The current status and future direction of fetal gene therapy

Review Article

Anna L David¹, Michael Themis², Simon N Waddington², Lisa Gregory², Suzanne MK Buckley², Megha Nivsarkar², Terry Cook³, Donald Peebles¹, Charles H Rodeck¹, Charles Coutelle²

¹Department of Obstetrics and Gynaecology, Royal Free and University College London Medical School, London WC1E 6HX
²Gene Therapy Research Group, Section of Cell and Molecular Biology, Division of Biomedical Sciences, Imperial College School of Medicine, London SW7 2AZ
³Department of Histopathology, Imperial College School of Medicine, London W12 0HS

Correspondence: Dr A.L. David, Room 212, 2nd floor, Department of Obstetrics and Gynaecology, Royal Free and University College Medical School, 86-96 Chenies Mews, London, WC1E 6HX, UK. Telephone: +44-20-7679-6059; Fax: +44-20-7383-7429; e-mail: a.david@ucl.ac.uk

Key words: fetal gene therapy; adenovirus; retrovirus; lentivirus; adeno-associated virus; Sendai virus; liposome

Abbreviations: Cystic fibrosis (CF), Cystic Fibrosis Transmembrane Regulator (CFTR), and ornithine transcarbamylase (OTC), lysosomal storage disorders (LSDs), cerebrospinal fluid (CSF), Duchenne muscular dystrophy (DMD), Spinal muscular atrophy (SMA), survival motor neuron gene 1 (SMN 1), adeno-associated viral (AAV), severe combined immunodeficiency disorders (SCID), recessive adenosine deaminase deficiency (ADA), bone marrow transplantation (BMT), dystrophic form of epidermolysis bullosa (DEB), congenital diaphragmatic hernia (CDH), Intrauterine growth restriction (IUGR)

Received: 18 September 2003; Accepted: 29 October 2003; electronically published: November 2003

Summary

Application of gene therapy in utero has been considered as a strategy for treatment or even prevention of early onset genetic disorders such as cystic fibrosis and Duchenne muscular dystrophy. Prenatal gene transfer may target rapidly expanding stem cell populations that are inaccessible after birth, permit induction of immune tolerance against vector and transgene and allow permanent gene transfer by use of integrating vector systems. Application of this therapy in the fetus must be safe, reliable and cost-effective. Recent developments in the understanding of genetic disease, vector design, and minimally invasive delivery techniques have brought fetal gene therapy closer to clinical practice. Prenatal studies in animal models are being pursued in parallel with adult studies of gene therapy, but they remain presently at the experimental stage.

I. Introduction

Gene therapy uses the intracellular delivery of genetic material for the treatment of disease. A wide range of diseases including cancer, vascular and neurodegenerative disorders and inherited genetic diseases are being considered as targets for this therapy in adults. Application of gene therapy in utero has been considered as a strategy for treatment or even prevention of early onset genetic disorders such as cystic fibrosis and Duchenne muscular dystrophy (Coutelle et al, 1995). Gene transfer to the developing fetus may target rapidly expanding stem cell populations that are inaccessible after birth and may allow permanent gene transfer by use of integrating vector systems. The functionally immature fetal immune system may permit induction of immune tolerance against vector and transgene, and thereby facilitate repeated treatment after birth. Finally, and most importantly for clinicians, fetal gene therapy would give a third choice to parents following prenatal diagnosis of inherited disease, where currently termination of pregnancy or acceptance of an affected child have been the only options. Application of this therapy in the fetus must be safe, reliable and cost-effective. Recent developments in the understanding of genetic disease, vector design, and minimally invasive delivery techniques have brought fetal gene therapy closer to clinical practice. Prenatal studies in animal models are being pursued in parallel with adult studies of gene therapy, but they remain presently at the experimental stage. This review explores the latest developments in the field of in utero gene therapy and their implications for its future clinical application.
Table 1: Examples of candidate diseases for fetal gene therapy

<table>
<thead>
<tr>
<th>Disease</th>
<th>Therapeutic gene product</th>
<th>Target cells/organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic fibrosis (CF)</td>
<td>CF transmembrane regulator</td>
<td>airway and intestinal epithelial cells</td>
</tr>
<tr>
<td>Metabolic disorders:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>Ornithine transcarbamylase</td>
<td>hepatocytes</td>
</tr>
<tr>
<td>Glycogen storage disorders:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pompe disease</td>
<td>α1,4-glucosidase</td>
<td>hepatocytes, myocytes and neurons</td>
</tr>
<tr>
<td>Sphingolipid storage disorders:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tay-Sachs disease</td>
<td>β-N-acetylhexosaminidase</td>
<td>fibroblasts, neurons</td>
</tr>
<tr>
<td>Mucopolysaccharide storage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sly disease</td>
<td>β-glucuronidase</td>
<td>hepatocytes, neurons</td>
</tr>
<tr>
<td>Muscular dystrophies:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duchenne</td>
<td>dystrophin</td>
<td>myocytes</td>
</tr>
<tr>
<td>Neurological disorders:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinal muscular atrophy</td>
<td>survival motor neuron protein</td>
<td>motor neurons</td>
</tr>
<tr>
<td>Haemophilias:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemophilia B</td>
<td>human factor IX clotting factor</td>
<td>hepatocytes</td>
</tr>
<tr>
<td>Haemoglobinopathies:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-thalassemia</td>
<td>β-globin chains of haemoglobin</td>
<td>haematopoietic precursor cells</td>
</tr>
<tr>
<td>Immunodeficiency disorders:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-linked severe combined</td>
<td>γc cytokine receptor</td>
<td>haematopoietic precursor cells</td>
</tr>
<tr>
<td>Skin disorders:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dystrophic epidermolysis bullosa</td>
<td>type VII collagen</td>
<td>keratinocytes</td>
</tr>
<tr>
<td>Non-inherited perinatal diseases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia-ischaemia</td>
<td>neurotrophic factors</td>
<td>cortical neurons</td>
</tr>
<tr>
<td>Infectious diseases:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>herpes DNA</td>
<td>oral mucosa</td>
</tr>
<tr>
<td>Placental disorder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe pre-eclampsia</td>
<td>nitric oxide synthase</td>
<td>trophoblasts</td>
</tr>
</tbody>
</table>

II. The candidate diseases

Fetal 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 (Wilson and Wivel 1999). Some of the diseases that may be suitable for in utero treatment are listed in Table 1 and are discussed as examples for conditions with similar manifestations and/or target tissues.

A. Cystic fibrosis

Cystic fibrosis (CF) appears to be an ideal candidate for treatment with in utero gene therapy. Firstly it is the most common lethal autosomal recessive disorder in Caucasians with an incidence of 1 in 2000 livebirths in Western Europe and North America. Several mutations of the Cystic Fibrosis Transmembrane Regulator (CFTR) gene encoding the CFTR protein have been identified and the resulting disease is characterized by abnormal electrolyte transport in the epithelia of the airways, the ducts of the sweat glands and exocrine pancreas, and the intestine. The main sites of CFTR expression in the non-CF human bronchi are the submucosal glands (Engelhardt et al, 1992). In vitro studies where normal and CF airway cells were mixed, suggest that as few as 6-10% of cells expressing normal CFTR are required to correct the chloride transport defect of an epithelial cell monolayer (Johnson et al, 1992); thus, successful gene therapy may require only relatively low level epithelial airway transduction.

Phase I gene therapy trials directed towards pulmonary disease in CF have shown equivocal results and highlight the problems of present gene therapy approaches in adults (Bigger and Coutelle 2001). The lungs may already be severely damaged or obstructed, even in young adult patients, limiting delivery of gene therapy to the airway epithelium. Fluorocarbon liquids such as perfluor have recently been shown to improve distribution of adenoviral vectors and gene expression in normal and diseased adult lungs (Weiss et al, 1999a, 2001). Pretreatment of airways with detergents (Parsons et al, 1998) or the fatty acid sodium caprate (Gregory et al, 2002) or EGTA (Wang et al, 2000) also improves adenovirus-mediated airways transduction. A comparison of agents to modulate paracellular permeability showed that pretreatment of adult murine airways with sodium caprate had a good safety profile, and enhanced adenovirus-mediated gene transfer to the trachea more efficiently than sodium laurate, another fatty acid sodium salt or EGTA, a calcium chelator (Johnson et al, 2003). Immune responses to the vector, particularly in the case of adenoviral vectors, limit the dose that may be safely administered, and reduce the duration of expression.

The CFTR gene has been proposed to play an important, albeit still unknown, physiological role in normal fetal development (Gaillard et al, 1994; Tizzano et al, 1994). Furthermore the cystic fibrosis disease process appears to begin during development of CF fetuses since by the mid-trimester a pro-inflammatory state exists in fetal CF airways (Hubeau et al, 2001) and there are abnormalities of the pancreas and small bowel (Boué et al,
B. Metabolic disorders

Inherited inborn errors of metabolism can affect a number of metabolic pathways. For example the urea cycle disorders are caused by defects in genes encoding enzymes or membrane transporters in ureagenesis. Their prevalence is approximately 1:30,000 births and ornithine transcarbamylase (OTC) deficiency is one of the most severe of these conditions (Summar and Tuchman, 2001). OTC deficiency is transmitted as a partially dominant X-linked trait. In patients with partial OTC deficiency, such as hemizygous males and heterozygous females, the first clinical episode is delayed for months or years with less severe hyperammonemia. However, patients with complete OTC deficiency present with life-threatening hyperammonemia within one week of birth and despite medical therapy to reduce the ammonia levels, 50% of the children are dead by the age of 4, and of those surviving, the mean IQ is less than 50 (Maestri et al, 1999). Since the urea cycle is principally sited in the liver, gene therapy directed towards hepatocytes has the potential to correct the metabolic abnormality. Indeed the success of orthoptic liver transplantation in long-term treatment of this condition supports the concept (Lee and Goss, 2001).

Adenoviral vectors have been shown to transiently correct OTC deficiency in the sparse fur murine model after neonatal and adult treatment (Stratford-Perricaudet et al, 1990; Ye et al, 1996). In a phase I human clinical trial in patients with partial OTC deficiency, adenoviral vectors expressing the human OTC-cDNA were administered. There was evidence of dose-related toxicity to the adenovirus and the last patient treated suffered a systemic inflammatory response syndrome that lead to his death (Raper et al, 2002).

Because of its early onset, severity and present difficulties in postnatal gene therapy, OTC deficiency is an interesting candidate for in utero gene application targeted to the fetal liver (see chapter IV). Prenatal diagnosis for OTC deficiency by detection of the genetic mutation in fetal DNA is available in families with a known congenital abnormality. In non-informative families, deficiency of OTC enzyme can be detected in the fetal liver after liver biopsy (Holzgreve and Golbus, 1986). Other serious genetic diseases that would primarily require hepatocyte directed gene transfer are amino acid disorders (e.g. phenylketonuria, tyrosinaemia), carbohydrate disorders (e.g. galactosaemia) and fatty acid oxidation disorders (e.g. long-chain acyl-CoA dehydrogenase deficiency) (Preece and Green 2002).

C. Storage disorders

The lysosomal storage disorders (LSDs) are a group of congenital deficiencies of one or more lysosomal enzymes. In mucopolysaccharidosis type VII (MPS type VII) a deficiency of β-glucuronidase activity leads to accumulation of undegraded glycosaminoglycans in lysosomes. Clinically, patients develop hepatosplenomegaly, mental and growth retardation, hearing and vision defects, skeletal deformities and die of cardiac failure. Many of the LSDs present already during fetal life with hydrops fetalis and prenatal diagnosis can be performed by detection of β-glucuronidase deficiency in chorionic villi or fetal blood (Geipel et al, 2002). Although individually rare, as a group they occur in approximately 1 in 7500 live births and are one of the more prevalent groups of inherited diseases in humans (Wraith, 2002). Bone marrow transplantation and enzyme replacement therapy are being developed for many of the mucopolysaccharidoses. However, the short half-life of lysosomal enzymes in the circulation means that patients need biweekly parenteral administration which increases the risk of an immune response to the infused enzyme. In addition, systemically administered enzyme is unable to cross the blood-brain barrier and can therefore not be used to treat central nervous system disease manifestation.

The LSDs are considered to be good candidates for gene therapy and the liver may be the ideal site for gene transfer. Newly synthesized lysosomal enzymes are secreted into the systemic circulation and are recaptured by distant cells. Based on the observed enzyme levels in patients with mild late-onset disease, the amount of enzyme needed to correct the deficiency may only be 1-10% of normal levels (Cheng and Smith, 2003). Gene transfer to naturally occurring animal models of MPS type VII has been investigated using adeno-associated virus (Daly et al, 1999), adenovirus (Kamata et al, 2003) and lentivirus (McCray Jr et al, 2001). Intravenous administration of retroviral vectors containing canine β-glucuronidase to neonatal MPS type VII dogs prevented some bone and joint abnormalities, corneal clouding and heart valve defects that commonly occur in this animal.
model (Ponder et al, 2002). Some aspects of bone disease were not prevented however, which may be due to abnormal bone formation in utero. There was also concern that systemic gene therapy administration may not reach the brain even in neonatal dogs when the blood-brain barrier is still forming. The immature blood-brain and blood –cerebrospinal fluid (CSF) barrier is more permeable to small proteins than in mature brains and there is a developmentally regulated mechanism that selectively transfers some larger proteins from the blood to the CSF (Dziegielewski et al, 2001). Thus a prenatal gene transfer approach may be more effective and also applicable to other disorders that affect the brain, such as the glycosphingolipid lysosomal storage diseases (Gaucher and Tay-Sachs disease) (Jeyakumar et al, 2002).

**D. Muscular dystrophies**

Duchenne muscular dystrophy (DMD) is the commonest form of muscular dystrophy, a group of congenital disorders characterised by muscle wasting and weakness. This X-linked recessive disease has an incidence of 1 in 3500 live male births. Affected boys are usually diagnosed aged 3-4 years and characteristically, skeletal muscle degeneration after repeated rounds of necrosis is followed by the onset of fibrosis that eventually leads to muscle weakness and death (Emery, 1993). Patients are usually confined to a wheelchair by age 11 years, and although improved nursing care and positive pressure ventilation to aid breathing allows some patients to reach the 3rd decade, respiratory or cardiac failure is the common cause of death (Simonds et al, 2000). Prenatal diagnosis is available for almost all muscular dystrophies including Duchenne (Emery, 2002). Current treatment includes supportive measures such as surgery for correction of contractures and prevention of respiratory infections. The disease is caused by mutations in the DMD gene that encodes the 427kDA protein dystrophin, associated with the sarcolemma in muscle. Skeletal and cardiac muscle biopsies from DMD patients are characterized by absent or abnormal dystrophin. Gene transfer into muscle cells has been explored using naturally occurring animal models of muscular dystrophy that involve mutations in the DMD gene (Wells and Wells, 2000). The large size of dystrophin cDNA (14kb) precludes insertion into conventional vectors with the exception of gutless adenovirus. Consequently the majority of viral constructs incorporate mini or microdystrophin cassettes based on a 6.3kb truncated dystrophin gene resulting from a large intrame deletion in the rod domain which was isolated from a Becker muscular dystrophy patient with very mild symptoms. Adenoviral transfer of minidystrophin results in good transduction of neonatal mdx mouse muscle with reduced degeneration and improved muscle mechanics (Deconinck et al, 1996; Vincent et al, 1993). In the neonatal and adult mdx mouse, injection of an adeno-associated virus containing a minidystrophin into the leg muscle led to normal myofiber histology and protected membrane integrity (Wang B et al, 2000). The early onset of this disease, which begins to be visible histologically by the 18th-20th week of gestation (Vassilopoulos and Emery, 1977; Turkel et al, 1981) and presents clinically between 2-4 years of age, complicates postnatal gene therapy. Thus a prenatal approach to treatment might prevent the disease process.

Prenatal gene transfer may offer advantages over neonatal or adult treatment. Efficient gene delivery to several affected muscles groups is technically difficult and the alternative may be efficient gene transfer to a large percentage of existing and rapidly expanding muscle cells in utero. Postnatal gene delivery is also complicated by the risk of cellular immune responses against the transgenic proteins as demonstrated in the dystrophin-deficient mdx mouse model by loss of transgenic dystrophin-expressing fibres following dystrophin gene transfer (Wells and Wells, 2000; Chamberlain 2002). In contrast in utero gene transfer may avoid the development of immune reactions to the vector or transgene product and enable repeat injection postnataally. Furthermore immune responses have been reported in several adenovirus-mediated gene transfer studies although it was not possible to determine the relative contribution of the immune response to the vector or transgene. In most DMD patients, there is a lack of dystrophin expression which could lead to a functional copy of the dystrophin protein being recognised as a foreign antigen. Gene transfer during fetal life could lead to immunological tolerance to the dystrophin or allow repeated injection post-natally. Similar conditions such as the congenital Emery-Dreifuss and Fukuyama muscular dystrophies (Emery, 2002) could also potentially be treated using a prenatal gene transfer approach.

**E. Neurological disorders**

Spinal muscular atrophy (SMA) is one of the most common inherited causes of childhood mortality, with an incidence of 1 in 10,000 live births. It is characterized by progressive degeneration of alpha motor neurons within the spinal cord and results in proximal, asymmetrical limb and trunk muscle paralysis that leads to death (Crawford and Pardo, 1996). SMA is caused by homozygous loss or mutation in the survival motor neuron gene 1 (SMN 1) which is telomeric. Humans and primates also have a centromeric copy called the SMN 2 gene but this fails to provide sufficient full-length SMN protein to maintain motor neurons. Evidence from family studies and animal models of SMA suggest that the number of copies of the SMN 2 gene may modify the severity of the disease. Gene therapy strategy would have to provide and express a functional copy of the SMN gene in the relevant neuronal cells. Efficient expression of the SMN gene was demonstrated recently after adenovirus-mediated delivery of the SMN gene to human primary fibroblasts from SMA patients in vitro (DiDonato et al, 2003). Intraspinal or intramuscular application of a vector targeting neuronal cells will be required for in vivo therapy and other diseases requiring this targeting include amyotrophic lateral sclerosis.

Immunohistochemical analysis of normal fetal tissue has demonstrated that the expression of SMN protein is relatively high in skeletal muscle, heart and brain and...
undergoes a marked drop in the postnatal period. In contrast, SMN protein is greatly reduced in all tissues from fetuses affected with SMA (Burlet et al, 1998). These observations suggest that SMN protein may be required during embryo-fetal development and as such, prenatal gene transfer may be more effective than adult treatment. Prenatal diagnosis is available using deletion analysis of the SMN 1 gene (Matthjis et al, 1998).

F. Haemophilias
The haemophilias A and B are also particularly suitable for gene therapy in utero. Both are X-linked hereditary haemorrhagic disorders which occur in 1 in 10,000 and 1 in 25,000 males respectively and are caused by the absence or dysfunction of the respective human factor VIII (hFVIII) or IX (hFIX) clotting factors (Furie et al, 1994). Current treatment uses replacement therapy with hFVIII or hFIX. Unfortunately, a number of patients develop antibodies to therapy leading to ineffective treatment and occasional anaphylaxis (Lusher, 2000). Indeed, the complications of haemophilia have treat in some cases been far worse than the diseases themselves, increasing their morbidity and mortality (Soucie et al, 2000).

As the coagulation factors are required in the blood and can be secreted functionally from a variety of tissues, the actual site of production is not so important as long as therapeutic plasma levels are realized. Adult gene therapy strategies have therefore concentrated on application to the muscle or the liver. Successful delivery and expression of FIX has been achieved in adult animal models of haemophilia B following portal intravascular administration of adenviral (Kay et al, 1994) and retroviral vectors (Kay et al, 1993). Sustained FIX expression was also observed after intramuscular injection of adult haemophilic dogs with adeno-associated viral (AAV) vectors expressing canine FIX (Chao et al, 1999; Herzog et al, 1999) and after intravenous injection of adult haemophilic mice with AAV vectors expressing hFIX (Snyder et al, 1999). These results have culminated in the first clinical trial in humans that shows promising results although only low level hFIX expression has so far been observed (Kay et al, 2000). Successful delivery and expression of therapeutic hFIX without formation of antibodies has been achieved following administration of retroviral vectors in neonatal animal models (Xu et al, 2003). Prenatal gene therapy could be applied to the fetus via a number of routes including muscle, peritoneal, hepatic, intravascular or skin application. More recently our group has demonstrated that in utero application can provide long-term postnatal correction of the haemophilic phenotype in FIX deficient mice (Waddington et al, submitted). Prenatal diagnosis is available early in pregnancy (Ljung, 1999).

G. Haematopoietic diseases
1. The thalassaemias
The thalassaemias are inherited anaemias caused by over 200 mutations and globally are the commonest monogenic disorders. They are most prevalent in the Mediterranean region, the Middle East, the Indian subcontinent and South-East Asia where gene frequencies reach 3-10% of the population (Weatherall and Clegg, 1996). β-thalassaemia is characterized by insufficient production of the β-globin peptide by erythroid cells which results in low levels of the major form of adult haemoglobin, HbA, made up of two α- and two β-globin chains. The excess α-globin chains then precipitate in the erythroid cells, impair their maturation and this leads to haemolysis and anaemia. Homozygotes or compound heterozygotes suffer with the most severe form of the disease, β-thalassaemia major. Similarly α-thalassaemia results in excess β-globin chains due to different degrees of α-globin chain deficiency. In the most severe form, αβ-thalassaemia, all four α-globin chains are defective or absent which leads to hydrops fetalis and intrauterine death. Patients with thalassaemia require regular lifelong blood transfusions to survive although this leads to iron overload that affects the liver, heart and endocrine organs. Prevention of iron overload with iron-chelating therapy such as parenteral deferoxamine is the mainstay of current patient management. Therapies aimed to increase the production of fetal haemoglobin have had disappointing results (Olivieri and Weatherall, 1998). Allogeneic haematopoietic stem cell replacement offers the only definitive cure and has been successful in over 1000 patients worldwide (Olivieri, 1999). Outcomes depend on whether the patient has hepatomegaly, portal fibrosis and has effective chelating therapy before transplantation. The 3 year disease-free survival falls from over 90% to 60% in children with the above risk factors.

Gene therapy approaches have aimed to stably introduce a regulated human globin gene into haematopoietic stem cells. Recently high expression of erythropoietin was found to improve the anaemia of β-thalassaemia in a mouse model by induction of high levels of HbF synthesis (Johnston et al, 2003). Expression of transgenic globin sequences would need to be sustained, finely regulated and at high levels since haemoglobin synthesis represents 95% of all protein synthesis in reticulocytes. Initial attempts at gene therapy using the β-globin gene and a minimal locus control region (LCR) incorporated into a retroviral vector showed low levels and short-term expression of β-globin after transplantation of transduced haematopoietic stem cells into lethally irradiated mice (Rafitopoulos et al, 1997; Sadelain 2002). More recently lentiviral vectors containing the β-globin gene and larger LCR elements have been used to transfect bone marrow from β-thalassaemic mice. This was then transplanted into β'-thalassaemic heterozygote mice and resulted in therapeutically relevant levels of circulating haemoglobin (May et al, 2000). An advantage of prenatal gene therapy application in this context could be the access to rapidly dividing stem cell populations. Prenatal diagnosis for haemoglobinopathies can be done by assessment of globin-chain synthesis in fetal blood or by direct analysis of fetal DNA obtained by chorionic-villus sampling or amniocentesis.

Sickle cell disease, another inherited disorder of haemoglobin may also be amenable to prenatal gene therapy. In this condition missense mutations in the β-
globe, the earlier trials did not use conditioning of the transduced with a retroviral vector containing ADA
CD34
trials have used infusion of autologous peripheral T-cells, treatment of other genetic diseases. In ADA-SCID, clinical
mount an effective immune response to the transgene non-corrected cells. In addition, patients are unable to
precursors should have a selective survival advantage over the concept that genetically corrected autologous T-cell
abnormalities and prevent life-threatening opportunistic infections leading to death within the first year of life (Cavazzana-Calvo et al, 2001).

Histocompatible bone marrow transplantation (BMT) has been used to treat both conditions with some success. Survival after transplantation with HLA-identical bone marrow is over 90% but matched sibling donors are usually not available. Haploidentical BMT with T-cell depletion is commonly performed instead, with survival rates of up to 78% although many patients require lifelong immunoglobulin replacement therapy because of inadequate humoral activity (Buckley RH et al, 1999). In utero haematopoietic stem cell transplantation has been achieved in fetuses with XI-SCID by ultrasound guided intraperitoneal or intravenous injection (Flake et al, 1996; Touraine 1992; Wengler et al, 1996; Westgren et al, 2002). A selective T-cell and natural killer cell reconstitution can be achieved but B cell engraftment has not been detected. In ADA deficiency, a long-circulating form of bovine ADA conjugated with polyethylene glycol (PEG-ADA) has been used to correct the metabolic abnormalities and prevent life-threatening opportunistic infections.

The strategy for gene therapy of SCID is based on the concept that genetically corrected autologous T-cell precursors should have a selective survival advantage over non-corrected cells. In addition, patients are unable to mount an effective immune response to the transgene which has proved to be a major problem in gene therapy treatment of other genetic diseases. In ADA-SCID, clinical trials have used infusion of autologous peripheral T-cells, CD34+ bone marrow or umbilical cord blood cells transduced with a retroviral vector containing ADA cDNA. The earlier trials did not use conditioning of the bone marrow and PEG-ADA treatment was continued in all patients during and after treatment which made it difficult to evaluate immune function (Blaese et al, 1995; Bordignon et al, 1995; Kohn et al, 1995). Some patients showed long term persistence of the transduced cells although at low level. A more recent trial was performed in two infants with nonmyeloablative conditioning using busulfan and without concurrent PEG-ADA treatment. Both patients showed sustained engraftment of genetically corrected haematopoietic stem cells with differentiation into multiple lineages and improvement in their clinical condition (Aiuti et al, 2002).

In a similar way XI-SCID has been treated using autologous transplantation of CD34+ bone marrow transduced ex vivo with retroviral vectors containing the γc gene. Fifteen patients have now been treated and effective immune reconstitution has been achieved in thirteen patients (Friedmann, 2003). Unfortunately because of a serious adverse event in two of the patients, all gene therapy trials involving retroviral vectors in haematopoietic stem cells were initially halted in the US (Gansbacher and European Society of Gene Therapy 2003) (see VI Ethical and safety issues) and have now been restricted to case by case reviewed permission (Friedmann, 2003). Nevertheless this study has shown the ability of gene therapy to cure such conditions. Because of the survival advantage of genetically corrected cells and the ineffective immune response in SCID patients, it is unlikely that prenatal gene transfer would provide a particular benefit over postnatal treatment of this condition.

H. Skin disorders
Fetal gene delivery into the amniotic cavity may have unique benefits for treatment of inherited skin disorders. Epidermolysis bullosa is a group of inherited blistering diseases characterized by epidermal-dermal separation resulting from mutations that affect the function of critical components of the basement membrane zone. The dystrophic form of epidermolysis bullosa (DEB) is due to mutations in COL7A1, the gene encoding type VII collagen and has a prevalence of up to 2.4 per 100,000 population (Horn and Tidman, 2002). The clinical presentation varies from a mild dominantly inherited disease characterized by skin and oral blisters and nail dystrophy to a severe recessive subtype in which patients suffer from contractures, severe dental caries, dysphagia, anal fissures and squamous cell carcinoma. Current therapy involves management of the disease manifestations with proper wound care, surgical release of skin contractures, balloon dilatation of oesophageal strictures and graft skin therapy (Pai and Marinkovich, 2002).

Easy accessibility and visualization of skin make it an attractive target for gene therapy. Gene delivery can be in vivo by direct introduction to the skin by injection, electroporation or a ‘gene gun’. Alternatively a skin sample could be removed from the patient, and epidermal keratinocytes cultured and transduced ex vivo to insert genetic material and the genetically engineered cells
used to treat the underlying cause of the pulmonary bowel herniates through the diaphragmatic defect. Fetal effusion associated with congenital cardiac defects and hypoplasia. Examples of such conditions include pleural within the chest cavity also result in pulmonary 2003). Space occupying lesions that compress the lungs some success but has a high complication rate (Tan et al, 2003). Non-viral gene transfer approach has been used for junctional epidermolysis bullosa (JEB) in which there is severe laminin-5 deficiency. Integration of an attB-containing laminin 5 B3 expression plasmid using φC31 integrase into human keratinocytes from JEB patients produced skin tissue with no histological evidence of subepidermal blistering when regenerated on SCID mice (Ortiz-Urda et al, 2003).

Epidermolysis bullosa however, is a generalized disorder affecting the entire skin and the extracutaneous tissues. Prenatal therapy delivered into the amniotic fluid would bathe the entire skin surface and reach the gastrointestinal system by fetal swallowing. Injection into the amniotic cavity can be performed safely at relatively early gestation, but the timing of intra-amniotic delivery will be important from developmental considerations. Even at 20 weeks gestation, the fetal epidermis is incompletely keratinized and this would aid gene transfer. However there is a high rate of apoptosis in fetal keratinocytes and therefore the ideal strategy would be to target stem cells (Haake and Cooklis, 1997). Prenatal diagnosis for epidermolysis bullosa can now be performed with a 98% success rate in at risk families, paving the way for preliminary studies into prenatal treatment (Pfendner et al, 2003). Disorders of defective keratinisation such as harlequin ichthyosis, an autosomal recessive severe and usually fatal congenital ichthyosis (Akiyama, 1998), may also be amenable to prenatal gene transfer.

I. Perinatal disease

Pulmonary hypoplasia is another important cause of neonatal morbidity and mortality. In this condition, the fetal lungs fail to develop resulting in respiratory insufficiency at birth. Current neonatal management is supportive and involves surfactant replacement, careful mechanical ventilation avoiding barotrauma and treatment of pulmonary hypertension. Pulmonary hypoplasia can occur when there is reduced or no liquor surrounding the fetus (oligo or anhydramnios) prior to 22 weeks gestation, most commonly because of preterm premature rupture of the membranes (PPROM). Serial amniinfusion has been used for the prevention of pulmonary hypoplasia with some success but has a high complication rate (Tan et al, 2003). Space occupying lesions that compress the lungs within the chest cavity also result in pulmonary hypoplasia. Examples of such conditions include pleural effusion associated with congenital cardiac defects and congenital diaphragmatic hernia (CDH) in which the bowel herniates through the diaphragmatic defect. Fetal interventions such as drainage of pleural effusions can be used to treat the underlying cause of the pulmonary hypoplasia. Temporary occlusion of the trachea with an expandable balloon for treatment of CDH results in impressive expansion of the hypoplastic lung with tracheal fluid. However ‘plugging’ has yet to be shown to improve outcome in the long term (Harrison et al, 1998). Studies suggest that pulmonary hypoplasia in CDH begins during embryogenesis as an abnormality in growth factor signalling and actually precedes the development of the anatomical defect (Jesudason 2002). Prenatal gene therapy could be envisaged in the future to enhance antenatal lung growth and maturation by the targeted delivery of growth factors at specific times during lung development.

J. Infectious disease

Infectious diseases with pathogens such as Group B streptococcus, human immunodeficiency virus, hepatitis B virus and herpes simplex virus are a major cause of neonatal morbidity and mortality. Transmission of these diseases from mother to infant often occurs shortly before, during, or after birth by early rupture of the amniotic membranes or direct contact with infectious secretions during labor and delivery. Delivery by caesarean section to prevent such contact, and antibiotic and maternal antiviral treatments have been used with some success, particularly in the prevention of vertical HIV transmission. Immunisation of the fetus with DNA vaccines in late pregnancy has been proposed as an alternative approach to prevent neonatal infection (Gerds et al, 2000; Sarzotti et al, 1996; Watts et al, 1999). The mucosal surfaces of the eyes, respiratory and gastrointestinal tract are the primary site of entry for infectious agents during birth and the neonatal period. Thus intra-amniotic or intra-oral delivery of antigen would probably provide the best disease protection. Studies in the fetal mouse (Sarzotti et al, 1996), sheep (Gerds, et al, 2000) and baboon (Watts et al, 1999) have shown that fetal immunisation can induce active immunity in the newborn. In particular, in the fetal sheep, intra-oral administration of hepatitis B surface antigen DNA resulted in a higher protective antibody titre than an intramuscular injection of the recombinant protein vaccine (Gerds, et al, 2003). The timing of such an intervention is crucial since exposure of the fetus to the antigen before immune competence is reached may result in tolerance. In addition a single in utero injection may not be sufficient to maintain immunity. At present there is no clinical indication for such a prenatal immunization strategy.

K. Placental disorders

Pre-eclampsia/eclampsia is one of the leading causes of maternal and fetal morbidity and mortality. The underlying defect is believed to be inadequate deep placentation that fails to transform the spiral arteries into uteroplacental vessels and thus limits placental blood flow (Brosens et al, 2002). Secondary damage such as fibrin deposition and thrombosis then limit placental perfusion further and there is also widespread activation of the maternal vascular endothelium leading to decreased formation of vasodilators such as nitric oxide (Walker, 2000). Gene therapy could be used to improve uteroplacental perfusion by for example, temporary expression of nitric oxide synthase or placental growth factor. This
could prolong the pregnancy until fetal maturity was attained and reduce the likelihood of long-term complications in the mother and fetus.

Intrauterine growth restriction (IUGR) affects up to 8% of all pregnancies. It commonly occurs in pregnancies complicated by pre-eclampsia but can also arise in normotensive pregnancy. As well as leading to neonatal problems, the long-term consequences are serious since IUGR infants exhibit higher rates of coronary heart disease, type 2-diabetes, hypertension and stroke as adults (Barker et al, 1993). Abnormalities in placental development are believed to adversely affect placental function and deprive the fetus of the nutrients required for optimal growth. Transport of amino acids and essential fatty acids across the placenta is altered in IUGR fetuses and impaired oxygenation and acid base balance may be seen in severe cases (Pardi et al, 2002). Prenatal gene therapy could target placental transport mechanisms and increase the availability of essential nutrients to the fetus.

III. Vectors for in utero gene delivery

The development of efficient vector systems is crucial for the success of gene therapy. The ideal vector for fetal somatic gene therapy would introduce a transcriptionally regulated therapeutic gene into all organs relevant to the genetic disorder by a single safe application. Although none of the present vector systems meet all these criteria, many of them have characteristics that may be beneficial to the fetal approach.

A. Non-viral vectors

Cationic liposome/DNA complexes have the advantage of being relatively non-toxic and non-immunogenic but are still very inefficient in vivo. Another drawback with these vehicles is that the DNA introduced as plasmid molecules remains episomal and will be lost over time following cell division. This is a particular disadvantage in the fetus where cell populations are rapidly dividing. However, short term transgene expression has been shown to be a promising approach to maintain a patent ductus arteriosus prior to surgery for congenital heart defects in neonates (Mason et al, 1999). Liposomes containing plasmid expressing a decoy RNA designed to sequester fibronectin mRNA binding protein were delivered to the ductus arteriosus in fetal sheep at 90 days of gestation, prior to the onset of intimal cushion formation at 100 days of gestation. Fibronectin synthesis was inhibited resulting in a 60% reduction in intimal thickness and increased ductal patency at term.

More recently, non-viral systems have been developed that integrate into the host genome and could thus in principle provide long term gene expression, but these vectors are still at an early stage of experimental design (Olivares et al, 2002).

B. Viral vectors

Studies of in utero gene therapy have therefore concentrated on viral vectors, many of which have been designed to deliver reporter genes such as the β-galactosidase gene (lacZ). These allow tracking of the transduced cells and to define tissue expression by biochemical staining assays. Alternatively, use of vectors carrying therapeutic genes allows the assessment of potentially curative levels of the expressed protein and, in animal models of disease, even the observation of phenotype correction. The hFIX gene for instance, can be used both as a marker gene, allowing the analysis of blood levels of the hFIX protein over time in non-haemophilic animals, and to study the correction of the blood clotting parameters in animal models of haemophilia. Postnatal readministration of hFIX protein or the hFIX vector to fetally treated animals can be used to examine whether immune tolerance has been achieved.

1. Retrovirus

Vectors that are able to integrate into the host genome such as retroviruses, lentiviruses and to a lesser extent adeno-associated viruses, may offer the possibility of permanent gene delivery. Although only fairly low virus titres can be produced, virus gene transfer may be improved by complexing vectors with cationic agents, (Themis et al, 1998) or by the administration of retrovirus producer cells in vivo to allow localised gene delivery close to the site of cell transfer (Douar et al, 1997; Russel et al, 1995).

Retroviruses require dividing cells for gene transfer (Miller DG et al, 1990) which suggests that they may be better suited for use in fetal tissues where cells are rapidly dividing rather than in adult applications. Other problems include reports of premature promoter shutdown (Palmer et al, 1991; Challita and Kohn 1994) leading to transcriptional shutoff. Human serum can almost completely inactivate some retroviral particles (Welsh et al, 1975) which limits their use in vivo although increased resistance to serum inactivation can be achieved by generating retroviruses from particular human packaging cells (Cosset et al, 1995) or by pseudotyping, which replaces the natural envelope of the retrovirus with a heterologous envelope (Engelstadter et al, 2001). A particular problem with in utero application is that amniotic fluid has also been shown in vitro to have a mild inhibitory effect on retrovirus infection (Douar et al, 1996). A further difficulty is the relatively short half-life of the retroviral particles in vivo which may hinder transduction because fetal cell division is non-synchronized and only those cells undergoing cell division at the time of infection will become transduced.

Retroviruses were used in the first successful gene therapy trial, where bone marrow stem cells transduced ex vivo with retroviral vectors expressing the correct cDNA were delivered to infants suffering from an X-linked form of severe combined immunodeficiency (SCID) (Cavazzana-Calvo et al, 2000). The infants were able to leave protective isolation, discontinue treatment and appear to be developing normally (Hacein-Bey-Abina et al, 2002). However two of the fifteen patients treated for X-linked SCID have developed leukemia which has been shown to involve insertional mutagenesis. An expanded clonal population of T-cells was demonstrated to be
carrying the transgene inserted at 11p13 in the region of LMO2, an oncogene frequently overexpressed in T cell leukemias (Marshall 2002). Insertional mutagenesis is an acknowledged potential complication with retroviral mediated gene transfer because gene integration occurs randomly into the genome. This is the first report of malignant change in humans following retroviral gene therapy and only one example has been found in extensive animal studies using this vector (Li et al, 2002). Investigations are ongoing to determine whether any other factor contributed to the development of insertional mutagenesis and clonal expansion in these particular patients (Friedmann 2003).

2. Lentivirus

Because of the limitation of infection to dividing cells by retroviruses, alternative vectors such as lentiviruses have been developed to circumvent this restriction. Significant progress has been made in recent years in the development of lentiviral vectors, a retroviral sub-group based on the Human Immunodeficiency Virus (HIV) (Trono, 2000) or Equine Infectious Anaemia Virus (EIAV) (Mitrophanous et al, 1999). HIV vectors are capable of transferring genes into nondividing cells such as neurons (Naldini et al, 1996) and quiescent haematopoietic progenitor cells, (Case et al, 1999) which will be particularly useful for these tissue targets. Lentiviral vectors integrate into the genome randomly and are therefore theoretically able to cause insertional mutagenesis.

Lentiviruses can be made more stable by pseudotyping which allows virus titres to be improved by ultracentrifugation. This offers the opportunity of infecting a greater number of cells in vivo and different envelopes allow targeted gene transfer to specific tissues, for example to the nervous system (Mazarakis et al, 2001) and airways (Kobinger et al, 2001). Both the EIAV vector, a vector derived from non-primate animal lentiviruses, (Mitrophanous et al, 1999) and Feline Immunodeficiency Virus (FIV) (Wang, et al, 1999) have been developed in an attempt to create vectors for use in human treatment which are not associated with any human pathology. Our recent work has shown that high level sustained transgene expression can be achieved in a variety of tissues using the EIAV vector in fetal mice after intravascular administration (Figure 1) (Waddington et al, 2003).

3. Adeno-associated viral vectors

Adeno-associated virus (AAV) is also a promising novel vector system. It is a common human parvovirus that is not associated with any human pathology. AAV naturally requires co-infection with adenovirus as a helper virus, but the latest AAV vectors circumvent the need for adenovirus and therefore make the production of pure AAV particles easier (Xiao et al, 1998). AAV is also able to infect non-dividing cells and to achieve long-lasting gene correction in vitro and in vivo (Herzog et al, 1999; Wang et al, 1999; Kay et al, 2000). The basis for long-term transgene expression is not quite clear. Integration of the wild type virus is predominantly at an apparently specific functionally unimportant location on human chromosome 19 reducing the theoretical risk of insertional mutagenesis; however recombinant vector appears to integrate at low levels and non-specifically (Monahan and Samulski, 2000). AAV vectors have a limited capacity for the insertion of foreign genes that is about 4.7kb, although recently 'split AAV vectors' have been designed where large genes are split between two AAV genomes to increase AAV packaging capacity. After concatemerisation of these genomes in the host cell mRNA, splicing allows the removal of intervening ITR sequences and restoration of the split coding sequence to yield wild-type functional protein (Sun et al, 2000). Because the extent of AAV integration is still in question, this vector system may not give the permanent gene expression ideal for in utero gene therapy without repeat treatment, although long term transgene expression after intraperitoneal delivery in mice has recently been reported (Lipshutz et al, 2003). Some caution has also been expressed as AAV integration appears to induce chromosome deletions (Nakai et al, 2003).

4. Adenovirus

Adenoviral vectors have been used as attractive vectors for proof of principle studies in fetal gene therapy since they have continually achieved highly efficient gene transfer in vivo. The adenoviral coding sequences necessary for viral replication are deleted, rendering them replication defective. They are relatively stable and can be obtained at high titre making systemic administration in humans and large animal models feasible. The adenovirus genome replicates outside the chromosome, which avoids the risk of insertional mutagenesis but results in only transient gene expression. Their broad host range and tropism to most cells of the human body, including the respiratory epithelium, has made them very useful in initial pathfinder studies on vector delivery and transgene expression. They are particularly useful for exploring different technical approaches to fetal gene therapy.

Factors that determine the kinetics of transgene expression include vector elimination, since adenovirus is not an integrating vector, and promoter shutdown. Adenoviral vectors are also highly immunogenic. Major concerns about the safety of adenoviral vectors were raised following the death of Jesse Gelsinger from a systemic inflammatory response to a first generation adenovirus vector used for a phase I clinical trial towards gene therapy of the inherited metabolic disorder, ornithine transcarbamylase deficiency (Lehrman, 1999). Even fetal administration of adenoviral vectors has been associated with an immune response (McCray, et al, 1995) particularly after postnatal repeat exposure to the vector (Iwamoto et al, 1999). Attempts to reduce the immunogenicity and toxicity of the vector and to increase its insert capacity have led to the generation of the so called 'gutless vectors’ in which essentially all adenoviral coding sequences have been eliminated (Chen et al, 1997; Schiedner, et al, 1998).
Figure 1. Upper panel. Representative sections of fetal livers harvested at 72h, 7, 14, 28, 79, 168 days and 1 year after yolk sac injection of high titre titre EAIV SMARTZ (equine infectious anaemia virus vector expressing the β-galactosidase gene driven by the CMV promoter) lentiviral vector (n=1, 1, 3, 1 and 1, respectively). Uniform hepatocyte staining is observed after 72 h followed by the emergence of clusters of β-galactosidase-stained hepatocytes to day 79. Macroscopic appearance of liver sections (top row, x 10). Microscopic analyses (bottom row, x 400). Age matched noninfected control livers of 3 day old and 1-year-old animals are shown in the lower panel. Lower panel. Representative sections of fetal tissues harvested at 72 h, 7, 14, 79 days and 1 year after yolk sac injection of high titre EAIV SMARTZ lentiviral vector (n=1, 1, 3 and 1, respectively). High-level staining is observed after 72 h and 79 days in brain, 7, 14 and 79 days in heart and 14 and 79 days in skeletal muscle. Low-level expression is shown in lung and kidney at 79 days postinjection. Macroscopic appearance of tissues (left columns, x 10). Microscopic analysis (right column, x 400). (Waddington et al 2003). Republished with permission from Nature Publishing Group.
Because adenoviruses provide highly efficient gene transfer yet transient expression, novel hybrid vectors have been developed to take advantage of adenovirus infectivity and the permanent nature of integrative vectors such as retroviruses and lentiviruses (Murphy et al., 2002; Kubo and Mitani, 2003). Hybrid vectors may offer efficient gene expression to fetal organs such as the lung in which it has so far proved difficult to achieve high level gene transfer with integrating vectors.

5. Sendai virus

Recently, the negative strand RNA cytoplasmically replicating Sendai virus, a member of the paramyxovirus family was developed as a gene transfer vector. Early vectors still capable of self-propagation, were found to provide very high levels of marker gene expression in a wide range of tissues including bronchial epithelium (Yonemitsu et al., 2000), skeletal muscle (Shiotani et al., 2001) and vascular endothelium (Masaki et al., 2001). Second generation vectors, although still capable of intracytoplasmic replication of the RNA genome, are incapable of intercellular propagation. In these vectors, genes encoding surface glycoproteins including the haemaglutinin-neuraminidase (HN) protein or the fusion (F) protein, which are responsible for cell binding and infection, have been deleted from the viral genome (Inoue et al., 2003). Injection of F-deficient Sendai virus vector into the fetal mouse via various routes including intravascular, intra-amniotic, intra-muscular, intra-peritoneal and intra-spinal resulted in expression of marker gene in gut wall, lung, muscle, peritoneal mesothelia and dorsal route ganglia respectively. Further optimisation will be needed to develop these first generation constructs into clinically applicable vectors (Waddington et al., submitted).

IV. Fetal gene therapy studies

Since the initial attempts in the early 1990s, in utero gene therapy has been investigated in a range of different animals using a variety of techniques. The possible routes of administration are illustrated in Figure 2.
A. Animal models

Small animals are the most commonly used because they offer a number of advantages. Transgenic mouse models exist for many genetic diseases such as cystic fibrosis and haemophilia and this allows the therapeutic effect of the gene therapy to be studied. Small animals are also cheaper to maintain and have short breeding cycles with large litters which permit studies over several generations e.g. on germline transmission. However, their size precludes their use for the development of minimally invasive techniques for gene therapy delivery as required in human application.

Studies in large animals have mainly used sheep, since they are well established as an animal model relevant to human fetal physiology, have a good tolerance to in utero manipulations and a consistent gestation period of 145 days, which is approximately half that of the human. There are some differences between ovine and human biology (Newnham and Kelly 1993). In late gestation the fetal growth rate in sheep is over double that in humans (Fowden, 1995) and the placental weight declines from 90 days gestation while it remains static in the human (Barcroft and Barron 1946). However the major difference is in the structure of the placenta. In sheep the synepitheliochorial placenta consists of six tissue layers, three from the mother and three from the fetus, and it is the most complete barrier possible (Benirschke and Kaufmann 1990). The maternofetal interdigitations (placentomes) are spread throughout the uterine cavity and may be difficult to avoid during ultrasound-guided uterine interventions. In humans, there is only a single discoid placenta and there is extensive invasion of the endometrium by the trophoblast that removes the three maternal tissue barriers and results in a hemomonochorial placenta at term. Probably as a result of these structural differences, γ-globulin does not pass from the mother to the fetus in the sheep, but is able to cross the placenta in humans.

Nonhuman primates are close physiologically to humans with menstrual cycles of similar length and hormonal control, comparable cellular and endocrine processes of implantation, and similar timetables of prenatal development. The placental structure in some nonhuman primates is also the same, for example in the rhesus monkey the placenta is hemomonochorial and bidiscoidal (Benirschke and Kaufmann 1990). For this reason they are used as an animal model in studies of teratology, developmental biology, infertility and contraception (Hendrickx and Peterson 1997). Ultrasound guided injection techniques as used in fetal medicine have also been applied extensively in the fetal nonhuman primate with comparable results (Tarantal, 1990). However nonhuman primates are more costly than sheep and are difficult to maintain.

The rabbit has been studied in some prenatal gene therapy studies. Minimally-invasive percutaneous ultrasound guided injection and fetoscopic procedures are also being developed (Brandt et al, 1997; Papadopulos et al, 1999). Because of the small size of the fetus and litter number however, technically this is only possible from late gestation. The guinea pig has the same placential structure as humans but they are not commonly used in prenatal gene therapy studies because of the small fetal size and lack of transgenic models of disease.

There are unfortunately few large animal models of human genetic disease available for testing of gene therapy. Efforts to produce transgenic domestic animals are continuing particularly in the pig, sheep and cow (Piedrahita 2000). There are however, some dog models including mucopolysaccharidosis type VII, Duchenne muscular dystrophy and haemophilia B, which are useful for investigating the therapeutic effect of gene therapy. The dog is also a suitable model for minimally invasive delivery techniques and studies on prenatal gene transfer have used ultrasound guided intraperitoneal or yolk sac injection through the exposed uterus (Lutzko et al, 1999; Meertens et al, 2002).

B. Application routes in fetal medicine

Invasive surgical techniques such as maternal laparotomy or hysterotomy must be performed to access the fetus in small animal models, but have also been applied in large animal studies such as in the sheep (Tran et al, 2000; Vincent et al, 1995). Surgery carries a high morbidity from wound infection and haemorrhage and the risk of mortality is significant.

Minimally invasive procedures with fibreoptic telescopes are currently in use in fetal medicine and are being adapted for application of gene therapy in large animal fetuses. Fetoscopy was developed in the late 1970s for examination of 2nd trimester fetuses and for fetal blood sampling (Rodeck, 1980). The morbidity from fetoscopy is significant however, because of the relatively larger diameter of the puncture site in the fetal membranes which leads to premature rupture of the membranes and preterm labour and its associated problems. With the improvement in ultrasound technology in the 1990s, more detailed anatomical survey of the fetus could be performed and fetal blood sampling by ultrasound guided injection became routine practice. Operative fetoscopy has recently re-emerged for use together with ultrasound in endoscopic fetal surgery for conditions such as twin reversed-arterial-perfusion sequence (Quintero et al, 1994), severe feto-fetal transfusion syndrome (Ville et al, 1997) and congenital diaphragmatic hernia (Harrison et al, 1998).

Percutaneous ultrasound-guided injection is the least invasive technique for accessing the fetus and is used frequently in the clinical setting. Coelocentesis uses ultrasound to guide a needle into the extraembryonic coelom in the early first trimester. It has a success rate of >95% at 6-11 weeks of gestation, and has been suggested as a possible technique for stem cell engraftment in early gestation (Wilson and Wivel 1999). It may be of little use, however for in utero gene therapy because of the limited transfer from the extraembryonic coelom via the amniotic membrane to the amniotic cavity (Jauniaux and Gulbis 2000). Studies on the risk of miscarriage in ongoing pregnancies beyond the 1st trimester following coelocentesis gave controversial results (Makrydimas et al, 1997; Ross et al, 1997; Santolaya-Forgas et al, 1998).
Amniocentesis is mainly used clinically for prenatal diagnosis. Although it is one of the safest intrauterine procedures, intra-amniotic application of vectors may be only of limited use in fetal gene therapy because of vector dilution by the large volume of amniotic fluid, although it would be the ideal application route for in utero gene therapy of skin diseases.

Accessing the systemic circulation has greater potential. In fetal medicine, fetal blood can be obtained in the second trimester under ultrasound guidance either from the placental cord insertion, the fetal heart or more safely from the intrahepatic umbilical vein (Chinnaiya et al., 1998). The procedure has a good success rate clinically, is low risk and is used commonly for rapid karyotyping or fetal blood transfusion (Nicolini et al., 1990). From 12 weeks of gestation ultrasound-guided intracardiac puncture for fetal blood sampling has been performed on patients undergoing surgical termination of pregnancy (Jauniaux et al., 1999). Similarly, radiolabelled fetal liver cells were successfully injected into the heart of 13 week old fetuses under ultrasound guidance (Westgren et al., 1997) prior to prostaglandin termination of pregnancy. No fetal heart rate abnormalities were detected and all fetuses were alive at least 6 hours after the procedure. Intraperitoneal injection has been applied for in utero stem cell transplantation in humans from 14 weeks of gestation (Touraine 1999; Muench et al., 2001) and is an alternative route for blood transfusion before 18 weeks of gestation (Rodeck and Deans 1999). Ultrasound guided intramuscular injection has been used to deliver corticosteroid therapy for maturation of preterm infant lungs and vitamin K to the fetus (Larsen et al, 1978; Ljubic et al, 1999).

C. Direct targeting of the fetal circulation

Delivery of vectors to the systemic fetal circulation appears to be a highly effective route for targeting gene therapy to a range of fetal tissues and particularly to the liver for treatment of diseases such as the haemophilias and the metabolic and storage disorders. This can be accomplished in small animals such as the mouse by intracardiac injection (Christensen et al., 2000; Wang et al., 1998) or by injection into the yolk sac vessels (Schachtner et al., 1996). Indeed, yolk sac vessel injection of adenoviral vectors containing the hFIX gene into fetal mice resulted in therapeutic levels of hFIX expression (Waddington et al., 2002). Long-term transgene expression was observed in the liver, heart, brain and muscle up to a year after delivery of lentiviral vectors containing the β-galactosidase gene into yolk sac vessels of fetal mice (Waddington et al., 2003) and was then used to achieve correction of the haemophilic phenotype in factor IX deficient mice (Waddington, submitted).

In larger animals such as in the sheep, intravascular delivery can be achieved by injection via the umbilical vein (Yang et al., 1999). Adenoviral vectors containing the lacZ or hFIX genes were delivered into the umbilical vein of late gestation fetal sheep using ultrasound-guided percutaneous injection from 102 days gestation (term = 145 days) (Themis et al., 1999). Positive lacZ expression was seen in about 30% of fetal hepatocytes, and hFIX expression in fetal and neonatal plasma by ELISA analysis reached therapeutic levels within a week of delivery in two animals.

In early gestation, delivery of adenoviral vectors into the umbilical vein of fetal sheep at 60 days of gestation via hysterotomy resulted in widespread transduction of fetal tissues (Yang et al., 1999). Our group has attempted ultrasound-guided umbilical vein injection of adenoviral vectors in fetal sheep at the earlier time of 53 days of gestation but this was unsuccessful due to procedure-related mortality (David et al, 2003a).

Ultrasound-guided intracardiac injection has been used to deliver adenoviral vectors to the late gestation fetal rabbit (Wang et al., 1998). Transgene expression was observed in up to 40% of fetal hepatocytes and was transient as expected. A fetal immune response to the vector and transgene was detected. Unfortunately the procedure also had a 25-40% mortality rate, comparable to other studies on fetal blood sampling in rabbits (Moise et al., 1992). Although technically straightforward, ultrasound-guided intracardiac delivery of adenoviral vectors to fetal sheep in early gestation resulted in 100% mortality due to haemorrhage (David et al, 2003a).

D. Alternative routes for targeting the fetal circulation and liver

Due to the peculiarities of the fetal anatomy, vector delivery via the umbilical vein or yolk sac vessels will preferentially target the liver, which is an important organ for treatment of many genetic diseases. However in early pregnancy this not been technically possible and alternative approaches to reach the liver and the circulation have been tried.

1. Intrahepatic injection

Fetal intrahepatic injection has been performed in mice using adenoviral vectors (Lipshtutz et al, 1999a, b, 2000; Mitchell et al, 2000), adeno-associated vectors (Mitchell et al, 2000; Sabatino et al, 2002) and lentiviral vectors (MacKenzie et al, 2002). In these studies, high levels of transgene expression in fetal hepatocytes were observed as well as gene transfer to other organs such as the heart, spleen, lung, intestine and brain suggesting haematogenic spread.

Ultrasound guided intrahepatic injection has been performed in a few large animal models. In the late gestation fetal rabbit, X-gal staining of the fetal hepatocytes was seen 2 days after ultrasound guided intrahepatic injection of adenoviral vectors containing the β-galactosidase gene in late-gestation fetal rabbits (Baumgartner et al, 1999). Similarly, strong expression of transgenic enhanced green fluorescent protein was observed in hepatocytes one month after ultrasound-guided intrahepatic delivery of adeno-associated viral vectors to the late-gestation rhesus monkey (Lai et al., 2002). Ultrasound guided intrahepatic injection in early gestation sheep fetuses has also been performed with fetal survival rates of 81% (David et al, 2003a). Only low level
hepatocyte transduction however was observed after adenoviral and retroviral mediated gene transfer into fetal sheep (David et al, 2003a) and primates (Tarantal et al, 2001b).

2. Intraperitoneal injection

Intraperitoneal injection has also been used for successful gene transfer to multiple tissues including the liver in fetal mice (Lipshutz et al, 1999b, c) rats (Hatzoglou et al, 1990, 1995) and sheep (Tran et al, 2000). Persistent peritoneal expression was observed 18 months after intraperitoneal injection of adeno-associated virus serotype 2 (AAV2) vectors containing the luciferase gene in fetal mice (Lipshutz et al, 2001). Recent studies in the fetal mouse have shown that transgene expression could be increased by intraperitoneal injection of AAV5 serotype vectors rather than AAV2 serotype vectors and by changing from the elongation factor 1α or CMV promoter to the woodchuck hepatitis virus posttranscriptional regulatory element (Lipshutz et al, 2003).

In large animal models, retroviral vectors containing the α-L-iduronidase gene were delivered by ultrasound guided injection after exteriorisation of the uterus into the peritoneal cavity or yolk sac of mid-gestation fetal dogs with canine α-L-iduronidase deficiency (mucopolysaccharidosis type 1). Low level tissue transduction was observed but expression of the transgene did not persist beyond the neonatal period (Meertens et al, 2002). In early gestation fetal primates, ultrasound guided intraperitoneal injection of Moloney murine leukemia virus amphotropic and vesicular stomatitis virus-G protein (VSV-G) pseudotyped retrovirus and VSV-G pseudotyped HIV-1 lentiviral vectors resulted in only low level tissue transduction (Tarantal et al, 2001b). In contrast long-term transduction of hematopoietic stem cells in the bone marrow and blood could be demonstrated 5 years following delivery of retroviral vectors into the peritoneal cavity of early gestation fetal sheep at laparotomy (Porada et al, 1998). Delivery of adenoviral vectors containing the hFIX gene to early gestation fetal sheep by ultrasound guided intraperitoneal injection had good fetal survival of 77% and therapeutic levels of hFIX were also obtained after injection of adenovirus hFIX vector (Figure 3).

Figure 3. Time course of transgene expression after ultrasound guided intraperitoneal, intramuscular, intrahepatic or intramniotic delivery of an adenoviral vector containing the human factor IX gene to early gestation sheep fetuses. Concentrations of human factor IX in fetal or lamb plasma were determined by ELISA analysis. Fetal samples were collected at post mortem (David et al 2003a). Republished with permission from Mary Ann Liebert Inc, Publishers.

E. Intramuscular injection

The main aim of intramuscular injection is to target the muscle for treatment of muscular dystrophies but this route may also be used for ectopic production of proteins such as hFIX in the treatment of haemophilias. In the fetal mouse, injection of adenoviral vectors containing the β-galactosidase gene into the shoulder or hindlimb musculature resulted in persistent muscle and liver transgene expression for 16 and 8 weeks respectively after injection (Yang et al, 1999). Intramuscular injection of lentiviral vectors led to transduction of myocytes and cardiomyocytes indicating systemic spread of the virus from the site of injection (MacKenzie et al, 2002).

Our group successfully achieved in vivo expression of hFIX after injection of adenovirus and AAV hFIX vectors in adult and fetal mice (Schneider et al, 2002). A recent study using EIAV lentivirus containing the lacZ gene combined intrathoracic, supracostal, intraperitoneal and intramuscular injection of three limbs and a single flank in the fetal mouse. This resulted in widespread gene expression in all injected muscles and also the diaphragm and heart which are the essential muscle groups to be reached for successful gene therapy of DMD (Gregory et al, 2003).

Finally, delivery of adenoviral vectors into the hindlimb musculature by ultrasound guided injection has been explored in one study in the early gestation fetal sheep. Fetal survival was 91% and therapeutic levels of hFIX were also obtained after injection of adenovirus hFIX vector (Figure 3).
Expression of β-galactosidase by immunohistochemistry 2 days after intraperitoneal or intra-amniotic delivery of an adenoviral vector containing the β-galactosidase gene to early gestation fetal sheep. Original magnifications are as indicated. Intraperitoneal injection at 52 days of gestation, positive staining is seen in (A) fetal small bowel serosa, (B) surface of umbilical cord and (C) fetal subcapsular hepatocytes.

Immunohistochemistry for β-galactosidase showed strong staining of the hindlimb musculature and occasional positively stained hepatocytes after injection of adenovirus lacZ vector. PCR analysis of vector presence in fetal tissues confirmed that broad haematogenic spread of vector had occurred (David et al, 2003a).

F. Targeting the fetal airways

1. Intra-amniotic injection

Intra-amniotic application has been investigated extensively in small animal models. Adenoviral vectors expressing the lacZ gene have been delivered to the fetal rat (Sekhon and Larson, 1995), mouse (Holzinger et al, 1995; Sekhon and Larson, 1995; Douar et al, 1997; Larson et al, 1997; Larson et al, 2000a; Mitchell et al, 2000) and guinea pig (Senoo et al, 2000) while adeno-associated viral vectors have been applied to the fetal mouse (Mitchell et al, 2000). In general, transgene expression is maximal in those tissues in contact with the amniotic fluid, namely the amniotic membranes and the fetal skin with less transduction of the gut and the mucosae. Indeed, therapeutic plasma concentrations of hFIX were achieved in fetal mice after intra-amniotic injection of adenoviral vectors carrying the hFIX gene (Schneider et al, 1999) and the transgenic protein remained detectable after birth. Intra-amniotic delivery of retroviral producer cells to the fetal mouse resulted in only low level transduction of the amniotic membranes and fetal skin and no airways or gut transduction (Douar et al, 1997).

In larger animals such as the fetal sheep, ultrasound guided intra-amniotic injection of an amphotropic retroviral producer cell line encoding the lacZ gene resulted in inefficient tissue transduction (Galan et al, 2002). Amniotic fluid was found to have an inhibitory effect on retroviral mediated tissue transduction, and this effect increased as gestational age progressed (Bennett et al, 2001). Better results have been obtained with adenoviral vectors. Low level transgene expression was seen in the fetal oesophagus and trachea after injection of adenoviral lacZ vectors at laparotomy in late gestation fetal sheep (Holzinger et al, 1995). Attempts to deliver adenoviral vectors into the amniotic cavity of fetal sheep using catheters placed at laparotomy had high mortality (Iwamoto et al, 1999). Ultrasound-guided intra-amniotic delivery of adenoviral vectors containing the lacZ or hFIX genes has been achieved in the early gestation fetal sheep (33 - 39 days of gestation, term = 145 days) equivalent to 8 – 10 weeks gestation in humans with 86% fetal survival (David et al, 2003a). Therapeutic plasma concentrations of hFIX were detectable up to 11 days after injection (Figure 3) and immunohistochemical analysis showed positive expression of β-galactosidase in the fetal skin and nasal cavities (Figure 4 D-F). This suggests that transduction of keratinocytes in utero may be able to deliver proteins to the circulation as well as to treat hereditary skin disease such as epidermolysis bullosa.

Gene transfer to the fetal airways is important for in utero treatment of cystic fibrosis.

However, no significant airway or gastrointestinal tissue transduction was seen after ultrasound-guided intra-
amniotic delivery of adenoviral vectors to early gestation fetal sheep (David et al, 2003a). Similarly ultrasound-guided intra-amniotic injection of adenoviral vectors in mid-trimester rhesus macaque fetuses resulted in significant transgene spread to tissues coming into contact with amniotic fluid but low level transgene expression in the fetal airways and intestine (Larson et al, 2000b).

Similar findings were observed in fetal rabbits (Boyle et al, 2001). Low levels of airway transduction are probably due to dilution of the vector by the relatively larger volume of the amniotic fluid as well as the lack of fetal breathing movements or fetal swallowing at this early gestation. It may be possible to enhance fetal breathing movements in later gestation using agents such as theophylline (Moss and Scarpelli, 1981) that lead to an intake of amniotic fluid to the lungs against the continuous outflow of tracheal fluid (Badalian et al, 1993; Kalache et al, 2000). Indeed increased intake of marker dye and some enhancement of adenovirus mediated marker gene expression was observed in mouse fetuses after theophylline administration. However other still unknown factors appear to influence the level of gene transfer to the fetal airways more effectively (Buckley, in preparation).

Recent work in our laboratory aimed to reproduce the iconoclastic report by Larson et al, (1997) that the CF-phenotype in CFTR-knockout mice can be cured by short-term prenatal expression of CFTR from an adenovirus vector, could not substantiate this claim (Buckley et al, 2003). We are, therefore, constructing integrating expression vector systems under tissue specific promoter control to achieve long-term postnatal CFTR-gene expression after in utero gene delivery.

2. Direct lung parenchymal injection

Direct injection of the lung parenchyma has been attempted to access the fetal airways but with poor results. In mid-gestation fetal primates, ultrasound guided injection of lentiviral vectors into the lung resulted in low level transgene expression in the fetal airways (Tarantal et al, 2001a). However, in the mid-gestation sheep fetus, ultrasound-guided delivery of an adenoviral vector to the lung parenchyma elicited only localized gene transfer and no spread within the airways could be detected (unpublished results).

3. Tracheal injection

Direct instillation of vector into the trachea has been more successful. Placement of catheters in the tracheae of fetal sheep can be performed by highly invasive techniques at laparotomy (McCray et al, 1995; Pitt et al, 1995; Vincent et al, 1995) or fetoscopically (Sylvester et al, 1997; Yang et al, 1999). Low level transduction of the proximal airways can be achieved using adenoviral or retroviral vectors, and occlusion of the trachea with a balloon improves distal airway transduction. These techniques however, carry a significant morbidity and mortality.

Recently a percutaneous transthoracic route of injection of the fetal trachea has been developed in midgestation sheep using ultrasound guidance to target the

**Figure 4 D-F.** Intra-amniotic injection at 33 days of gestation, positive staining is seen in (D) surface of umbilical cord, (E) fetal nasal cavity and (F) fetal skin (David AL et al 2003a). Republished with permission from Mary Ann Liebert Inc, Publishers
fetal airways as illustrated in Figure 5 (David et al., 2003b). Using this technique we achieved good transgene expression in the fetal trachea and airways following intratracheal delivery of an adenovirus containing the β-galactosidase gene (Peebles et al., 2003). Transgene expression was enhanced by pretreatment of the fetal airways with sodium caprate, a fatty acid that opens the tight junctions between airways epithelial cells. This allows the vector to reach the basolateral surface where the coxsackie-adenovirus receptor (CAR receptor) responsible for binding adenovirus is located. Further enhancement of transgene expression was achieved by complexing the adenoviral vector with DEAE dextran, a polycation that neutralizes the negative charge on the vector, improving vector binding to the CAR receptor (Figure 6 and Figure 7).

Instillation of perflubron, an inert fluorocarbon, resulted in a redistribution of expression from the upper to the peripheral airways and is most likely due to flushing of the vector solution further down the airways by the water immiscible perflubron (Weiss et al., 1999b). These results show proof of principle for the relatively safe and minimally invasive in utero delivery of a gene therapy vector to the fetal airways that resulted in levels of transgene expression in the airway epithelia that may be relevant to a therapeutic application in cystic fibrosis gene therapy.

G. Targeting the fetal gut

Intrapharyngeal delivery has been attempted once in fetal rabbits at laparotomy to target the fetal gastrointestinal system as a model for the treatment of meconium ileus due to cystic fibrosis (Wu et al., 1999). Gene transfer to the small bowel enterocytes was achieved but there was significant maternal and fetal loss related to anaesthesia and the invasive surgery used. Ultrasound-guided injection of barium into the fetal stomach of rabbits has been performed successfully (Brandt et al., 1997) and this technique could be extended to deliver gene to the fetal gut. Gene delivery to the gut of fetal mice has been observed after intra-amniotic vector application and was most likely a result of fetal swallowing (Douar et al., 1997).

H. Delivery to the placenta

Targeting the placenta could be used in the treatment of placental disorders such as pre-eclampsia or intra-uterine growth restriction. Low level gene transfer to the placenta has been achieved using angiographically guided injection of non-viral vectors into the uterine artery (Heikkilä et al., 2001). The intraplacental route has been attempted in mice, rats, guinea pigs and rabbits. Somatic gene transfer to the fetal heart and liver was achieved in some studies using mice (Woo et al., 1997; Türkay et al., 1999), but others have found little or no fetal gene transfer in mice and guinea pigs (Senoo et al., 2000) or rats (Xing et al., 2000). Commonly, the placenta showed the most transfection, but maternal tissues also demonstrated transgene expression, which although not unexpected, is undesirable in therapy aimed at the fetus.

V. Development of the fetal immune system

A major restriction in adult gene therapy is the immune response to vector and/or transgene. In utero application, on the other hand, aims to circumvent this by treatment before maturity of the functional immune system and this depends critically on the time at which fetal tolerance might be induced. The human immune system develops progressively through the first trimester and is not fully functional until 1-2 years after birth (Riley, 1998). Lymphoid cells appear first in the fetal liver from 8 weeks of gestation, with B lymphocytes and natural killer cells predominating over T cells (Pahal et al., 2000). T lymphocytes increase in number in the fetal liver and circulation from 12 weeks of gestation.
Figure 6: Na-caprate stimulation of DEAE dextran complexed adenovirus mediated airway transduction. Panel 1: Examples of staining in the peripheral lungs after virus alone (a) and DEAE complexed virus (b) and of the trachea after Na-caprate pre-treatment and uncomplexed virus administration (c) in fetal sheep injected between 102 and 109 days of gestation. Panel 2a: Na-caprate pre-treatment followed by DEAE dextran complexed virus in a 108 day sheep. Widespread gene expression was seen in the small (a), medium (b) and large (c) airways and also the main bronchi (d) and trachea (e). Panel 2b: Similar results were observed in a fetus injected at 81 days of gestation. Expression was seen in the airways (a & b) and trachea (c). Panel 3: Na-caprate pre-treatment followed by DEAE dextran complexed virus followed by perflubron. Staining of the peripheral airways in transverse sections (a & b) and longitudinal section showing gene expression was limited to the terminal branches of the bronchial tree (c). Some staining of the bronchioles (d) and trachea (e) was also observed, although less than in the absence of perflubron. Scale bar = 5mm in all cases. (Peebles et al 2003).
Although they are not capable of producing a definitive cytotoxic response until 18 weeks of gestation (Mackenzie and Maclean, 1980) natural killer cells and some T cell lines may provide a limited immune response earlier in gestation (Miyagawa et al, 1992; Phillips et al, 1992). The fetal lamb is able to produce detectable circulating antibodies in response to some antigenic stimuli from 66 days of gestation (Silverstein et al, 1963) and to reject skin grafts after 77 days of gestation (Silverstein et al, 1964). This would suggest a 'window of opportunity' in the first third to half of pregnancy during which time introduction of foreign genetic material may not produce an immune response. No humoral immune response to the transgene was observed in early gestation fetal sheep, although antibodies to the adeno viral vector were detected for each route of injection (David et al, 2003a). Similarly, umbilical vein injection of adeno viral vectors into fetal sheep at 60 days of gestation via hysterotomy resulted in widespread transduction of fetal tissues with no humoral immune response to the adeno viral vector (Yang et al, 1999). Expression of a foreign antigen during early fetal development may also result in its recognition as "self" where exposure of the fetus to foreign antigen is maintained (Billingham et al, 1956; Binns, 1967) thus allowing development of tolerance. Evidence to support induced tolerance has been reported after in utero intraperitoneal delivery of retroviral vectors in fetal sheep (Tran et al, 2001).

Induction of tolerance to transgene in adults although possible, is expensive, therefore, prenatal induction of tolerance may provide an excellent alternative. For example, a single injection of adenovirus expressing the factor IX gene into the fetal mouse was shown to provide long term, albeit diminishing expression over five months. Furthermore, 56% of these adult mice remained tolerant to repeated challenges with hFIX protein (Figure 8). In contrast, a group of mice which received adenovirus for the first time as adults developed high levels of anti-hFIX antibodies (Waddington et al, 2002). This provides proof of principle that gene therapy application in utero may allow induction of immune tolerance.

However the paradigm of self/non-self immune tolerance and sensitisation has been recently challenged by the hypothesis of Matzinger (2002). This suggests that immunity arises as a consequence of cellular alarm signals from distressed or injured cells stimulating antigen presenting cells. A recent study examined the idea that the fetus is particularly susceptible to induction of tolerance; the study concluded that, rather than being due to ignorance, timing-based tolerance or properties of naïve T cells in early life, tolerance induction in fetus may arise from differences in fetal antigen presentation; this remains to be identified (Anderson, et al, 2001).

VI. Ethical and safety issues

There are various ethical issues in relation to in utero gene therapy that need to be addressed before such therapy could be applied clinically (Fletcher and Richter, 1996; Recombinant DNA Advisory Committee 2000). One major concern is that fetal gene therapy has potential adverse effects such as injury, infection, severe immune reactions or preterm labour on the fetus as well as on the mother. Furthermore, many parents decide to terminate an affected pregnancy, and therefore the option of in utero treatment must be at least as safe for the mother, and should also reliably treat the disease (Coutelle and Rodeck, 2002).

There is a theoretical risk that the therapeutic gene product or vector that is required at a certain stage during fetal development could cause oncogenesis. In addition, insertion of vector sequences may cause developmental aberrations to occur.

While one of the aims of prenatal gene therapy is to achieve immune tolerance to the transgene and delivery system, vectors must be designed to be sufficiently different to the wild type so that the immune system remains able to mount an effective immune response against wild-type virus infection.

The problem of insertional mutagenesis as a potential risk of retroviral gene therapy has been debated for some years. This serious adverse event has now been identified in a trial of gene therapy for X-linked severe combined immunodeficiency syndrome in which CD34+ haemopoietic stem cells were transduced ex vivo with the γc gene using retroviral vectors. Two patients out of fifteen treated developed acute lymphoblastic leukemia (ALL) three years after successful gene therapy treatment. Analysis of the lymphocytes showed that the transgene had been inserted adjacent to an oncogene, LMO2, the product of which has been implicated in the pathogenesis of ALL (Juengst, 2003). Further work is needed to address this issue and to devise strategies to determine and possibly direct integration sites.

Germline transmission is another risk that raises ethical concerns. Fetal somatic gene therapy does not aim to modify the genetic content of the germ-line but inadvertent gene transfer to the germ-line could occur. Compartmentalisation of the primordial germ cells in the gonads is complete by 7 weeks of gestation in humans and it is unlikely therefore that any therapy applied after this time would result in germ-line transduction. Examination of germ cells after delivery of retroviral vectors (Porada et al, 1998; Tran et al, 2000) or adeno viral vectors to early gestation fetal sheep has not shown any detectable transmission (David et al, 2003a). Following intravascular administration of adeno viral vectors to late gestation fetal sheep, vector DNA was detectable by PCR in the gonads, but extensive investigation by RT-PCR could not detect any gene expression. A similar risk of germline transduction occurs with AAV that can integrate into the genome. No AAV sequences were detectable in the germline tissues of fetal mice receiving injection of AAV vectors via the intraperitoneal route nor the tissues of their progeny (Lipshtutz et al, 2001). Many of these issues are not confined to in utero or even adult gene therapy and concerns regarding germ-line transmission can be raised in particular for chemotherapy and infertility treatment (Schneider and Coutelle 1999). Finally there is the concern that fetal gene therapy research poses special challenges to informed consent (Burger and Wilfond 2000).
Figure 7: Na-caprate stimulation of DEAE dextran complexed adenovirus mediated β-galactosidase expression. **Panel 1**: Na-caprate pre-treatment followed by DEAE dextran complexed virus. Widespread X-gal staining (a-c) and immunohistochemical localisation (d-f) of β-galactosidase expression in the trachea (a & d), bronchial epithelium (e) and airway epithelium (b,c & f). **Panel 2**: Na-caprate pre-treatment followed by DEAE dextran complexed virus followed by perflubron. X-gal staining (a-c) and immunohistochemical localisation (d-f) of β-galactosidase expression in the peripheral airways. All fetuses were injected between 102 and 116 days. Scale bar = 5mm in all cases. (Peebles D et al 2003).
Figure 8: Durability of expression and tolerance of exogenous and expressed hFIX. Prenatal and adult mice were injected intravenously with adenoviral vectors expressing the hFIX gene (AdhFIX) and repeatedly rechallenged, as adults, with intraperitoneal hFIX protein then intravenous AdhFIX while hFIX concentrations were measured. The y axis shows blood hFIX concentrations (µg/ml) after in utero or adult injection of AdhFIX (Phase I), repeated injection of hFIX protein to the adult mice (Phase II) and repeated injection of AdhFIX to the adult mice (Phase III). The x-axis shows the experimental time course in days. Arrows indicate injection points. Groups I and II are mice initially injected in utero with AdhFIX at days 15 and 17 of gestation, respectively. Group III contains mice initially injected intravenously with AdhFIX as adults. Group IV did not receive prior injection of AdhFIX. Group V received neither prior injections of AdhFIX or hFIX protein. A line representing a therapeutic threshold of 40 ng/ml hFIX is included. Points are mean±S.D. (Waddington et al 2002). Reprinted with permission from the American Society of Hematology.
The decision to participate in a fetal gene therapy trial would occur close to the time of prenatal diagnosis of the condition. The parents may hear information in a highly biased way and not consider the risk to future pregnancies. It will be important to ensure that parents are adequately counselled and understand these issues before agreeing to take part in any future research. The general public remains concerned that ethical discussion about issues such as gene therapy, cloning and the Human Genome Project are falling behind the technology (Brown, 2000). It is therefore important to provide adequate information which will allow the public to understand the risks and benefits of these novel techniques and to enable an educated involvement in the decision-making process along with health professionals. This will also help individuals to give informed consent as these procedures become used in clinical practice.

VII. Conclusions

Fetal gene therapy offers the potential for obstetricians and gene therapists not only to diagnose but also to treat inherited genetic disease. However, for the treatment to be acceptable, it must offer advantages over postnatal gene therapy, be safe for both mother and fetus, and preferably avoid germ-line transmission. Currently, in utero gene therapy remains an experimental procedure. But in the future, better understanding of the development of genetic disease in the fetus, and improvements in vector design and targeting of fetal tissues should allow this technology to move into clinical practice.

References


mucopolysaccharidosis type VII using nonprimate lentiviral vectors. Mol Ther 3, 850-856.


Miller DG, Adam MA, Miller AD (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 10, 4239-4242.


Dr. Anna David