultrasound, *IM* intramuscular, *IP* intraperitoneal

\* animal model of disease, *vg* vector genomes, *p* particles, *pfu* plaque forming units, *sc* self-complementary vector

issue now that blood donors are screened effectively, have in some cases been far worse than the diseases themselves, increasing their morbidity and mortality (16). The clotting proteins are required in the blood and can be secreted functionally from a variety of tissues, thus the actual site of production is not so important as long as therapeutic plasma levels are realized.

Adult gene therapy strategies have concentrated on application to the muscle or the liver, achieving sustained FIX expression in adult hemophiliac dogs or mice after intramuscular or intravascular injection of adeno-associated virus (AAV) vectors (17–19). Chao et al. observed that the AAV serotype is important. In mice, injection of AAV serotype 1 resulted in tenfold higher levels of canine FIX when compared with serotype 2 (19). In clinical trials using AAV2hFIX vectors in hemophilia B patients, only short-term and low level FIX expression was observed, however, which was probably caused by a cell-mediated immune response to transduced hepatocytes (20, 21). A clinical trial using a self-complementary AAV8 vector serotype, which may stimulate less of an immune response, is currently underway in adults with hemophilia B (22).

Waddington et al. demonstrated permanent phenotypic correction of immune-competent hemophiliac mice by intravascular injection of a lentivirus vector encoding the human Factor IX (hFIX) protein at 16 days of gestation (term = 22 days) (9). Plasma factor levels remained at 10–15% of normal in treated animals for their lifetime. Sabatino et al. subsequently demonstrated induction of tolerance after AAV-1-hFIX administration in Factor IX-deficient fetal mice (23).

Translation to large animals has been slower because of the need for longer-term gene transfer and a higher vector dose when compared to small animals, but recent studies demonstrate the potential for this route of delivery. Long-term transduction of hematopoietic stem cells in the bone marrow and blood could be demonstrated 5 years after delivery of retroviral vectors into the peritoneal cavity of early gestation fetal sheep at laparotomy (24). In early gestation, delivery of adenovirus vectors into the umbilical vein of fetal sheep via hysterotomy resulted in widespread transduction of fetal tissues (25). Using ultrasound-guided injection, systemic vector spread and widespread tissue transduction was demonstrated after first trimester intraperitoneal injection of adenovirus vectors into fetal sheep, although direct injection of the umbilical vein was limited by the procedure-related high mortality in late first trimester (26).

More recently, using a self-complementary AAV8 vector expressing the hFIX gene which has a high transduction capacity in mice and macaques (27), long-term hFIX expression has been demonstrated after ultrasound-guided intraperitoneal injection of fetal sheep in early and late gestation (28). No functional antibodies could be detected against the vector or transgene product and



there was no liver toxicity observed. Antibodies to the therapeutic gene were detectable when the animals were challenged at 6 months of age postnatally with the hFIX recombinant protein, showing that induction of immune tolerance was not achieved. This is probably due to the fall in hFIX expression that was undetectable by 1 year after birth and a higher initial vector dose may be required to maintain hFIX levels. Umbilical vein delivery in fetal non-human primates of a tenfold higher dose of the same self-complementary AAV system in late gestation produced clinically relevant levels of hFIX sustained for over a year, with liver-specific expression and a non-neutralizing immune response (29).

Hemophilia A and B do not usually manifest until after birth. Deficiency of some clotting factors, however, can lead to life-threatening neonatal central nervous system hemorrhage. For example, congenital factor VII deficiency, the most common autosomal bleeding disorder, is typified by severe or lethal bleeding in around 20% of patients with a homozygous or compound heterozygous genotype. They present with <1% of normal levels and often present bleeding in the central nervous system (30, 31). A therapy delivered during the fetal period could avoid long-term pathology and provide therapeutic transgene expression for life. Moreover, increasing expression even above 1% would be considered enough to substantially improve the risk and incidence of spontaneous hemorrhage (31, 32).

A second bleeding diathesis, which would benefit from a fetal or early neonatal gene therapy approach is congenital factor X deficiency. As per factor VII, factor X is synthesized by hepatocytes and constitutes a central component of the coagulation cascade. In its severest form it can present at birth as severe and fatal intracranial hemorrhage (33). The Rosen laboratory generated a strain of mouse with a disruption of the factor X gene, which manifests as partial embryonic lethality or fatal neonatal bleeding (34). The same laboratory went on to perform fetal injection of wild-type hepatocytes into factor X-deficient recipients and were able to achieve phenotypic rescue (35).

#### **4.2. The Thalassaemias and Sickle Cell Disorders**

Inherited abnormalities of hemoglobin (Hb), a tetramer of two  $\alpha$ -like and two  $\beta$ -like globin chains, are a common and global problem. Over 330,000 affected infants are born annually worldwide, 83% with sickle cell disorders and 17% with thalassaemias (36). Screening strategies can be premarital and/or antenatal depending on socio-cultural and religious customs in different populations and countries. Prenatal diagnosis is available in many countries from 11 weeks of gestation using chorionic villus sampling or amniocentesis from 15 weeks.

The  $\beta$ -thalassemias, including the hemoglobin E disorders, are not only common in the Mediterranean region, South-East Asia, the Indian subcontinent, and the Middle East but have spread to

much of Europe, the Americas, and Australia owing to migration of people from these regions. Approximately 1.5% of the global population are heterozygotes or carriers of the  $\beta$ -thalassemias. In the most severe form of  $\beta$ -thalassaemia, Cooley's anemia or  $\beta$ -thalassaemia major, there is a profound anemia that if untreated, leads to death in the first year of life. Even a mild correction of the globin chain imbalance in a fraction of maturing erythroblasts reduces the morbidity caused by ineffective erythropoiesis, and improves outcome (37). Affected children become dependent on regular blood transfusions, leading to iron deposition, organ failure, and death. Chelation therapy to remove the circulating iron is not effective. Postnatal allogeneic haematopoietic stem cell transplantation (HSCT) has been developed over the last 30 years and can cure the condition with recent results of 90% survival and 80% thalassaemia-free survival (38). However, HSCT is only available in approximately 30% of cases because of the lack of a suitable matched donor (37), and it is associated with complications such as Graft Versus Host Disease.

In alpha-thalassemia there is a deficit in the production of the Hb  $\alpha$  globin chains. Underproduction of  $\alpha$  globin chains gives rise to excess  $\beta$ -like globin chains which form tetramers, called Hb Bart's in fetal life and HbH in adult life. Compound heterozygotes and some homozygotes for  $\alpha$ -thalassaemia have a moderately severe anemia with HbH in the peripheral blood. Finally, some individuals who make very little or no  $\alpha$  globin chains, have a severe anemia, termed Hb Bart's hydrops fetalis syndrome which is commonly diagnosed prenatally and which, if untreated causes death in the neonatal period (39).

Sickle cell disorders are caused by Hb gene variants that similar to thalassaemia reduce mortality from falciparum malaria in carriers, and leads to high carrier levels in malaria endemic countries. The abnormal HbS cells in the circulation leads to recurrent painful sickle cell crises. Current treatment relies on a number of strategies to prevent crises from occurring using, for example, prophylactic antibiotics, pneumococcal vaccination and good hydration, and effective crisis management using oxygen and pain-relief (40).

Effective gene therapy in thalassaemia and sickle cell disease will depend on understanding the regulation of the globin genes. In  $\beta$ -thalassaemia, for example, the tissue and developmental-specific expression of the individual globin genes is governed by interactions between the upstream  $\beta$ -globin locus control region ( $\beta$ -LCR) and the globin promoters (41). Amelioration or even cure of mouse models of human sickle cell disease (42) and  $\beta$ -thalassaemia major (43–45) has been achieved using lentivirus vectors that contain complex regulatory sequences from the LCR region. Injection of this optimized lentiviral vector into the yolk-sac vessels of fetal mice at mid-gestation resulted in human alpha-globin gene expression in the liver, spleen, and peripheral blood in

newborn mice and expression peaked at 3–4 months reaching 20% in some recipients (46). Expression declined at 7 months of age (normal lifespan 2–3 years) possibly due to insufficient HSC transduction or the late stage of mouse gestation at which the vector was introduced. Work is continuing using a lentivirus vector system that has a natural tropism for the haematopoietic system by way of a ubiquitous chromatin opening element (UCOE) augmented spleen focus forming virus (SFFV) promoter/enhancer (47, 48). This provides reproducible and stable function in bone marrow stem and all differentiated, peripheral haematopoietic cell lineages (49) and may improve long-term expression.

The potential for an ex vivo gene transfer approach for the treatment of thalassaemia was recently demonstrated in an adult patient with severe  $\beta$ -thalassaemia who had been dependent on monthly transfusions since early childhood. After autologous transplantation of gene-modified haematopoietic stem cells using a lentivirus encoding  $\beta$ -globin, he became transfusion independent for at least 21 months. Blood hemoglobin was maintained between 9 and 10 g/dl, of which one-third contained vector-encoded  $\beta$ -globin. The therapeutic effect is due to dominance of a myeloid-based cell clone. Integration of the vector into the host chromosomal site AT-hook 2 (HMGA2) of this clone resulted in transcriptional activation of high mobility group protein (HMGA2) expression in erythroid cells and caused truncation of an inhibitory microRNA-binding site in this gene. The observed cell expansion could be a stochastic and fortuitous event or the result of dysregulation of the HMGA2 gene expression in myeloid stem/progenitor cells (50). Further research is needed to determine whether this clone remains homeostatic or whether its development may be a prelude to multistep leukemogenesis, a problem that has been observed in ex vivo autologous stem cell gene therapy for severe combined immunodeficiency (51).

### 4.3. Cystic Fibrosis

The fetal lung is an ideal target for prenatal gene therapy because transduction of the fluid-filled fetal lungs may be achieved more easily than in postnatal life, where there is an air-tissue interface. Postnatal lung damage also reduces gene transfer (52). Candidate diseases for lung-directed prenatal gene transfer include cystic fibrosis (CF), alpha-1 antitrypsin deficiency, surfactant protein B deficiency, and pulmonary hypoplasia.

Cystic fibrosis (CF) is a common lethal autosomal recessive disease in which tissue injury begins in the prenatal period (53). The potential targets for CF lung manifestations are the ciliated epithelial cells and ducts of the submucosal glands, where the wild-type CF transmembrane conductance regulator (CFTR) protein is expressed. Correction of as few as 6% of the defective cells may be sufficient to correct the chloride transport defect (54). Although CF is a multisystem disease, much of the morbidity and mortality

derives from the diseased lung. Here the classical gene therapy target is the ciliated epithelial cells and ducts of the submucosal glands in the lungs where the wild-type CFTR protein is normally expressed (55). Gastrointestinal manifestations of CF are now increasingly recognized as an important contributor to morbidity in those patients who reach adulthood (56), as well as affecting 15% of neonates with the life-threatening condition of meconium ileus (57). With the advent of prenatal screening for CF, the possibility of offering treatment to couples whose fetus is affected becomes more real (58).

Around 400 CF patients have been given gene therapy postnatally using viral and nonviral gene transfer agents through mainly nebulized systems (59). Early trials established the safety of adenovirus and nonviral vectors but CFTR expression was hindered by the low transduction efficiency of both vector classes on the respiratory epithelium, partly due to the location of the adenovirus receptor in the basolateral membrane of the respiratory epithelium which is isolated from the lumen by tight junctions (60). In addition, a robust immune response caused a dose-dependent inflammation and pneumonia related to the immunogenicity of the viral proteins that prevented repeat administration (61). Based on these shortcomings other vectors were developed for clinical use, such as AAV2 which in clinical trials has reduced toxicity and immunogenicity (62, 63). Unfortunately, these phase I/II trials were in general unsuccessful due to neutralizing antibodies that prevented reliable repeat vector administration. Other vector systems that have been investigated for gene transfer to the lung include AAV1 and five in the rat (64) and helper-dependent adenovirus vector, incorporating a human epithelial cell-specific expression cassette in the rabbit lung (65).

The only present clinical trial for CF uses nonviral gene transfer agents such as polyethylenimine (PEI), cationic lipid 67 (GL67), and DNA nanoparticles which may generate less of an immune response. Proof-of-principle studies on the nasal epithelium show a 25% correction of the molecular defect (66) and expression of hCFTR is seen in sheep transfected with a human CFTR plasmid, complexed with GL67 (67).

One of the barriers to effective gene transfer to the airways in the adult or neonate with CF is that inflammation and damage of the lung precludes effective gene delivery. This could be overcome if gene therapy is applied at a stage of prenatal life where no or minimal lung damage has occurred. Importantly, the fetal lungs are fluid filled and transfection of the fluid-filled fetal airways may be more easily achieved. Fluorocarbon liquids such as perflubron have been used to push vector into the distal fetal airways from injection at the trachea (68) and have been shown to enhance adenovirus-mediated gene expression in normal and diseased rat lungs (52). The proliferating cell population in CF airways are

mainly basal cells (69) and these would be the best target in any gene therapy approach.

Initial studies appeared promising, with a report that CFTR-knockout mice could be cured by prenatal adenovirus administration into the amniotic fluid (70). Since the fetus draws amniotic fluid into the lungs during fetal breathing movements, intra-amniotic delivery could provide an efficient route of gene transfer to the airways. Two further studies using the same vector, delivery method and mouse strain as well as a different CFTR-knockout mouse strain have, however, been unable to replicate these findings (71, 72). The high spontaneous survival rate of the CF-mouse strain used in Larson's experiments may explain the initial enthusiasm for the results observed (70). In addition, the inability to cure CF in this model might be due to the strain of mice used, the vector construct which only gives short-term gene transfer (73), or on account of insufficient fetal breathing movements. Nevertheless, gene transfer to human fetal lungs is achievable in a xenograft model in SCID mice with long-term expression in the surface epithelial and submucosal gland cells observed up to 4 weeks and 9 months after administration of adeno-associated and lentiviral vectors, respectively (74, 75).

Transgene expression in the fetal mouse lung can be improved by increasing fetal breathing movements using a combination of intra-amniotic theophylline administration and exposure of the dam to elevated CO<sub>2</sub> levels (76). Theophylline has a similar effect on breathing movements in fetal sheep (77). Much of the vector was diluted in the amniotic fluid volume and not concentrated in the organ(s) required for CF therapy as strong gene transfer to the skin occurred after intra-amniotic delivery (76).

In large animals such as the fetal sheep we were unable to produce significant airways gene transfer after intra-amniotic adenovirus vector injection in the first trimester although the nasal passages were transduced (26). Fetal breathing movements are not present in the first trimester human or sheep fetus, and the large amniotic fluid volume even at this gestation means that a more targeted approach to the lung may be required in clinical practice.

Several studies have applied adeno-associated virus vectors (AAV), many using the amniotic route. Injection of AAV2 into rabbit amniotic fluid transduced the trachea and pulmonary epithelium of the fetus (78) and prolonged gene expression was seen in further studies in mice, rats, and macaques (79). Gene delivery to the lung parenchyma can also be achieved by indirect means using AAV, by intraperitoneal injection, for example (80). Similarly, injection of AAV1 and AAV2 into mouse muscle, peritoneal cavity, and intravenously gave lung expression of the transgenic protein (81).

Local injection of the lung parenchyma is an alternative to the amniotic route but gives only local gene transfer in fetal rats (82, 83) and non-human primates (84). The stage of gestation is important,

with transgene expression more local to the lung after vector injection in early second trimester (pseudoglandular stage) when compared to the late first trimester (embryonic stage) (85).

In larger animals, injection of the fetal trachea by transthoracic ultrasound-guided injection (86) targets gene transfer to the medium to small airways (68). Increased transgene expression in the fetal trachea and bronchial tree was seen after complexation of the virus with DEAE-dextran, which confers a positive charge to the virus, and pretreatment of the airways with sodium caprate, which opens tight junctions in the airway epithelia thereby improving vector access to the coxsackie-adenovirus receptors (68, 87). For gastrointestinal CF pathology widespread intestinal transduction was achieved using ultrasound-guided gastric injection in the early gestation fetal sheep (88) that had an associated low morbidity and mortality. Transgene expression was enhanced after pretreatment of the fetal gut with sodium caprate after adenovirus complexation with DEAE-dextran. In addition, instillation of the fluorocarbon perflubron after virus delivery resulted in tissue transduction from the fetal stomach to the colon.

Another potentially important application of prenatal gene therapy to the lung lies in fetuses affected with congenital diaphragmatic hernia (CDH), a condition where lung hypoplasia causes significant morbidity and mortality. Adenovirus-mediated prenatal CFTR expression enhanced saccular density and air space in the lungs of a rat model of CDH (89). Short-term expression of growth factors at a critical stage of lung growth may be useful for this serious condition. After surgical creation of CDH in fetal sheep, nonviral vector expression of keratinocyte growth factor in the trachea lead to increased surfactant protein B synthesis in the lungs suggesting better maturation of the regrowing lung (90).

#### **4.4. Diseases of the Nervous System**

Early lethal genetic diseases of the nervous system are individually rare, yet collectively lead to a large disease burden, and in some populations have a high prevalence (91). Conditions can directly affect the nerves themselves, such as spinal muscular atrophy (SMA), a disease primarily of the peripheral nervous system. Alternatively, enzyme deficiencies can lead to a damaging buildup of lysosomal substrates that damage neurons, as well as other organs in the body. Examples include the lysosomal storage diseases such as acute neuronopathic (Type II) Gaucher disease, neuronal ceroid lipofuscinoses, and Niemann–Pick disease type C. In some cases, these conditions are not recognized during fetal life on prenatal ultrasound examination. For example, there are a few case reports that some fetuses with SMA have increased nuchal translucency; however, a recent study in 12 women with affected fetuses did not find any association (92). In Niemann–Pick disease type C, however, in utero splenomegaly, hepatomegaly, ascites, fetal growth restriction, and oligohydramnios (reduced liquor volume) are common (93). Screening programs are available in populations



with high prevalence such as the Ashkenazi Jews, where triple disease screening for Tay–Sachs disease, type 1 Gaucher disease, and cystic fibrosis (CF) are commonly performed together (94). Prenatal diagnosis is available for these conditions via chorionic villus sampling assuming the gene defect is known.

#### 4.4.1. *The Lysosomal Storage Diseases*

The lysosomal storage diseases are inherited deficiencies of lysosomal enzymes that lead to intracellular substrate accumulation. In mucopolysaccharidosis type VII (MPS type VII), for example, a deficiency of  $\beta$ -glucuronidase activity leads to accumulation of glycosaminoglycans in lysosomes (95) leading to enlarged liver and spleen, growth and mental retardation, and death from cardiac failure. Lysosomal storage diseases can manifest during intrauterine life as nonimmune hydrops (96). Although rare, MPS VII has been a disease of choice to investigate gene therapy because of the availability of a mouse and dog model. Correction of the MPS phenotype theoretically requires only low levels of the therapeutic gene product (97). Neonatal injection of a retrovirus vector in MPS VII dogs and mice resulted in hepatocyte transduction, with uptake of the enzyme from the circulation by other organs. The treated animals did not develop cardiac disease or corneal clouding and skeletal, cartilage, and synovial disease was ameliorated (98). Nonviral mediated gene transfer to the liver of MPS I and VII mice also improved the phenotype (99). Still, the major challenge remains to target the brain which currently requires multiple brain injections with accompanying risks (100, 101) and immunosuppression to prevent pan-encephalitis that develops secondary to an immune response to the transgene (101). Widespread correction of the pathological lesions in an MPS VII mouse was recently observed with adeno-associated virus gene transfer (102), a vector which elicits less of an immune response. Prenatal gene delivery is an alternative strategy. Injection of adenovirus into the cerebral ventricles of fetal mice led to widespread and long-term gene expression throughout the brain and the spinal cord (103). In the same study, delivery of a therapeutic gene to the cerebral ventricles of fetal MPS type VII mice prevented damage in most of the brain cells before and until 4 months after birth. A similar study using an AAV vector had comparable results but with longer expression (104).

From a translational perspective, direct vector administration into the fetal brain or ventricles for prenatal gene transfer is unappealing. There are technical difficulties in injecting the fetal brain through the skull using minimally invasive injection techniques, although this has been achieved in non-human primate (105) and sheep (A.L. David, unpublished work) under ultrasound guidance. In contrast, ultrasound-guided access to the human fetal circulation is commonly used for fetal blood sampling and transfusions in clinical practice, with minimal fetal loss rate or complications (106). This triggered the hunt for vectors that could cross the blood/brain barrier.

Recently, AAV vectors of serotypes 2/9 have been shown to have an astonishing ability to transduce cells of the nervous system, achieved not by intracranial but via intravenous injection in neonatal mice (107, 108), cats (108), and non-human primates (109). The ability of the vector to cross the blood–brain barrier may depend on specific populations of receptors within the brain that facilitate transfer for particular AAV serotypes (110). A recent study describing fetal intravenous injection of AAV 2/9 in either single-stranded or self-complementary format showed comprehensive transduction of the central nervous system, including all areas of the brain and retina, and the peripheral nervous system including the myenteric plexus. Interestingly, the single stranded version, containing a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) achieved far higher and more comprehensive levels of expression than the self-complementary vector lacking WPRE (111).

Prenatal gene transfer has also been applied with some success in glycogen storage disease type II (GSDII), which is caused by a deficiency in acid  $\alpha$ -glucosidase (GAA). This leads to lysosomal accumulation of glycogen in all cell types and abnormal myofibrillogenesis in striated muscle with death from respiratory failure. Delivery of the AAV-*GAA* vector by intraperitoneal injection to the mouse embryo in knockout models gave high-level transduction of the diaphragm and restoration of its normal contractile function (12).

Neuronal ceroid lipofuscinoses (NCLs), known collectively as Batten disease, are autosomal recessive lysosomal storage diseases which have lead to significant central nervous system pathology. Infantile NCL, caused by mutations in the *CLN1* gene, results in deficiency in palmitoyl protein thioesterase 1 (PPT1). Patients with the disease are born with no pathological manifestations but by 12 months of age they show signs of mental retardation, motor dysfunction, and visual problems (112) and survive only to 6 years of age, on average. A mouse model of this disease shows many of the same pathological symptoms and premature death occurs by 8.5 months (113). Although there have been no attempts as yet at treating this model by fetal gene therapy, there is a strong case to be made for this in a preclinical and clinical setting.

#### 4.4.2. Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is characterized by degeneration of the lower motor neurons in the anterior horn of the spinal cord and the brainstem. Although rare (incidence 1:10,000) (114), SMA is invariably fatal after a course of progressive muscle weakness and atrophy. It is caused by homozygous loss or mutation in the telomeric survival motor neuron gene 1 (*SMN1*) with subsequent neuronal cell death through apoptosis. Affected individuals can be partially protected by the presence of an increased copy number of the *SMN2* gene (114), a nearly identical copy gene of



SMN1, that produces only 10% of full-length SMN RNA/protein. This suggests that SMN2 may play a disease-modifying role and could be a target for gene therapy of the disease.

The childhood forms which are all autosomal recessive can be divided into three types depending on their severity (115). The fetal form of the disease, type 0, presents in utero with diminished fetal movements and arthrogryposis (116). Neuronal degeneration and loss in SMA type I begins during intrauterine life which makes prenatal gene therapy an attractive option (117). Vectors derived from adenovirus, herpes simplex virus (HSV), adeno-associated virus (AAV), and lentivirus are capable of transducing neurons in vitro and in vivo (115). Neuroprotective factors such as cardiotrophin 1 (118) or anti-apoptotic proteins such as Bcl-xL can be used, for example, adenovirus Bcl-xL has been shown to inhibit neuronal cell death in rat cell cultures (119). SMN gene replacement is also possible. Multiple intramuscular injections of a RabG-EIAV lentivirus vector containing the human SMN gene increased SMN protein levels in SMA type I fibroblasts and in SMA mice and reduced motor neuron death (120).

Lower motor neurons can be targeted by direct injection of the spinal cord which, although successfully achieved in the rat and mouse postnatally (121, 122), is technically risky in the adult human. Injection of an AAV8 containing the human SMN gene into the CNS of SMA mice improved mortality (123) although they still died prematurely despite continual, high-level expression from the viral vector, which may have been due to a failure to correct the autonomic system that regulates cardiac function.

An alternative is remote delivery, and motor neuron gene expression has been achieved by intramuscular or intra-axonal injection with subsequent retrograde axonal transport in small animals (124, 125). A therapeutic effect was documented after intramuscular injection of adenovirus-cardiotrophin 1 in a mouse model of SMA (118). It is not clear, however, if remote delivery will be effective in larger animals or an affected human where the peripheral nerves are much longer and retrograde transport is impaired secondary to the disease. Fetal application in this context may provide the advantage of a shorter and healthy axon and recent results in mice suggest that a fetal approach is feasible. Using lentivirus vectors, that are efficient at infecting nondividing cells Rahim et al. observed transduction of multiple dorsal root ganglia and efferent nerves following intrathecal injection of an EIAV (equine infectious anemia virus) lentivirus vector into fetal mice at 16 dpc (126).

Systemic delivery using AAV vectors is probably the way forward and might also correct the cardiac dysfunction that occurs. Foust et al. incorporated SMN cDNA into the 2/9 vector serotype and showed that neonatal intravenous injection of this vector into the corresponding mouse model of SMA resulted in an unprecedented improvement in survival and motor function (127). Using

a self-complementary AAV9 vector containing a codon-optimized SMN1 sequence injected intravenously on day 1 postnatal, Dominguez et al. achieved 100% rescue of a mouse model of severe SMA, completely corrected motor function and reduced the weight loss associated with this model (128).

#### 4.4.3. *The Urea Cycle Defects*

The inherited inborn errors of metabolism result from enzyme deficiencies in different metabolic pathways. One of the first metabolic disorders targeted by gene therapy is the defect in the urea cycle, ornithine transcarbamylase deficiency (OTC), an X-linked condition which results in accumulation of ammonia with resultant repeated episodes of hyperammonemia within 1 week of life, damaging the central nervous system and jeopardizing life (129, 130). A phase I trial targeting the liver through intra-arterial adenovirus injection ended with low level gene transfer and a fatal immune reaction in one of the 17 patients (131, 132). Subsequent investigation in small animals focused on less immunogenic vectors and showed that long-term correction of the metabolic defect in OTC deficiency could be achieved using a helper-dependent adenovirus vector (133) and AAV (134). Because of the difficulties with postnatal OTC deficiency gene therapy and the severity and very early onset of the complete form, fetal gene therapy may be a good approach.

A notable success in small animal models is in the long-term correction of bilirubin UDP-glucuronyltransferase deficiency in fetal rats using a lentivirus vector (11). Humans who suffer from this defect are classified as having Crigler–Najjar type 1 syndrome and suffer severe brain damage early in childhood due to the inability to conjugate and excrete bilirubin. A rat model of Crigler–Najjar was injected with a lentivirus vector carrying the gene for bilirubin UDP-glucuronyltransferase. The treated rats sustained a 45% decrease in serum bilirubin levels for more than a year, a level that would be considered therapeutic in the human (11). Despite the long-term expression, these rats developed antibodies against bilirubin UDP-glucuronyltransferase (135), which may be related to the fact that the fetal injection was done late in fetal life and to the unusual immunogenicity of the transgenic protein.

Intravascular vector delivery in small animals can give excellent liver transduction but in larger animals such as the fetal sheep, the intraperitoneal route seems to be the best route to target gene transfer to the fetal liver (26), and no immune response to the transgenic protein was detected after injection of adenovirus vectors in early gestation. Direct intrahepatic injection resulted in low level gene transfer with necrosis of the liver around the injection site, which was thought to be due to a direct toxic effect of adenovirus vector on hepatocytes (26). Studies of intrahepatic injection using other vectors such as lentivirus or AAV show better results.

In the non-human primate, intrahepatic or intraperitoneal (IP) vector injection resulted in widespread gene transfer and particularly to the liver, with no transplacental transfer to the mother (136–139). In one of these studies, however, IP injection of lentivirus vector at the end of the first trimester showed that a subset of female but not male germ cells were transduced (137). In the ovary, meiosis begins in the innermost areas of the cortex during the 12th and 13th weeks of gestation, while proliferating primordial germ cells forming the oocytes are found in the most superficial areas of the cortical region of the developing ovary, and these may be vulnerable to lentiviral gene transfer when delivered early in gestation via the IP route. Since IP injection is a relatively safe and well-studied ultrasound-guided fetal injection method (140), this is likely to be the route of choice when compared to liver injection, which is used rarely in fetal medicine for diagnosis of congenital liver disease. The risk of germline gene transfer in female fetuses will need to be evaluated carefully.

Metabolic diseases other than ornithine transcarbamylase deficiency and bilirubin UDP-glucuronyltransferase deficiency that could benefit from fetal gene therapy to the liver are phenylketonuria, galactosemia, and long-chain acyl-CoA dehydrogenase deficiency.

#### **4.5. Muscular Dystrophy**

Targeting the muscle for gene delivery could be a successful strategy for treatment of muscular dystrophies. Duchenne muscular dystrophy (DMD) is the commonest form and is X-linked. Abnormal or absent dystrophin leads to progressive muscle weakness in early childhood, culminating in death secondary to respiratory or cardiac failure during the third decade of life. For a one step prenatal gene therapy, the striated muscles in the limbs and chest, and the cardiac muscle would need to be transduced. Prenatal diagnosis is available, and carriers can be screened for the presence of a male fetus using noninvasive prenatal diagnosis, avoiding the need for invasive tests and the associated miscarriage risk in 50% (141).

In adult clinical trials, dystrophin gene transfer to striated muscle using viral (142) and nonviral vectors (143) has been hampered by low efficacy because of the development of cellular and humoral immunity to the transgenic dystrophin gene (144, 145). This could be avoided by prenatal application, which would also target a rapidly proliferating population of myocytes that are present in the fetus. Satellite cells that are capable of regenerating muscle fibers are transduced after intramuscular lentivirus vector delivery to fetal mice (146). Importantly, inducible dystrophin expression begun during fetal life corrected the phenotype in a DMD mouse model, where postnatal expression did not (147), supporting a fetal approach.

Intramuscular fetal injection of an adenovirus containing the full-length murine dystrophin gene in the mdx mouse model of DMD conferred effective protection from cycles of degeneration and regeneration normally seen in affected muscle fibers (148), but gene transfer level was low. More efficient gene transfer to all necessary muscle groups was seen after delivery of lentivirus vectors to fetal mice using multiple routes of injection. Systemic delivery targeted the heart, direct injection transduced the limb musculature and intraperitoneal injection reached the diaphragm and innermost costal musculature. Expression lasted for over 15 months and did not stimulate any immune response (149).

Large animal muscle gene transfer has been investigated. Gene delivery to the hindlimb musculature of the early gestation fetal sheep using ultrasound-guided injection of adenovirus vectors resulted in highly efficient gene transfer with a low procedure complication rate (26). A clinically relevant method for respiratory muscle gene transfer has also been evaluated in early gestation fetal sheep and showed that transduction of intercostal muscles occurred after ultrasound-guided creation of a hydrothorax into which adenovirus vectors were introduced (150).

There has been considerable recent success using AAV vectors to transduce fetal musculature. Early studies on AAV showed long-term local transgenic protein expression following direct injection into fetal mouse muscle (151) and transduction of the diaphragm after IP injection (152). Using this route to administer AAV1, Rucker and colleagues restored acid  $\alpha$ -glucosidase activity to the diaphragm in a mouse model of Pompe disease (12). This was the first demonstration that fetal gene transfer could correct a model of congenital muscle pathology. More recently, studies on intraperitoneal delivery of AAV8 into normal fetal mice show high levels of marker gene expression in all the muscle groups affected by congenital muscular dystrophies (153), and in the mdx mouse model of DMD, delivery of an AAV containing dystrophin significantly improved the dystrophic phenotype (154). Postnatal application of AAV6 containing full length and micro-dystrophins in neonatal mice can almost entirely prevent and partially reverse the pathology associated with DMD, but only near the site of injection (155). In late-gestation macaques, umbilical vein injection of AAV9 results in very high levels of transgenic protein expression in many tissues including skeletal and cardiac muscle (29). Systemic delivery of AAV vectors in the fetus may finally provide a solution to target the necessary muscle groups for muscular dystrophy therapy. However, the packaging capacity of AAV is restricted to delivery of truncated dystrophin minigenes which may negatively counteract the efficiency of this vector system.

Exon skipping is another strategy that is proving quite successful. An antisense oligonucleotide is used to modify splicing,

such as skipping of the mutated exon 51, which allows a partly functional dystrophin protein to be produced from the muscle. This therapy has been successful in the *mdx* mouse and a dog model of DMD, and there are currently three phase III trials internationally (156).

#### **4.6. The Genodermatoses**

The genodermatoses are a group of genetic skin diseases that may be associated with significant morbidity and mortality. Examples include the epidermolysis bullosa (EB) disorders, the ichthyotic disorders, and disorders of pigmentation such as oculocutaneous albinism. Methods of prenatal diagnosis are varied. Where the molecular defect is known, amniocentesis or chorionic villus sampling is commonly used. X-linked ichthyosis is associated with low levels of unconjugated estriol, one of the markers used for Down's syndrome screening, and this can prompt prenatal diagnosis in the mother. When the gene mutation is unknown, however, fetal skin biopsy is necessary that unfortunately carries a slightly higher fetal loss than other invasive tests (1–3%), may result in scarring, and may need to be performed at quite late gestations (157). Ultrasonography can be used in the diagnosis of a few of these disorders. In harlequin ichthyosis, for example, typical sonographic features include echogenic amniotic fluid, large joint and digital contractures and facial dysmorphism, including flat face and wide mouth with thickened lips.

The genodermatoses may be good candidates for prenatal gene therapy, where gene transfer to the skin via the amniotic fluid may provide obvious advantage to cumbersome postnatal therapy. Transgenic protein expression is seen in the skin after intra-amniotic delivery of adenoviral vectors to mice (12 days postconception (dpc)) (76), and sheep in the early first trimester (day 33 of 145 days of gestation) using ultrasound-guided injection (26). In a mouse model of Herlitz junctional epidermolysis bullosa, a lethal skin disease, a combination of adenovirus and AAV vectors injected into the amniotic cavity of fetal mice (14 dpc) led to expression of the laminin-5 transgenic protein although only a minor increase in the lifespan of treated mice was seen (158). In all these studies, only the most superficial layers of the skin, the periderm and epidermis were transduced. Several strategies have been used in small animals to target the deeper layers, such as intra-amniotic injection with subsequent electroporation (159) or application of microbubble-enhanced ultrasound (shot-gun method) (160, 161). Translation to clinical practice will be challenging. Earlier in gestation, epidermal stem cell populations are accessible for gene transfer using the intra-amniotic delivery route. Injection of lentivirus vectors between day 8 and 12 dpc in fetal mice resulted in long-term transgenic protein expression in basal epidermal stem cells into adulthood (162). Using a skin-specific keratin 5 promoter instead of the cytomegalovirus promoter also improved epidermal gene transfer.

#### **4.7. Primary Immune Deficiencies**

The primary immune deficiencies result from inherited mutations in genes required for the production, function or survival of specific leukocytes such as T, B or NK lymphocytes, neutrophils and antigen-presenting cells, or are caused by cytotoxic metabolites. The leukocytes are produced from the pluripotent hematopoietic stem cells (HSC) in the bone marrow, and therefore allogeneic bone marrow transplantation (BMT) from a healthy donor into an affected patient can restore the immune system. Successful BMT has been achieved in deficiencies such as Wiskott–Aldrich Syndrome (WAS), Chronic Granulomatous Disease (CGD) and Adenosine Deaminase Deficiency where there is toxicity rather than a defect of the proliferation gene and in X-linked Severe Combined Immunodeficiency (SCID). With the exception of X-SCID all of the other primary immune deficiencies require extra therapeutic steps such as pre-transplant conditioning, marrow cytoablation to “make space” in the marrow for the transplanted HSC and immune ablation to prevent rejection of the donor HSC. These strategies carry risks for the patient and in some cases, a haploidentical donor is unavailable. Gene therapy has therefore been developed for treatment of some patients.

Postnatal gene therapy using in vitro transduced autologous HSCs with subsequent transplantation into the same patient has been used successfully in adenosine deaminase deficient SCID (163, 164), X-linked SCID (165, 166), and CGD (167). Despite the encouraging results, 4 out of 26 subjects subsequently developed a T-cell leukemia-like condition which may have been related to integration of the retroviral vector near a suspected proto-oncogene (168). Newer approaches to decrease this risk have used lentivirus vectors that have been studied in non-human primates (169). Also, semi-viral systems have been developed with the aim to offer stable gene transfer along with a favorable pattern of integration (170, 171). These semi-viral systems are still limited by their low transduction efficiency as in the case of the sleeping beauty transposon (170, 171).

Prenatal gene therapy in early gestation before the maturation of the immune system could, theoretically, eliminate the need for marrow conditioning or the restriction to an HLA-matched donor. Prenatal treatment with hematopoietic stem cell transplantation has been attempted for a variety of immunodeficiencies and hemoglobinopathies using IP transfer of paternal or maternal hematopoietic cells or fetal liver (172); however, the clinical successes were mainly in cases of X-linked SCID (173, 174) where no immune response to the transplanted cells could be mounted.

An autologous stem cell gene transfer approach (175) using fetal stem cells from a number of sources within the fetus including the blood, liver, amniotic fluid (AF), and placenta could be adopted. Fetal liver or blood sampling at an early gestational age carries a significant risk of miscarriage (176, 177). It is now apparent that



pluripotent stem cells can be readily derived from fetal samples collected at amniocentesis (178) or chorionic villus sampling (179, 180), procedures that have a low fetal mortality. Human AFS cells have the potential to differentiate into a variety of cell types and can be transduced easily without altering their characteristics (178, 181, 182). Recent work in sheep described good fetal survival after autologous AF mesenchymal stem cell (MSC) transplantation using ultrasound-guided amniocentesis, and subsequent IP injection of selected, expanded, and transduced AFMSCs into the donor fetus. Widespread cell migration and engraftment, particularly in the liver, heart, muscle, placenta, umbilical cord, and adrenal gland was seen (183).

Prenatal diagnosis of the primary immune deficiencies is available where the gene mutation is known. For example, it has been applied in families that have been identified to be at risk of these conditions, such as those harboring mutations in both of the recombination-activating genes RAG1 and RAG2 that are involved SCID syndromes (184).

#### **4.8. Diseases of the Sensory Organs**

Prenatal gene delivery looks promising in eye and ear diseases but translation to man will be challenging. In animal models of Leber congenital amaurosis, a severe retinal dystrophy, fetal gene therapy using AAV or lentivirus vectors resulted in an efficient transduction of retinal pigment epithelium and restoration of visual function (10, 185). Similarly, AAV was able to efficiently transfect the developing cochlea in fetal mice (186). All these previous studies relied on injection into the developing sensory organ itself, which will be difficult to achieve in clinical practice. Vector delivery via the amniotic cavity early during embryonic development depends critically on the stage of gestation. For example, intra-amniotic delivery of lentivirus specifically at day 8 dpc resulted in gene transfer to the mouse retina (187) but later delivery time points were only able to target the lens and cornea. Greater tissue specificity and safety can probably be accomplished by the use of tissue-specific promoters, or regulated transgene expression, but there will still be the need for accurate prenatal diagnosis at a time of gestation equivalent to 3–5 weeks in human pregnancy, something that will be difficult to achieve with current diagnostic techniques.

#### **4.9. Fetal Growth Restriction**

Prenatal gene therapy is also being investigated for treatment of obstetric disorders. Severe fetal growth restriction (FGR) affects 1:500 pregnancies and is a major cause of neonatal morbidity and mortality. The underlying abnormality in many cases is uteroplacental insufficiency, whereby the normal physiology process of trophoblast invasion that converts the uterine spiral arteries into a high-flow large conduit for blood, fails to occur. Currently, there is no therapy available that can improve fetal growth or delay delivery to allow fetal maturity. FGR is commonly diagnosed on routine

fetal ultrasound when the fetal growth velocity falls below the expected gestational age charts. Abnormally low uterine artery Doppler blood flow and increased vascular resistance is also classically seen in mid-gestation.

A targeted approach to the uteroplacental circulation is needed, since intravascular infusion of sildenafil citrate, a nitric oxide donor, causes a drop in systemic blood pressure and had detrimental effects on growth-restricted sheep fetuses (188). In the pregnant sheep local over-expression of VEGF mediated via adenovirus injection into the uterine arteries increased uterine artery blood flow and significantly reduced vascular contractility (189). VEGF expression was confined to the perivascular adventitia of the uterine arteries, together with new vessel formation, supporting the local effect of gene transfer. Further work suggests these effects are long term, lasting from mid-gestation (80 days) through to term (145 days) (190) with reduced intima to media ratio suggesting vessel remodeling, and adventitial angiogenesis demonstrated. Recent work in an FGR sheep model in which uterine blood flow is reduced by 35% in mid-gestation, demonstrates that uterine artery injection of the same dose of Ad. VEGF significantly improved fetal growth in late gestation (191).

#### **4.10. Fetal Structural Malformations**

Fetal structural malformations are another potentially important application of prenatal gene therapy. Although individually rare, collectively up to 1% of all fetuses are affected by a structural malformation that for some are lethal or are associated with significant morbidity. Congenital diaphragmatic hernia (CDH), for example, is a condition where there is a defect of the diaphragm resulting in herniation of some or all of the intra-abdominal organs into the fetal chest. This compresses the fetal lungs preventing adequate growth, which results in poor lung function at birth. With surgical correction of the diaphragmatic defect, many neonates do well. Current management of severe CDH involves fetoscopic placement of an inflatable balloon in the fetal trachea to block outflow of the tracheal fluid, which encourages lung growth (192). There is, however, an underlying lung defect which may contribute to the lung pathology, and gene transfer may play a part in correcting this problem.

Short-term expression of growth factors at a critical stage of lung growth may be useful for this serious condition. In a rat model of CDH, adenovirus-mediated prenatal CFTR expression enhanced saccular density and air space in the lungs (89). After surgical creation of CDH in fetal sheep, non-viral vector expression of keratinocyte growth factor in the trachea lead to increased surfactant protein B synthesis in the lungs suggesting better maturation of the regrowing lung (90).



## 5. Conclusions

Presently, candidate diseases for prenatal gene therapy would be monogenic diseases that are lethal, during the perinatal period or in early childhood, as well as obstetric conditions or structural malformations of the fetus, in which treatment before birth provides a therapeutic advantage when compared with neonatal or adult gene transfer. Clinically, severe phenotypes may be rescued, in particular increasing the likelihood of intact neurological and other key functions at birth. The broader application of therapy during fetal life will require the establishment of suitable screening programmes for particular conditions, and suitable accurate diagnostic procedures, to allow therapy to be applied in time to have a therapeutic effect.

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