GENE THERAPY FOR OBSTETRIC CONDITIONS

INTRODUCTION

The first clinical trials of gene therapy in the 1990s offered the promise of a new paradigm for the treatment of genetic diseases. Over the decades that followed the challenges and setbacks which gene therapy faced often overshadowed any successes. Despite this, recent years have seen cause for renewed optimism. In 2012 Glybera™, an adeno-associated viral vector expressing lipoprotein lipase, became the first gene therapy product to receive marketing authorization in Europe, with a licence to treat familial lipoprotein lipase deficiency (1). This followed the earlier licensing in China of two gene therapies: Gendicine™ for head and neck squamous cell carcinoma and Oncorine™ for late-stage nasopharyngeal cancer. By this stage over 1800 clinical trials had been, or were being, conducted worldwide, and the therapeutic targets had expanded far beyond purely genetic disorders (2). So far no trials of gene therapy have been carried out in pregnancy, but an increasing understanding of the molecular mechanisms underlying obstetric diseases means that it is likely to have a role to play in the future. This review will discuss how gene therapy works, its potential application in obstetric conditions and the risks and limitations associated with its use in this setting. It will also address the ethical and regulatory issues that will be faced by any potential clinical trial of gene therapy during pregnancy.

WHAT IS GENE THERAPY?

Gene therapy is the introduction of genetic material into a cell so that it produces a therapeutic protein product. A gene therapy must therefore consist of genetic information, the transgene, and a way of introducing it into the cell, the vector. The genetic information contained in a transgene can include the code for the desired protein and promoters to regulate its expression. Depending on the therapeutic aim, the protein, regulation and vector may be designed to act in various ways.
Types of transgene

The actions of proteins generated by gene therapy, like all therapeutic proteins, can be divided into several broad categories (3). The most simple and intuitive strategy is to introduce the gene for a protein that is missing or defective, for example the β-haemoglobin chain for treatment of β-thalassemia. There are numerous other single gene disorders that would be amenable to this strategy, including cystic fibrosis, haemophilia, Duchenne muscular dystrophy, and X-linked severe combined immunodeficiency (SCID-X). However, while monogenic disorders have been targeted since the first clinical trials of gene therapy, achieving long term, safe, therapeutic expression has proven difficult (2). In other fields, most notably oncology, a variety of therapeutic actions have been exploited. Many of these involve interfering with existing pathways that contribute to tumour growth, such as the formation of new blood vessels. Vascular endothelial growth factors (VEGFs) are important mediators of angiogenesis and their overexpression has been associated with poor prognosis in ovarian cancer (4). Anti-angiogenic gene therapy, which produces soluble VEGF receptors to bind excess VEGF, has improved survival in a mouse model of ovarian cancer and is planned to undergo a phase I clinical trial (5). Proteins with novel functions can also be produced, for example enzymes that convert a nontoxic pro-drug into a cytotoxic metabolite, known as suicide gene therapy. One example is herpes simplex virus 1 thymidine kinase (HSV1TK), which phosphorylates ganciclovir (CGV) and converts it into the toxic CGV-triphosphate. Combination of HSV1TK gene transfer using adenovirus vectors followed by CGV therapy has also been studied in animal models as a potential treatment for uterine leiomyomata (fibroids) (6). Given the wide range of actions of protein messengers, receptors and enzymes, there are many obstetric conditions in which manipulation of protein pathways, particularly in relation to growth factors and angiogenesis, could prove beneficial.
Gene Therapy for Obstetric Conditions

Figure 1: Gene therapy involves using a vector, either viral or non-viral, to introduce a transgene, which in turn codes for a therapeutic protein.

Types of vector

An ideal vector would introduce the transgene efficiently and specifically to the target cells, both dividing and non-dividing, and cause it to be expressed for as long as desired, with no adverse effects. For obstetric conditions the length of time would most likely be short, limited by gestational length. The vector would also be able to carry a large amount of genetic material and be simple and cheap to manufacture (7). In reality the non-viral and viral vectors currently available each have their own advantages and limitations (8).

Non-viral methods involve introducing the transgene either physically or chemically into the target cell(s). The most widely used non-viral vectors in clinical trials are liposomes, which are artificial lipid vesicles containing the transgene that introduce it into the cell by binding with the cell membrane.

Membrane binding can be enhanced by incorporating viral envelope proteins into the lipid vesicle,
creating a kind of hybrid vector known as a virosome. Other methods to enhance gene transfer include: microinjection of DNA directly into the nucleus, which can be used in the research setting but is not feasible clinically; hyperdynamic injection using a high volume over a short period of time; a gene gun, which penetrates cells with microparticles coated in DNA; and electroporation or ultrasound, which uses electrical pulses or high frequency sound waves to make the cell membrane temporarily permeable to genetic material. Non-viral vectors are associated with fewer side-effects than viral vectors but also with reduced efficacy and only short-term expression (9, 10).

Viruses are a natural choice of vector because, as obligate intracellular parasites, they have evolved to introduce their own DNA or RNA into host cells and use the cell machinery to replicate (Figure 2). To produce a viral vector, the genes governing viral replication and protein production are removed and replaced by the chosen transgene, incorporating a promoter to regulate its expression. Viral vectors can be divided into two main categories: those where the transgene forms an episome within the cytoplasm of the cell, such as adenoviruses, and those that integrate the transgene into the host DNA, such as retroviruses and lentiviruses. Adeno-associated virus (AAV) vectors share characteristics of both groups, mainly forming episomes, but over time have the potential to integrate as well (11). The choice of viral vector, therefore, depends on the therapeutic aim.

Adenoviruses, which normally cause respiratory and gastrointestinal infections, infect a wide range of dividing and non-dividing cells. They also trigger a host immune response, with B cells generating antibodies to their surface proteins and T cells targeting infected cells by recognising the presence of functional virus proteins (8). Adenoviral vectors are efficient, have widespread action, and can carry large transgenes. Their usefulness in treating monogenic disorders is limited by their short-term expression, however this makes them of particular interest for use in obstetric conditions, where short-term expression may be desirable.
Figure 2: Similarities and differences between wild-type adenoviral replication and replication-deficient adenoviral vector action. (1) Fibre proteins on the viral capsid bind to coxsackievirus adenovirus receptors (CARs) on the cell surface (2) The virus is taken up via endocytosis (3) Within the endosome the fibres are shed and the viral core enters the cytosol (4) Microtubules transport the viral core to the nucleus, where DNA enters via a nuclear pore (5) Infection with a wild-type adenovirus results in DNA replication and synthesis of new viral structural and function proteins. These are assembled into new viral particles, which are then released from the cell (6) Transfection with a replication-deficient adenoviral vector results in transgenic protein expression.

Immunogenicity has been a problem, especially with early vector backbones. In 1999 a systemic inflammatory response to a high dose of adenovirus vector resulted in multi-organ failure and the death of an 18 year-old participant in a clinical trial of adenoviral gene therapy for ornithine transcarbamylase deficiency, an inborn error of urea synthesis (12). The participant’s enrolment in
the trial was subsequently criticised, as the inclusion criteria specified an ammonia level of <70μM, while his ammonia level had fluctuated above and below this cut off before enrolment. Furthermore, there was criticism that adverse events in previous participants in the trial had not been properly reported. Detailed investigations suggested that the participant had an exaggerated vector-induced activation of innate immunity, which was not predicted based on the preclinical and clinical data available at the time (13). By removing more of the viral genes, newer generation helper-dependent adenoviral vectors have been developed that produce less of an immune response (14). Even so, their expression is limited to weeks or months, in part because episomes are not replicated and so are gradually lost from dividing cell populations.

Retroviruses and lentiviruses generate little immune response and integrate into the host genome. Consequently they have the potential to provide long-term expression (15). This is important for the correction of monogenic diseases, but also carries with it the risk of insertional mutagenesis. Transgenes can include their own promoters, to improve and regulate expression after they have been introduced. Integration of a transgene into the host genome has the potential to influence the expression of nearby genes and if inserted upstream of a proto-oncogene, this can lead to malignancy. There are concerns that the fetal genome may be more susceptible to insertional mutagenesis due to the higher proliferation that takes place in utero (16). Insertional mutagenesis and subsequent leukemia has been seen during some haematopoietic stem cell gene therapy trials, such as for SCID-X (17). Both retroviruses and lentiviruses have a tendency to integrate in actively transcribed genes, with retroviruses favouring the transcription start regions (8). Retroviruses but not lentiviruses are limited by the fact that they can only transduce dividing cells because the preintegration complex cannot cross the nuclear membrane (18). This could be an advantage for targeting the placenta for example, which is a highly replicating organ.

Like adenoviruses, AAV infects a wide range of cells, but without causing any disease in humans. The natural virus has a tendency to integrate into a safe region of chromosome 19. This tendency is lost
when the viral genes are removed to convert it into a vector, but the theoretical possibility of insertion mutagenesis remains (8, 10). Insertion occurs much less frequently than with retroviruses, however, and the transgene mainly forms episomes. This means that long-term expression is best achieved in non-dividing cells (14). Although AAV vectors do not produce the immune response associated with adenoviral vectors, they are limited in the size of transgene that they can carry and the presence of neutralizing antibodies from previous natural infection can limit their gene transfer.

**Targeting delivery with viral vectors**

One of the advantages of viral vectors is that they generate more efficient and widespread introduction of the transgene than non-viral vectors. On the other hand it can be harder to target delivery into specific cells, increasing the risk of harmful production of the protein in inappropriate organs and the introduction of the transgene into germ cells, both of which are particular concerns when considering gene therapy for obstetric conditions. Germline gene therapy, deliberate gene transfer to oocytes or sperm cells to produce changes in future generations, was not considered to be acceptable for ethical and safety reasons by the Gene Therapy Advisory Council in 1998 (19). More recently mitochondrial DNA replacement (20) has been achieved in human oocytes using spindle transfer and in January 2015 the UK government approved regulations to govern application of these techniques to eradicate the transmission of serious mitochondrial disease from mother to child (21). Inadvertent germline transmission remains a risk with some types of vector, either to the pregnant woman, or, if there were a significant chance of the vector crossing the placenta, to the fetus. In reality this risk is very low and is discussed in detail later. The targeting of viral vectors to specific cells can be improved by using *ex vivo* gene transfer, by modifying the vector or transgene, and by altering the route of administration.

With *ex vivo* gene transfer some of the target cell population, commonly stem cells, are removed, transduced outside the body and then returned to the patient (10, 17). This avoids the risk of
inappropriate vector spread and allows higher multiplicities of infection of the viral vector to be used without the risk of systemic side-effects. It is particularly suited to congenital haematological conditions, for example the primary immunodeficiency SCID-X, where the stem cell population is easily accessible from the blood or bone marrow (22). However, it has also been used to generate skin grafts from epidermal stem cells in xeroderma pigmentosum and to modify hepatocytes for autologous transplantation in familial hypercholesterolaemia. In the case of obstetric disorders arising from the placenta, chorionic villi could be sampled under ultrasound guidance, with stem cells isolated and transduced ex vivo (23). The amniotic fluid provides an alternative source of multipotent stem cells that would be matched to the placenta (24-27).

Alternative ways of targeting gene therapies include: using vectors created from viruses that naturally target certain cell types, such as herpes simplex viral vectors to transduce the central nervous system (28); modifying the virus vector envelope proteins to target certain cell types (pseudotyping); and using tissue-specific promoters (8, 14). In the case of adenoviruses, the fibre proteins that form part of their capsid normally allow them to enter cells by interacting with the coxsackievirus and adenovirus receptor (CAR). This receptor is found on a range of cells, making delivery with adenoviral vectors normally quite non-specific. The fibre-mutant adenovirus vector carrying the Arg-Gly-Asp peptide sequence (Ad-RGD) has altered fibre proteins which allow it to enter cells via integrin receptors. This increases its tropism for certain cell types/organs, including the placenta (29), endometriotic cells (30), and human leiomyoma cells, suggesting it could be used to deliver targeted treatment for uterine fibroids (31). Protein expression could also be increased in endometriotic cells lines by using promoters that are specific to endometriosis cells: secretory leukocyte protease inhibitor (SLPI) and heparanase.

In obstetric disorders, targeting may also be achievable by altering the route of administration. The therapeutic targets during pregnancy can be broadly considered to be the fetus, the placenta, or the mother. Fetal gene therapy generally relies on direct administration, for example to the fetal
peritoneal cavity, umbilical vein, or amniotic fluid. However, attempts have been made to deliver viral vectors to the fetus via intraplacental injection in pre-clinical studies (32-36) with variable success, depending in part on the stage of gestation (32) (See Table 2). These studies have shown that direct intraplacental injection results in effective local gene transfer to the placenta. However, in situations where the target was the placenta rather than the fetus, unwanted spread to the fetus and the mother could be an issue. An alternative method is to deliver gene therapy to the placenta by injection into the uterine arteries, targeting the maternal side of the uteroplacental circulation. In rabbits, low levels of gene transfer to the placenta were achieved using angiographically guided injection of non-viral vectors into the uterine artery (37). Targeted gene transfer to the uterine artery can also be achieved using direct external vascular application of the vector in combination with Pluronic gel (38). This is a thermolabile gel which is liquid when cold and solidifies upon warming, allowing the vector to coat the external vessel wall.

**FETAL GENE THERAPY**

When prenatal diagnostic methods identify a fetus with a life-threatening genetic disorder that manifests *in utero*, fetal gene therapy is an appealing idea with several potential advantages over postnatal gene therapy, including: the ability to target cells and tissues that are inaccessible postnatally; the relative immaturity of the fetal immune system, allowing tolerance of the transgene product; the possibility of preventing or limiting the complications of diseases that develop before or shortly after birth; and the need for lower doses of the vector, thus reducing production costs (7, 14, 39-41). For these reasons fetal gene therapy has been the focus of much research, including many *in vivo* pre-clinical animal studies that have shown efficacy to cure disease such as in mice haemophilia (42) metabolic storage disorders (43, 44) and muscular dystrophy (45), retinal blindness in mice (46) and birds (47), and in mice to prevent the development of fetal anomalies such as cleft palate (48). There are no studies performed in large animal models of disease but plasma levels of therapeutic transgenic factor IX protein expression have been achieved in normal sheep (49) and non-human
primates (50) after fetal gene therapy using adeno-associated virus factor IX vector injection, and neuronal transduction of the central and peripheral nervous systems is seen after systemic AAV vector injection in non-human primates (51). Target diseases are considered to be those that are life-threatening, that have pathology beginning in utero where accurate prenatal diagnosis is feasible, a postnatal treatment would be too late or is not available, and a disease where there is a close correlation between the genotype and the phenotype (52). At present no disease fits the criteria and with current vector systems still being developed for postnatal treatment, it may be some years before a fetal gene therapy may be translated into the clinic. An alternative strategy is to gene correct fetal stem cells that are available in the amniotic fluid or placenta for example, which can then be delivered back into the fetus as an autologous transplant (53). This avoids the fetal immune system which is responsible for the failure of in utero stem cell transplantation for non-immune deficiency congenital disease such as thalassaemia and metabolic storage disorders (54). Pilot studies in sheep demonstrate the feasibility of this approach (24, 55) whereby CD34+ stem cells with hematopoietic potential were derived from fetal sheep amniotic fluid, transduced and underwent autologous transplantation into fetal sheep and immunodeficient mice. It has advantages over systemic fetal gene transfer since transduction is limited to selected fetal cells, is performed outside the fetus in well controlled conditions and it allows for detailed safety testing such as insertion site analysis.

MATERNAL GENE THERAPY

The use of transgenic proteins in cell cultures and pregnant animals has helped to elucidate the molecular mechanisms underlying normal pregnancy and obstetric diseases. This has the potential for translation into gene therapies given to the pregnant woman rather than the fetus. Due to the inherent interdependence that occurs in pregnancy, maternal gene therapy has the prospect of providing both maternal and fetal benefit for a number of conditions. Furthermore, because pregnancy represents a limited time period, the challenges of producing long-term gene expression
would not apply. Maternal gene therapy has not been explored as widely as fetal gene therapy, but it may have a role in both obstetric and non-obstetric conditions.

**Fetal growth restriction**

Fetal growth restriction (FGR) is a serious condition affecting 8% of all pregnancies and contributing to 50% of stillbirths (56). In this condition, the fetus fails to achieve its growth potential and is born smaller than anticipated. The causes are heterogeneous and can include structural abnormalities of the fetus, aneuploidy, congenital viral infection or maternal medical disorders. More commonly, impaired uteroplacental function restricts delivery of nutrients to the fetus, resulting in slowing or even cessation of fetal growth. In about 1 in 500 cases, FGR is severe and early in onset, occurring before 28 weeks gestation. Current management is to deliver the fetus before death or irreversible organ damage occurs, particularly to the brain. However, early delivery in severe early onset FGR adds additional risks to the baby from extremely preterm birth, with its own attendant short- and long-term complications (57). Furthermore FGR may be detected while the fetal weight is far below 500g, a situation considered by many to be non-viable. Even modest increases in birthweight (e.g. from 500 to 600g) and gestation at delivery (e.g. from 26 to 27 weeks) are associated with significant improvements in mortality and morbidity (58). It is in these severe early-onset cases of FGR that maternal gene therapy is initially being considered, where the benefit of gaining gestation or fetal weight outweighs any potential risks of a novel therapy. If it is found to be safe and efficacious there is potential to use maternal gene therapy in more moderate FGR, which affects a larger number of pregnancies.

In normal pregnancy, trophoblast invasion of the maternal spiral arteries produces a low resistance, high flow maternal uterine circulation. These changes are enhanced by the placental production of vasoactive substances such as VEGF, which causes vasodilation and angiogenesis. In uteroplacental FGR, trophoblast invasion is impaired, resulting in a relative reduction in uterine artery blood flow (59, 60). There is also a reduction in available maternal VEGF and an increase in its soluble receptor,
soluble fms-like tyrosine kinase 1 (sFlt1) (61, 62). Our research over the last eight years has demonstrated that adenovirus vector (Ad) mediated manipulation of VEGF expression in the uterine arteries produces an increase in uterine artery blood flow (63-65) and ameliorates the effects of FGR in animal models (66, 67).

There are currently seven proteins within the VEGF family, of which five occur naturally in humans: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF). These proteins bind to various combinations of the three VEGF receptors to produce different angiogenic and lymphangiogenic effects (68). VEGF-A occurs in a variety of isoforms depending on pre-RNA splicing, including VEGF-A165. In contrast VEGF-D is naturally produced in an inactive long form, and has to be shortened to the active form, VEGF-D\(^{\text{ΔNΔC}}\). With a viral vector, however, the pre-processed active form can be produced directly. Both VEGF-A\(_{165}\) and VEGF-D\(^{\text{ΔNΔC}}\) have angiogenic effects, although the effect of VEGF-D\(^{\text{ΔNΔC}}\) appears to be more diffuse (69).

The short and long-term effects of adenovirus vectors containing the A\(_{165}\) (Ad.VEGF-A\(_{165}\)) or pre-processed D isoform (Ad.VEGF-D\(^{\text{ΔNΔC}}\)) of VEGF were initially studied in normal sheep pregnancy. This involved performing a laparotomy in mid-gestation dams to inject the VEGF adenoviral vector into one uterine artery and a non-vasoactive control adenoviral vector coding for bacterial β-galactosidase (Ad.LacZ) into the contralateral uterine artery. Short-term studies comparing Ad.VEGF-A\(_{165}\) with Ad.LacZ found that uterine artery blood flow, as assessed by Doppler ultrasound, increased in both arteries 4-7 days after administration, as expected with increasing gestation. However, while the increased flow was not significant in the Ad.LacZ treated arteries, there was a significant three-fold increase in blood flow in the Ad.VEGF-A\(_{165}\) treated vessels. This was associated with a reduced contractile response and increased relaxation response of Ad.VEGF-A\(_{165}\) vs. Ad.LacZ treated vessels (63). Levels of endothelial nitric oxide synthase (eNOS) and VEGF receptor 2 (VEGFR-2) were also up-regulated at day 4-7 in vessels transduced with Ad.VEGF-A\(_{165}\) compared with those transduced with Ad.LacZ (64). Similar results were achieved in short-term studies comparing Ad.VEGF-D\(^{\text{ΔNΔC}}\) with
Ad.LacZ, in which vessels administered Ad.VEGF-D^{ΔNΔC} had a significantly reduced contractile response, a significantly increased relaxation response, and a significant increase in phosphorylated eNOS (64). Furthermore, compared with non-transduced vessels at the same gestational age, there was a significant increase in endothelial cell proliferation in the main branch of the uterine artery 4-7 days after transduction with Ad.VEGF-D^{ΔNΔC}.

**Figure 3:** Injection of Ad.VEGF-A_{165} (5x10^{11} vector particles) into the uterine artery of a pregnant ewe (day 98±2 of 140 day gestation) produces a significantly greater increase in uterine artery volume blood flow from baseline as measured by implanted flow probes than a control vector (Ad.LacZ) injected into the contralateral uterine artery (p=0.02). Change in uterine artery volume blood flow was calculated as a percentage change from baseline (measured over 3 days before administration; adjusted to 0) and presented as mean ± SE (64).

Longer-term effects on uterine artery volume blood flow were assessed using implanted flow probes. In vessels injected with Ad.VEGF-A_{165}, a significant increase in uterine artery volume blood flow persisted at 28 days after administration compared with vessels injected with Ad.LacZ (Figure 3) (64). The same trend was seen after injection with Ad.VEGF-D^{ΔNΔC} (65). In both cases this was associated with a persistent reduction in vascular contractile responses in the uterine arteries, as well as a significantly increased number of adventitial blood vessels at 30-45 days after administration (64, 65). At this stage, human VEGF expression was no longer detectable by enzyme-
linked immunosorbent assay (ELISA) in the uterine arteries or uterine wall and there was no ongoing up-regulation of VEGFR-2. These findings show that the beneficial effects of Ad.VEGF administration persist even after transgene expression has ceased, which is probably related to the formation of new vessels in the uterine artery adventitia (63-65).

Subsequent experiments were carried out using a sheep model of FGR, whereby adolescent ewes conceive through single sire donor embryo transfer (to minimise genetic heterogeneity and ensure optimise pregnancy rates) and are subsequently overnourished. The high dietary intake in these still-growing adolescent dams results in nutrient partitioning away from the gravid uterus, causing accelerated maternal tissue growth at the expense of the fetus (70). This is associated with a ≈42% reduction in uterine artery volume blood flow from mid-gestation, followed by placental and fetal growth restriction relative to the normally growing fetuses of adolescent dams fed a moderate (control) diet. In this situation approximately 52% of high-intake pregnancies demonstrate "marked" FGR, an accepted definition of which is a birth weight more than two standard deviations (SD) below the genetic potential (71), which can be estimated from contemporaneous control-intake pregnancies on a study-by-study basis. In these pregnancies fetal and placental weights are reduced by ≈48%, whilst the remaining pregnancies (48%) are considered "non-FGR" (72). This experimental paradigm replicates key clinical features of human uteroplacental insufficiency without requiring any surgical interference to the uteroplacental circulation (73). An asymmetrical pattern of growth restriction is observed, with prioritisation of brain growth (brain sparing) and increased umbilical artery Doppler indices (74, 75). There is also impaired placental vascularisation and secretory function, and reduced placental expression of VEGF (76-78).

Overnourished adolescent ewes were randomised to receive Ad.VEGF-A_{165} or a control treatment (Ad.LacZ or saline) injected into both uterine arteries in mid-pregnancy. The fetal growth velocity, as determined by serial ultrasound measurements of fetal abdominal circumference by a single masked assessor, was significantly increased in the Ad. VEGF-A_{165} group compared with the Ad.LacZ or saline
groups at 3-4 weeks after injection, and there appeared to be amelioration of fetal brain sparing (66, 79). At necropsy at 131 days gestation (term = 145 days), significantly fewer fetuses demonstrated marked FGR in the Ad.VEGF-A165 group than in the Ad.LacZ or saline groups. Increased fetal growth velocity was also demonstrated in a second experimental group, in which overnourished adolescent dams were similarly randomised to receive uterine artery injections of either Ad.VEGF-A165 or saline, however the pregnancies were allowed to continue until spontaneous delivery at term (Figure 4)(80). At birth Ad.VEGF-A165-treated lambs tended to be heavier (p=0.081) and demonstrated increased absolute postnatal growth velocity during the first three months of life (81). Fractional growth rates were not different, indicating appropriate growth relative to initial birth weight, and body composition analysis revealed greater lean tissue mass. No impact on the epigenetic status of key somatotrophic axis genes was observed.

![Figure 4: Serial ultrasound measurements of: a. fetal abdominal circumference (AC); and b. fetal biparietal diameter to AC ratio (a marker of fetal brain sparing) in 57 singleton-bearing adolescent ewes receiving a control (n=12) or high dietary intake (n=45) to promote normal or compromised fetal growth, respectively (80). Overnourished ewes were randomised to receive bilateral uterine artery injections of Ad.VEGF-A165 (n=18), Ad.LacZ (n=14) or saline (n=13) in mid-pregnancy. Control-fed ewes all received saline (n=12). Those time points at which there were significant differences between Ad.VEGF-A165 and Ad.LacZ/saline-treated overnourished groups are indicated.](image-url)
An alternative animal model of FGR is periconceptual nutrient deprivation in the guinea pig, which impairs placental functional development, reduces the placental exchange and trophoblastic surface, increases the thickness of the exchange barrier and causes a 40% reduction in fetal weight with brain sparing (82), all of which are features of human FGR. A further advantage of this model is the fact that guinea pig placentation is the most similar to human placentation with a haemochorial type of placenta, and a homologous process of trophoblast invasion (83) and trophoblast cell proliferation (84). Mid-gestation nutrient-restricted Dunkin Hartley guinea pigs underwent laparotomy at 30-34 days gestation (term = 65) and the uterine and radial arteries were transduced with Ad.VEGF-A165 or Ad.LacZ, using a thermosensitive Pluronic gel (67). At necropsy, 31-34 days later, administration of Ad.VEGF-A165 was associated with a significantly lower brain:liver weight ratio, suggesting an attenuation of brain sparing.

In 2013 the European Union awarded a Framework Programme 7 grant to the EVERREST consortium (85) which aims to conduct a phase I/IIa clinical trial to assess the safety and efficacy of Ad.VEGF gene therapy given into the uterine arteries of pregnant women with severe early onset FGR. The project is being undertaken by the EVERREST Consortium, a multinational, multidisciplinary group, including experts in bioethics, fetal medicine, fetal therapy, obstetrics and neonatology. The clinical trial protocol is currently under development, requiring input from a wide range of healthcare experts within the consortium to consider inclusion, exclusion criteria and adverse event reporting. The ethical and regulatory issues which are raised by a trial of this nature and the development of the clinical trial protocol will be discussed later in the article.

Another therapeutic strategy in FGR is to try and improve placental function instead of increasing uteroplacental blood supply. In normal pregnancy insulin-like growth factors (IGFs) I and II act within the placenta to promote fibroblast proliferation and survival, and increase placental uptake and transfer of glucose and amino acids (86). IGF-I also regulates cytotrophoblast and syncytiotrophoblast differentiation (86). In sheep, direct administration of IGF-I, via a continuous
maternal infusion or regular injections into the amniotic cavity, has been shown to increase fetal growth rate in a placental embolisation model of FGR (87, 88). Translating these routes of administration into clinical practice would prove challenging, making IGF gene therapy an attractive alternative.

The potential for IGF gene therapy to alter placental growth and function has been demonstrated in vitro in cell cultures. Human primary placental fibroblasts transduced with adenoviral vectors expressing either IGF-I (Ad.IGF-I) or IGF-II (Ad.IGF-II) showed significantly increased proliferation and cell migration compared with non-transduced controls (89). Transduction of the BeWo choriocarcinoma cell line by Ad.IGF-I also resulted in significantly increased amino acid uptake and significant increases in mRNA and protein expression of amino acid (90) and glucose transporters (91).

The effects of intraplacental IGF gene therapy have been studied in vivo using murine and rabbit models of FGR, resulting from reduced uteroplacental blood supply (90-92). In the mouse model, one of the two mesenteric branches of the uterine artery (MUAL) is ligated at laparotomy at day 18 of gestation (term = 19-20 days) (93). This results in reduced pup birthweight and a significant reduction in placental expression of IGF-I, IGF-II, large neutral amino acid transporters (LAT) 1 and 2, and glucose transporter 8 (GLUT8) compared with sham surgery (90, 91, 93). Intraplacental administration of Ad.IGF-I at the time of MUAL resulted in significantly increased transporter expression compared with MUAL alone, with levels comparable to a sham surgery control group (90, 91). The rabbit model harnesses the natural vascular watershed along each horn of the bicornuate uterus, which affects the third pup from the ovarian end (the runt). Dams underwent laparotomy at day 21, with PBS, Ad.IGF-I, or Ad.LacZ injected into the placenta of the runt in one horn (92). Where runts were administered PBS, the pups closest to the ovarian end of the same horn also received intraplacental PBS, to act as a control group. Fetal, placental, liver, lung, and musculoskeletal weight were significantly lower in runts administered PBS compared with controls. However, runts
administered intraplacental Ad.IGF-I had significantly higher fetal, liver, and musculoskeletal weights than runts receiving PBS or Ad.LacZ. In Ad.IGF-I runts these weights were comparable to the control group, indicating normalisation of fetal growth. A note of caution is raised, however, by the loss of all pups in four of the 14 dams administered intraplacental adenoviral vector.

Pre-eclampsia

Pre-eclampsia is a multisystem disorder, which manifests as maternal hypertension and proteinuria after 20 weeks gestation. It affects 2-8% of pregnancies and in its severe form can lead to eclampsia, multi-organ failure, and maternal and fetal mortality (94). Although the manifestations of pre-eclampsia can often be managed in the short term, the only cure is to deliver the fetus and placenta, sometimes very prematurely. Pre-eclampsia and FGR often co-exist, especially onset is early in gestation (before 28 weeks), which reflects an overlap in the pathophysiology of the two conditions. In pre-eclampsia, as in FGR, there is a relatively anti-angiogenic state, with an increase in sFlt-1 and a reduction in available maternal VEGF (95, 96). Excess sFlt-1 production may be caused by placental hypoperfusion and hypoxia secondary to inadequate trophoblast invasion, by inflammatory cytokines such as tumour necrosis factor α (TNF-α) secondary to oxidative stress, or by a combination of these factors. Either way, placental damage leads to the production of proinflammatory microparticles, cytokines, sFlt-1 and other factors which in turn cause maternal endothelial dysfunction and the clinical manifestations of the disease.

The role of sFlt-1 in the pathophysiology of pre-eclampsia has been examined in animal experiments using pregnant rats (97, 98) and mice (99). Increases in maternal sFlt-1 levels, achieved either using sFlt-1 infusion (98) or by systemic delivery of an adenoviral vector expressing sFlt-1 (Ad.sFlt-1) (97, 99) results in hypertension, proteinuria, renal pathology, and a significant reduction in fetal and placental weight. These changes can be ameliorated by the simultaneous administration of an adenoviral vector expressing VEGF-A_{165} (Ad.VEGF-A_{165}), which significantly reduces maternal plasma sFlt-1 levels compared with Ad.sFlt-1 alone (99). When pregnant mice were administered Ad.VEGF-
A165 concurrently with Ad.sFlt-1, there was no evidence of the renal pathology or heavy albuminuria that resulted from injection of Ad.sFlt-1 alone. Furthermore, in contrast to the significant rise in blood pressure produced by Ad.sFlt-1 compared to a control vector, simultaneous administration of Ad.sFlt-1 and Ad.VEGF-A165 resulted in a fall in blood pressure over the subsequent two days. A similar short-term fall in maternal blood pressure and reduction in sFlt-1 levels was seen in healthy pregnant mice administered a haemaglutinating virus of Japan envelope (HVJ-E) vector containing the transgene for VEGF-A165 directly into the extraamniotic cavity on day 14.5 of gestation (100).

For more long-term expression, application of adenovirus vectors containing VEGF-A121 (Ad.VEGF-A121) has been investigated in the BPH/5 inbred mouse strain, which develops a pre-eclampsia-like syndrome (101). Untreated pregnant BPH/5 mice demonstrate defective trophoblast invasion, decreased uterine artery end-diastolic flow, increased fetal resorption, and late-gestational hypertension and proteinuria. Serum VEGF and placental VEGF expression is lower in the strain compared with ‘normal’ mice but without an increase in sFlt-1. Systemic intravenous delivery of Ad.VEGF-A121 at 7.5 dpc in BPH/5 mice resulted in higher levels of VEGF when compared to mice receiving control Ad.LacZ vector, that were comparable to untreated C57 mice at the same gestation. Treatment also ameliorated the late gestation blood pressure rise, prevented the development of proteinuria, and reduced fetal resorption by 50% compared with BPH/5 mice given Ad.LacZ. These studies demonstrate the potential for manipulating VEGF to prevent the development of or to treat pre-eclampsia.

Another potential therapeutic protein system is heme oxygenase (HO), an enzyme that consists of two functional isoforms: the inducible form HO-1 and the constitutively active form HO-2, which is important in the maintenance of healthy pregnancy. Through production of both carbon monoxide and bilirubin, HO has the ability to block the production of both sFlt1 and reactive oxygen species, and thus could be a novel therapeutic agent in the management of preeclampsia (96). In an established mouse model of spontaneous miscarriage due to immunological mismatching (CBA/J x
Current studies have shown that DBA/2J combination intraperitoneal injection of an adenovirus containing the HO-1 gene at 5 dpc reduced fetal loss with evidence of diminished placental apoptosis (102). This suggests a protective effect of systemic HO-1 up-regulation on pregnancy outcome and the prevention of oxidative damage in the placenta.

Gene therapy may also have a role in developing novel therapeutics to manage pre-eclampsia. Calcitonin gene related peptide (CGRP), a neuropeptide and vasodilator, has increased levels in normal pregnancy, but plasma levels are lower in women with pre-eclampsia. Plasma CGRP levels rise in response to magnesium sulphate, a drug used clinically to prevent eclamptic fits in severe pre-eclampsia. Experiments in rat models of pre-eclampsia induced using L-NAME to chronically inhibit nitric oxide production found that CGRP co-administration with L-NAME prevented the development of gestational hypertension and reduced fetal mortality. Its potential for direct clinical application is limited by its short half-life of 10 minutes, but this may be surmountable with the use of short-term gene therapy (103).

Preterm birth

Preterm delivery is the last major complication of pregnancy to be considered, affecting at least 10% of pregnancies worldwide, and is increasing in prevalence. There have, as yet, been no studies published on the use of gene therapies to delay delivery in women who are in threatened preterm labour, or to prevent preterm labour in high risk women. This is probably due to the multifactorial nature of the condition for which some authors have described a “preterm parturition syndrome” (104). Known triggers to parturition that could be amenable to gene therapy interventions include intrauterine infection/inflammation, uterine ischaemia and progesterone.

Pre-pregnancy and early pregnancy
Gene therapy is also being explored in a number of conditions that have a direct effect on fertility and early pregnancy, including endometriosis (105), ovarian failure (106), uterine fibroids (9), and miscarriage (102, 107).

Uterine fibroids, or leiomyomas, are benign tumours of the myometrial smooth muscle, which can increase the risk of pre-term labour, fetal malposition, obstructed labour and post-partum haemorrhage. Two potential gene therapies have been developed to treat uterine fibroids, both based on adenoviral vectors. The first is a suicide gene therapy using HSV1TK/GCV, where herpes simplex virus 1 thymidine kinase is used to convert ganciclovir into a toxic metabolite (6). The second uses a transgene for a dominant negative estrogen receptor (DNER), which inhibits the action of ‘normal’ wild-type estrogen receptors (108). In a rat model that is characterised by a mutation in the tuberous sclerosis 2 tumour suppressor gene, injection of either gene therapy directly into the uterine fibroids significantly reduced their volume over 30 days compared with control injections.

Because of the potential impact on a fetus, either from the direct effects of these transgenes or from extensive fibroid degeneration, such gene therapies would need to be carried out before pregnancy.

Gene therapy has been used in early pregnancy in a mouse model of spontaneous miscarriage (102, 107). Successful pregnancy is dependent on changes in the maternal immune system, which allow it to tolerate the presence of the fetus, specifically up-regulation of Th2 cytokines relative to Th1 cytokines. Two groups have used maternal intraperitoneal adenoviral gene therapy at 5 dpc, resulting in beneficial effects on the Th2/Th1 cytokine ratio and significant reductions in fetal resorption. As described above, Zenclussen et al. showed that overexpressing the HO-1 enzyme in the maternal peritoneal cavity protected locally against rejection in transplantation models probably due to its anti-oxidant, anti-inflammatory and cytoprotective functions (102). A separate group investigated the effect of adenovirus vector containing the anti-inflammatory fusion protein CTLA4Ig transgene. Vector injection into the peritoneal cavity reduced fetal loss rate in a mouse model of miscarriage (CBA/J x DBA/2 cross) by skewing the Th2/Th1 cytokine balance, expanding peripheral
CD4(+) CD25(+) regulatory T cell populations and inducing indoleamine 2,3 dioxygenase (IDO) mRNA and Foxp3 expression at the maternal-fetal interface, molecules that are key regulatory factors for feto-maternal tolerance (107). The mechanism of action appears to be related to inhibition of maternal spleen lymphocyte proliferation and regulation of apoptosis in the feto-placental unit (109).

Non-obstetric conditions during pregnancy

Oncology accounts for over two thirds of gene therapy trials, and in the future maternal cancer may become an indication for gene therapy during pregnancy (2). Pregnant women diagnosed with cancer can face a painful choice between ending the pregnancy early to start treatment, through termination or iatrogenic preterm delivery, undergoing treatment known to have adverse fetal effects, or risking their own health by delaying intervention.

The field of cancer gene therapy is too wide to examine in detail but it is worth considering one study on the effect of the antioxidant manganese superoxide dismutase (MnSOD) in pregnant mice (110). MnSOD can protect against the side-effects of radiotherapy, but must be present for 24-48 hours after radiation exposure to be effective against on-going apoptosis. Administration of the protein, even on multiple occasions, has not been effective at achieving this. Liposome mediated gene transfer of MnSOD to mid-gestation pregnant mice 24 hours before total body irradiation significantly increased pup survival over 220 days compared with pups from untreated irradiated mothers. However, there was still a significant reduction in litter size and pup weight compared with non-irradiated mice. This may be in part because whole body irradiation exposed the fetuses directly to ionising radiation, rather than just the indirect effect of cytokines crossing the placenta. With the use of targeted radiotherapy and shielding, fetal outcome may be further improved.

RISKS AND LIMITATIONS OF MATERNAL AND PLACENTAL GENE THERAPY
Some of the risks associated with gene therapy have already been discussed, and are currently being ameliorated with developments in vector design. The risk of insertional mutagenesis with retroviral vectors may be reduced by switching from retroviruses to lentiviruses, which are less likely to integrate near actively transcribed genes, the development of self-inactivating vectors, and the use of tissue-specific promoters rather than viral promoters (17). The immune reactions seen with adenoviruses are being addressed using helper-dependent adenoviral vectors, which provoke less of a T-cell response, using serotypes to which patients are unlikely to have pre-existing immunity, and coating vectors with polymers like PEG to protect them from neutralising antibodies (111).

With maternal gene therapy, as with many obstetric or fetal interventions, an important risk is that the intervention will be ineffective or only partially effective. In conditions such as recurrent miscarriage, severe early onset FGR, and early onset pre-eclampsia this could mean that a pregnancy which may have otherwise ended in miscarriage, stillbirth or termination instead leads to the livebirth of a child with chronic health problems (52). Whether parents perceive this as a risk or a benefit will be an individual matter, but it would need to be discussed as part of any pre-procedure counselling. With the use of adenoviral vectors, which are well suited to the relatively short-term nature of most obstetric conditions, there is also a risk that the generation of antibodies may mean that an intervention could only be given once. This could have implications for future pregnancies and for the woman’s long-term health if she were later to require adenoviral gene therapy for another indication.

**Placental transmission**

As maternal gene therapy would target the maternal system, and possibly the maternal side of the placenta, placental transmission to the fetus would generally be undesirable. If significant placental transmission were to occur it could potentially lead to fetal gene transfer with the attendant risks of fetal germline transmission and insertional mutagenesis, as well as the adverse effects of the expressed protein on fetal development. This may be particularly important in the case of growth.
factors (112, 113). Even in the case of placental gene therapy with direct intraplacental administration, excessive spread to the fetus may not be desirable. The findings of pre-clinical studies using maternal and placental administration are summarised in Tables 1 and 2 respectively.

The majority of studies in pregnant animals have been performed using adenoviral vectors. Wildtype adenovirus is able to cross the placenta, as demonstrated by the presence of viral DNA in amniotic fluid at amniocentesis (114). Whether this ability is retained by adenoviral vectors may depend upon the animal used, the dose given, and the route of administration. Intravascular administration to pregnant sheep (63, 64), mice (29), and guinea pigs (38) produced transgenic protein expression in the placenta but not in the fetuses. The same pattern was seen when adenoviral vector was applied to the outer surface of the uterine and radial arteries in guinea pigs using a thermolabile Pluronic gel (38). In contrast, after intravascular administration in rabbits (37) and multiple dose intravascular administration in rats (33), transgenic protein expression was detected in both the placenta and fetal organs. Following maternal intraperitoneal administration, microscopy only demonstrated transgene expression in the maternal side of the placenta, and not in fetal tissue (102, 107). Using PCR, however, the transgene and adenovirus were detected in fetal homogenate (102). Direct intraplacental administration of adenoviral vectors has almost always been found to result in transgenic protein expression in the fetus and dam (32-36, 92), with the site of highest fetal expression depending on the gestation of administration (32).

In the case of hybrid and non-viral vectors, the evidence is more limited and variable. Following administration of plasmid/PEI or plasmid/liposome complexes into the uterine arteries of pregnant rabbits, gene transfer was detected in the placenta and fetuses (37). Injection of virosomes into the extraamniotic space in pregnant mice, however, produced expression in the decidua, basal lamina of the placenta, and amnion, but not in the fetus (115). In contrast, after intravenous administration of liposomes in mice, the vector was not detected in either placental or fetal tissue (110).
Given the variation in placental structure between animals, however, these findings may not be directly applicable to human pregnancy. Studies exposing human placental villous explants to high dose adenoviral vector found occasional transduction of the underlying cytotrophoblast when the overlying syncytiotrophoblast was deficient, but no evidence of the vector crossing the basement membrane (116). Given the importance of placental transmission to the safety of maternal gene therapy more evidence is required, and further studies in the ex vivo perfused human placenta are currently underway.

**Germline transmission**

The risk to the woman of oocyte transduction is probably no higher during pregnancy than at any other time. The risk of fetal germline gene transfer after maternal gene therapy, however, will depend on the degree of placental transmission and the gestation at which gene therapy is given. Germline transmission has been observed after in utero fetal gene therapy, particularly in early gestation. Intraperitoneal administration of a retroviral vector to fetal sheep resulted in low-level transduction of sperm cells, with lower levels at later gestations (117). However, unlike humans, where germ cell compartmentalisation is complete by 7 weeks gestation, germ cells in fetal sheep continue to migrate to the testes after birth. In non-human primates, oocyte transduction has been observed after fetal intraperitoneal administration of a lentiviral vector in the late first trimester, but this route of administration has not been studied at later gestations (118). Although, theoretically, maternal gene therapy in the second or third trimester should carry a very low risk of fetal germline modification, this will be an important safety consideration for clinical translation.

**Placental toxicity**

The future use of gene therapy in pregnancy would not be possible if it resulted in significant placental toxicity. So far, however, such toxicity has not been demonstrated. Administration of an adenoviral vector into the uterine arteries of pregnant rabbits did not produce any evidence of a
placental inflammatory response, reflected by the number of placental macrophages and T cells (37). Similarly when human placental villous explants were exposed to $5 \times 10^{10}$ vp/ml of adenoviral vector expressing VEGF or LacZ for 60 minutes there was no increase in human chorionic gonadotrophin or lactate dehydrogenase, both of which can be released by placental cell damage (116). Further work to study the effect of viral vectors on perfused human placental tissue is on-going.

ETHICAL AND REGULATORY ISSUES

Medical research involving pregnant women raises specific ethical and regulatory issues, particularly in relation to early phase trials. While maternal gene therapy has yet to be considered by national ethical and regulatory bodies, parallels may be drawn with regulatory reviews of hypothetical fetal gene therapy proposals.

Clinical research in pregnancy

Clinical research in pregnancy remains limited, despite recognition that the physiological and metabolic changes of pregnancy mean the results of clinical trials in non-pregnant subjects (often predominantly male) may not be transferrable. The exclusion of pregnant women from many clinical trials has been criticised as putting them at an unfair disadvantage by restricting their access to evidence-based medicine (119, 120). Despite the 2002 Council for International Organizations of Medical Sciences (CIOMS) recommendation that pregnant women be presumed eligible to participate in research, progress has been slow. It has been argued that the reluctance to conduct clinical trials during pregnancy may arise from a residing reaction to the thalidomide tragedy, coupled with the categorisation of pregnant women as a vulnerable group (121).

The categorisation of all pregnant women as vulnerable to coercion or undue influence, as laid out by the United States Code of Federal Regulations (122), has been challenged over recent years (121, 123). However, in clinical trials which involve risks to the mother for potential benefit to the fetus, it could be argued that the internal and external pressures a pregnant woman faces affect her ability
to freely decide about participation (121, 124). While this must be a consideration for clinical researchers, and careful thought should be given to the consent procedures in any obstetric trial, it should not be seen as an insurmountable obstacle to conducting research in pregnancy. There are many occasions in routine obstetric care where an intervention that has a risk to the pregnant woman is performed for the benefit of the fetus, including antenatal corticosteroids for fetal lung maturation, antiarrhythmics for fetal tachyarrhythmias, and Caesarean section for fetal acidaemia. There is also a precedent for carrying out clinical trials of such interventions, including laser ablation for twin-twin transfusion syndrome (125) or prenatal open repair of myelomeningocele (126).

In semi-structured qualitative interviews, 24 women in four European countries who had experienced a pregnancy complicated by severe early onset fetal growth restriction were asked about their attitudes towards a future clinical trial of maternal gene therapy for this condition (127). The majority of women had felt capable of making treatment decisions during their pregnancies, and while they recognised the challenges of deciding whether or not to participate in such a trial, felt that with time and information it would have been their decision to make. Similarly CIOMS recommends that, even where these risks are uncertain, it is up to the pregnant woman to decide whether she considers them acceptable (124).

**Early phase clinical trials in pregnancy**

The development of entirely novel obstetric therapies, such as gene therapies, requires phase I safety trials, where the risks and benefits are inherently uncertain. Furthermore, because the primary outcome is safety, phase I trials often use a dose-escalation design, where the starting dose may be so low that no beneficial effect is anticipated. The Council of Europe “Additional Protocol to the Convention on Human Rights and Biomedicine, concerning Biomedical Research” states that in research involving competent adults which does not produce a direct benefit, the risks should be acceptable to the participant (128). In pregnancy, however, it requires that such research should have only minimal risks. Similarly, the United States Code of Federal Regulations states that research
without direct benefit to the mother of fetus should involve only minimal fetal risks (122). It has been argued that while maternal and fetal risks should be minimised, they should not have to be minimal (129), as this constitutes an unreasonable barrier to obstetric research. Under the current regulations, however, the future of early phase trials for those interventions that can only be carried out in pregnancy may depend on the regulatory interpretation of potential benefit.

Implications from national reviews of in utero gene therapy

In February 1998 the UK Gene Therapy Advisory Committee (GTAC) New and Emerging Technologies group presented their report on the potential uses of gene therapy in utero (19). This was followed, in March 1999, by the US National Institute for Health (NIH) Recombinant DNA Advisory Committee report on the scientific, medical, and ethical issues relating to prenatal gene therapy (52). At the time both reports concluded that there was insufficient data to support a trial of fetal gene therapy. They recommended that the risk benefit analysis and informed decision-making process would be key elements of any future trials, and highlighted the psychosocial and emotional risks to pregnant women, including the risk of coercion. They also advised that informed consent for participation should include a clear explanation that the intervention would be experimental and that long-term follow-up would be needed. Gene therapy to the mother intended to benefit the fetus was not considered at the time, but any such application would need to weigh up the potential risks to the mother as with any other gene therapy treatment, but also the risk to the fetus if significant placental gene transfer were to occur.

CONCLUSION

An increasing understanding of the molecular basis of disease (allowing identification of potentially therapeutic transgenes) together with on-going developments in vector design (allowing targeted and regulated delivery) mean that gene therapy offers an ever-expanding range of future therapeutic possibilities. In order to harness these advances for the benefit of pregnant women it is
vital that investigation continues into the pathophysiology of obstetric diseases, and that this is translated into clinical research. Pregnancy offers unique ethical and practical challenges for the conduct of clinical trials, which would only be intensified in a trial of maternal gene therapy. It is only by addressing these challenges, however, that we can hope to provide evidence-based therapeutics to future generations of pregnant women.

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Table 1: Summary of vector spread and transgene expression following maternally administered gene therapy, pfu = plaque-forming units, vp = viral particles, PCR = polymerase chain reaction, ELISA = enzyme-linked immunosorbent assay.

<table>
<thead>
<tr>
<th>Author</th>
<th>Route of delivery</th>
<th>Animal</th>
<th>Vector(s)</th>
<th>Dose</th>
<th>Day of administration (term)</th>
<th>Days until harvesting</th>
<th>Methods of detection</th>
<th>Detected in placenta?</th>
<th>Detected in fetus?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heikkila</td>
<td>Uterine artery</td>
<td>Rabbit</td>
<td>Ad.LacZ</td>
<td>$1 \times 10^{10}$ pfu</td>
<td>14-28 (30)</td>
<td>3</td>
<td>X-gal staining and PCR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Plasmid/PEI</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Plasmid/liposome</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>David 2008</td>
<td>Uterine artery</td>
<td>Sheep</td>
<td>Ad.VEGF-A_{165} or Ad.LacZ</td>
<td>$5 \times 10^{11}$ particles</td>
<td>88-102 (145)</td>
<td>4-7</td>
<td>ELISA and PCR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Mehta 2012</td>
<td>Uterine artery</td>
<td>Sheep</td>
<td>Ad.VEGF-A_{165} or Ad.LacZ</td>
<td>$5 \times 10^{11}$ particles</td>
<td>80-102 (145)</td>
<td>30-45</td>
<td>ELISA and PCR</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Mehta 2011</td>
<td>Uterine artery</td>
<td>Guinea pig</td>
<td>Ad.LacZ</td>
<td>$1 \times 10^{10}$ vp</td>
<td>45 (65)</td>
<td>2-7</td>
<td>ELISA, X-gal staining and PCR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Internal iliac artery</td>
<td></td>
<td>Pluronic gel to outer surface of uterine and radial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Study</td>
<td>Route of Injection</td>
<td>Species</td>
<td>Vector 1</td>
<td>Concentration</td>
<td>Days to Effect</td>
<td>Days from First Dose</td>
<td>Methodology</td>
<td>Tested</td>
<td>Unclear if Tested</td>
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<tr>
<td>Xing 2000</td>
<td>Jugular vein</td>
<td>Rat</td>
<td>Ad.GFP-GT3 or Ad.CMV-GT3</td>
<td>2.3x10^{10} pfu</td>
<td>14 (22)</td>
<td>6</td>
<td>Immunofluorescence, PCR and Southern blot</td>
<td>No</td>
<td>Unclear if tested</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 doses, total 5.4x10^{10} pfu</td>
<td>14-16 (22)</td>
<td>6 days from first dose</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Katayama 2011</td>
<td>Tail vein</td>
<td>Mouse</td>
<td>Ad-RGD.Luc or WT-Ad.Luc</td>
<td>5x10^8 pfu</td>
<td>10.5 (20)</td>
<td>2</td>
<td>Immunofluorescence and PCR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Epperly 2011</td>
<td>Intravenous</td>
<td>Mouse</td>
<td>MnSOD plasmid</td>
<td>100 µg</td>
<td>13 (20)</td>
<td>1</td>
<td>Immunohistochemistry and PCR</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Zenclussen 2006</td>
<td>Maternal peritoneal cavity</td>
<td>Mouse</td>
<td>Ad.HO-1 or Ad.GFP</td>
<td>1x10^5 and 1x10^8 pfu</td>
<td>5 (20)</td>
<td>9</td>
<td>Fluorescent microscopy</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Li 2009</td>
<td>Maternal peritoneal cavity</td>
<td>Mouse</td>
<td>Ad.CTLA41g or Ad.GFP</td>
<td>1x10^5 pfu</td>
<td>5 (20)</td>
<td>9</td>
<td>Confocal laser scanning microscopy</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

1010 Vectors: Ad.CMV-GT3 = adenoviral vector expressing human glucose transporter 3 gene under control of a cytomegalovirus promoter, Ad.GFP = adenoviral vector expressing green fluorescent protein, Ad.CTLA41g = adenoviral vector expressing green fluorescent protein and cytotoxic T lymphocyte-associated antigen 4, Ad.GFP-GT3 = adenoviral vector expressing green fluorescent protein and human glucose transporter 3 gene under control of a cytomegalovirus promoter, Ad.HO-1 = adenoviral vector
expressing heme oxygenase 1, Ad.LacZ = adenoviral vector expressing β-galactosidase, Ad-RGD.Luc = modified adenoviral vector expressing firefly luciferase, Ad.VEGF-A165 = adenoviral vector expressing vascular endothelial growth factor A165 isoform, MnSOD = manganese superoxide dismutase, WT-Ad.Luc = wildtype adenoviral vector expressing firefly luciferase.
Table 2: Summary of vector spread and transgene expression following intraplacental administration of gene therapy, pfu = plaque-forming units, cfu = colony-forming units, PCR = polymerase chain reaction.

<table>
<thead>
<tr>
<th>Author</th>
<th>Animal</th>
<th>Vector(s)</th>
<th>Dose</th>
<th>Volume of injection</th>
<th>Day of administration (term)</th>
<th>Days until harvesting</th>
<th>Methods of detection</th>
<th>Detected in placenta?</th>
<th>Detected in fetus?</th>
<th>Detected in dam?</th>
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</thead>
<tbody>
<tr>
<td>Woo 1997</td>
<td>Mouse</td>
<td>Ad.RSVLuc</td>
<td>$5 \times 10^7$ pfu per placenta</td>
<td>3 μL per placenta</td>
<td>12.5 (19.5-20.5)</td>
<td>3</td>
<td>Luciferase activity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td></td>
<td>Ad.RSVLacZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X-gal staining</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Türkay 1999</td>
<td>Mouse</td>
<td>Ad.CMVntLacZ or Ad.RSVntLacZ</td>
<td>$5 \times 10^7$ to $1 \times 10^8$ pfu per placenta</td>
<td>500 nl per placenta</td>
<td>9.5 (20)</td>
<td>4</td>
<td>X-gal staining</td>
<td>Not examined</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td></td>
<td></td>
<td>Ad.CMVntLacZ or Ad.RSVntLacZ</td>
<td>$2.5 \times 10^6$ to $1 \times 10^8$ pfu per placenta</td>
<td>500 nl per placenta</td>
<td>11.5 (20)</td>
<td>2</td>
<td>X-gal staining</td>
<td>Not examined</td>
<td>Yes</td>
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<td></td>
<td>Ad.CMVntLacZ</td>
<td>$2 \times 10^8$ pfu per placenta</td>
<td>1μL</td>
<td>14.5 (20)</td>
<td>4</td>
<td>X-gal staining</td>
<td>Not examined</td>
<td>Yes</td>
<td>Unclear if tested</td>
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<td>Researcher</td>
<td>Species</td>
<td>Vector</td>
<td>Average pfu per placenta</td>
<td>Volume per placenta</td>
<td>CFUs</td>
<td>PCR</td>
<td>X-gal Staining</td>
<td>Not Examined</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Kremer</td>
<td>Rat</td>
<td>Ad.GFP-GT3 or Ad.CMV-GT3</td>
<td>0.75 to 1.125x10^8 pfu per placenta</td>
<td>5-7.5μL per placenta</td>
<td>14 (22)</td>
<td>5-6</td>
<td>PCR</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Senoo</td>
<td>Mouse</td>
<td>Ad.CALacZ</td>
<td>3x10^6 pfu per placenta</td>
<td>Not stated</td>
<td>14 (20)</td>
<td>3</td>
<td>X-gal staining</td>
<td>Not examined</td>
<td>No</td>
<td>Unclear if tested</td>
</tr>
<tr>
<td>Katz</td>
<td>Mouse</td>
<td>Ad.LacZ</td>
<td>1x10^8 pfu per placenta</td>
<td>5 μL per placenta</td>
<td>14 (20)</td>
<td>3</td>
<td>X-gal staining</td>
<td>Yes</td>
<td>Yes (1 of 9)</td>
<td>Not examined</td>
</tr>
<tr>
<td>Keswani</td>
<td>Rabbit</td>
<td>Ad.LacZ</td>
<td>1x10^7 pfu per placenta</td>
<td>40 μL per placenta</td>
<td>21 (30)</td>
<td>2</td>
<td>X-gal staining</td>
<td>Yes</td>
<td>Equivocal</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Vectors:** Ad.LacZ = adenoviral vector expressing β-galactosidase, Ad.CMV-GT3 = adenoviral vector expressing human glucose transporter 3 gene under control of a cytomegalovirus promoter, Ad.GFP-GT3 = adenoviral vector expressing green fluorescent protein and human glucose transporter 3 gene under control of a cytomegalovirus promoter, Ad.RSVLuc = adenoviral vector expressing firefly luciferase under a respiratory syncytial virus promoter, Ad.RSVLacZ = adenoviral vector
expressing β-galactosidase under a respiratory syncytial virus promoter, Ad.CMVntLacZ = adenoviral vector expressing β-galactosidase under a cytomegalovirus promoter, Ad.RSVntLacZ = adenoviral vector expressing β-galactosidase under a respiratory syncytial virus promoter, LZ.RSLacZ = lentiviral vector expressing β-galactosidase, Ad.CALacZ = adenoviral vector expressing β-galactosidase under a synthetic CAG promoter