# *In vitro* and *In vivo* Gene Transfer to Respiratory Epithelium in Experimental Models of Fetal Gene Therapy for Cystic Fibrosis

**By**

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# Abstract

Fetal gene therapy is a novel technique for correction of monogenic disorders, such as cystic fibrosis. The aims of this study were to: i) develop small animal models for fetal gene transfer to respiratory epithelium; ii) to determine the ideal gestational timing of transfection in our models; iii) evaluate *in vivo* gene transfer techniques which are directly applicable to human fetal therapy; and iv) assess the durability of *in vivo* fetal gene transfer postnatally.

Vesicular Stomatitis Virus-G (VSV-G) pseudotyped lentiviral vector was used, which contains Green Fluorescence Protein (GFP), as the reporter gene. Fetal tracheas from time-mated New Zealand White pregnant rabbits were harvested on gestational day 24, 25 and 26 (term=31d) and put in organ culture medium, then transfected with 1 x $10^6$ viral particles. Following in vitro transfection, fetal tracheas began to express marker gene as early as one day after infection, with peak expression noted by day 7 post transfection. Results were confirmed by Polymerase Chain Reaction (PCR) and Immunohistochemistry (IHC). Based on the observation that 4 days of in vitro culture was necessary to achieve substantial marker gene expression, we evaluated two *in vivo* injection techniques on or before day 26 of fetal gestation: i) fetal tracheal injection (TI) following hysterotomy and partial fetal delivery, and ii) amniotic injection (AI) without hysterotomy. After injection of 1 x $10^6$ virus particles, fetuses and their control littermates were delivered by caesarean section on gestational day 30. Fetal tissues (trachea, lung, gut, liver, kidney gonad) were harvested and marker gene expression was confirmed by fluorescent microscopy, PCR and IHC. By PCR, there was evidence of extensive transduction of fetal tissues by AI (trachea, lung, gut, liver, kidney, skin), yet

selective transduction of trachea and lung by TI. IHC localized airway expression of GFP to tracheal surface epithelium and pulmonary alveolar cells. Control fetuses expressed marker gene following TI, presumably through blood borne transmission via placental vascular connections, while comparatively few control fetuses were positive for marker gene after AI. One doe was found to have GFP DNA in lung after TI, suggesting that maternal infection is possible with this model.

Marker gene expression was also observed in mid-gestational fetal CD1 mice following AI. Transfected fetuses were survived and sacrificed at various post-natal time points to test the durability of gene expression by Quantitative real-time PCR. The marker gene was detectable as late as 21 days after birth; however there was evidence that although transgene was detectable by PCR in trachea 3 weeks after birth, there was no corresponding GFP mRNA, suggesting that transgene expression may be "switched off".

Lentiviral vector mediated gene transfer using direct amniotic injection techniques exhibited transduction of respiratory epithelium in fetal rabbits and mice *in vivo*. This experimental system should prove useful for future fetal gene therapy studies.

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# ABBREVIATIONS

| | |
|---|---|
| AAV | adeno-associated virus |
| ACOG | American College of Obstetrics and Gynecology |
| Ad | adenoviral vector |
| AF | amniotic fluid |
| AI | amniotic injection |
| BGS | bovine growth serum |
| CAR | coxsackie adenovirus receptor |
| CF | cystic fibrosis |
| CFTR | cystic fibrosis transmembrane conductance regulator gene |
| CMV | cytomegalovirus |
| CVS | chorionic villus sampling |
| DMEM | Dulbecco's Modified Eagle's medium |
| EGTA | ethyleneglycol-tetraacetic acid |
| EIAV | equine infectious anemia virus |
| FBS | fetal bovine serum |
| FIV | feline immunodeficiency virus |
| G | gestation |
| GFP | green fluorescent protein |
| GTAs | gene transfer agents |
| IL-8 | interleukin-8 |

| | |
|---|---|
| LPC | lysophosphatidylcholine |
| LTR | long-terminal repeats |
| LV | lentivirus |
| MoMLV | moloney murine leukemia virus |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PolyA | polyadenylation |
| PUBS | percutaneous uterine blood sampling |
| RCL | replication competent lentivirus |
| RCR | replication competent recombinant |
| SCID | severe combined immunodeficiency disease |
| VSV-G | vesicular stomatitis virus G protein |

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# CHAPTER 1

# INTRODUCTION

## Cystic Fibrosis

### 1. Overview:

Cystic fibrosis (CF) is one of the most common autosomal recessive disorders. Although it is found in all ethnic groups [1], CF is most common among Caucasians and occurs in approximately one in 2,500 newborns in this population [2]. About 1 in 25 Caucasians are carriers. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) located on chromosome 7, which was cloned in 1989 [3]. The mutations lead to the defective production of the protein which is a c-AMP regulated chloride channel located in the apical membrane of epithelial cells causing imbalanced ion and water movement across the epithelium, resulting in accumulation of sticky mucus [4], bacterial infection and inflammation. This creates a disease complex with a wide range of disorders including chronic obstructive pulmonary disease, liver fibrosis, diabetes mellitus, cholelithiasis and arthritis. Although gut, liver, pancreas, reproductive tract, bronchi and lung are all affected; respiratory infections and lung disease are the major cause of morbidity and mortality.

Advances in medical treatment have increased life expectancy of CF patients; the estimated median survival age for those born in the 1990's is now 40 years [5]. Current therapies for CF are all palliative in nature. With the development of gene transfer techniques, the potential for disease treatment through defective gene replacement becomes theoretically possible.

## 2. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene:

The CFTR gene was cloned in 1989 by teams led by Lap-Chee Tsui and John Riordan at the Hospital for Sick Children, Toronto [3]. The CFTR gene encodes for a transmembrane protein, a member of the ATP Binding Cassette (ABC) family, which acts as chloride ion channel in cells lining the respiratory system. It is regulated by cyclic AMP (cAMP) through $\beta_2$ adrenergic receptor stimulation [6], PKA- and PKC-dependent phosphorylation [7]. In addition to its regulatory role in chloride ion transport, CFTR regulates other secretory channels, including the epithelial amiloride-sensitive Na channel (EnaC) [8], outward rectifying chloride channel [9, 10], renal potassium channel [11] and calcium activated chloride channel [12]. The CFTR protein has also been shown to play roles in the mediation of vesicular trafficking [13] and affects glycosylation. Despite its many cellular functions, CF pathology is defined by mutations of CFTR which alter transepithelial chloride transport [14], leading to the dehydration of surrounding respiratory and intestinal mucosa and impaired sodium re-absorption of the sudoriferous glands. The net effect in organs such as the lungs, intestine, pancreas and liver is decreased mucociliary clearance leading to luminal inspissation which is considered to be the basic pathology of CF. The CFTR gene is located on chromosome 7q31.2 and has 27 exons over 250 kilobase pairs that code for 1,480 amino acids [15, 16]. Expression of 6.5kb CFTR mRNA has been detected in the lung, colon, pancreas, liver and sweat glands. To date nearly 1,000 mutations have been identified in the CF gene [http//:www.genet.sickkids.on.ca/cftr/]. Approximately 1 in 25 Caucasians carries a

mutation in CFTR [2]. Mutations can be divided into six classes based on the molecular fate of CFTR [17, 18]. (1) CFTR synthesis defects, which lead to the absence of CFTR production; (2) defective processing resulting in the production of abnormal CFTR; (3) disrupted activation and regulation at the cell membrane; (4) reduced chloride conductance; (5) partly defective production or processing; (6) defective regulation of other channels. About 70% of CF patients have a phenylalanine deletion at residue 508 of the CFTR protein (termed ΔF508), which is termed a Class II mutation. These mutations can cause quantitative and qualitative problems with CFTR and result in disease of variable severity [1]. The genotype/phenotype correlations are not always consistent, hence the exact role of the CFTR gene in the development of CF disease is not completely understood [19].

## 3. Carrier Screening and Prenatal Diagnosis:

The American College of Obstetrics and Gynecology (ACOG) and other organizations recommend that all Caucasian couples who are pregnant or are considering pregnancy be offered carrier screening for CF [20]. With a 1 in 25 carrier rate for defective CFTR, "carrier couples" face a 25 percent chance of having an affected child with each pregnancy. With the development of DNA analytical techniques, it is possible to detect mutations and polymorphisms in the CF gene. Genetic counseling is highly recommended for those "carrier couples" so that personal choices and options can be discussed. The options include preimplantation diagnosis, prenatal diagnosis through chorionic villus sampling or amniocentesis, and cord blood testing when the baby is born [21].

# Gene Therapy

## 1. Overview:

Gene therapy is a broad term that includes any strategy designed to correct heritable gene defects by introducing a gene, gene segments or oligonucleotides into the cells of an affected individual. Somatic cells have been the only targets of human gene therapy to date [22]. Two fundamentally different techniques have been applied in the gene transfer process: (1) ex vivo or autologous cell transfer: By this approach, target cells are obtained from an afflicted patient, and the replacement genes are inserted into these cells in vitro, and then reimplanted into the patient. Protocols based on this methods have been proposed for epithelial and endothelial cells [23, 24], fibroblasts [25, 26], lymphocytes [27], hematopoietic stem cells [28]; and (2) in vivo gene therapy which is currently the most commonly practised technique. With this technique, genetic material is transferred directly into cells within a patient. Many human disease are currently considered for somatic gene therapy, such as inherited single gene disorders, multifactorial disorders (e.g., cancer and cardiovascular disease), and infectious diseases [29]. Several vector systems which deliver DNA to the targeted cells followed by the therapeutic expression of transgene-encoded proteins are in use, including viruses (e.g., retroviruses and adenoviruses), pure DNA molecules and whole chromosomes [30].

## 2. Gene delivery vectors:

Gene transfer systems also known as "vectors" play a central role in target cell gene delivery, and a number of different vectors have been developed and are under

investigation for efficacy of in vitro and in vivo gene transfer. An ideal vector should have the following characteristics: (1) can be produced in large amounts at high concentration; (2) method of production is convenient and reproducible (3) capable of precise and stable chromosomal introduction of the transgene; (4) the inserted transgene is responsive to its regulatory elements; (5) can target specific cell types; and (6) does not elicit host immune response. Unfortunately, no vectors currently exist which possess all of these characteristics.

**Adenoviral vector (Ad)** was the first viral vector to be tested in primates and in human trials. This vector is derived from wild-type adenovirus which is a non-enveloped, linear, double-stranded DNA virus. Both adenovirus and adenoviral vectors (derived from wild-type adenovirus) can bind to the cell surface of dividing and non-dividing cells, following two receptor interactions: the Coxsackie Adenovirus Receptor (CAR) [31] and integrin cell surface receptor [32]. These receptors are located at the basolateral membrane of intact polarized airway epithelial cells [33]. This is one of the limitations of infection efficiency of adenoviral vectors when they are used to infect airway epithelial cells. Although this can potentially be overcome by replacing the receptor binding elements of the capsid, this has not yet been reasonably achieved [34-36]. Another problem is the generation of antibodies to adenoviral proteins by a cytotoxic T-cell response, which targets the host cells containing the vector [37]. Ad is also a common airway pathogen, so pre-formed antibodies in potential recipients may reduce vector transfection efficacy. Clinically, the application of adenoviral vector can cause acute and potentially life threatening inflammatory response due to antigen presenting cells [38-40], and an extensively reported fatality resulting from this inflammatory response [41] has

substantially reduced the interest in Ad as the "vector of choice" for gene therapy trials in humans.

**Adeno-associated virus (AAV)** is a non-pathogenic human parvovirus which is widespread in 80% of the human population. AAV genome is a linear single-stranded DNA molecule. AAV vectors can efficiently transfect dividing and nondividing cells, including human airway epithelial cells [42, 43]. AAV integrates into a defined region of the cellular genome which is thought to be safe since insertion does not appear to be associated with human disease, however, current AAV vectors, presumably as a result of gene deletion, appear to integrate non-specifically. The receptor for AAV is located on the basolateral membrane of airway epithelial cells [44]. To date, sub-type AAV6 vector appears to be the most effective in transfecting airway epithelium [45]. A major disadvantage with AAV vectors is its small genome, which limits the size of a transgene to 4-5 kb. This makes it difficult to transport CFTR (6.5 kb) under a cell specific promoter. This may be overcome by making use of homologous recombination between two partial vectors [46]. The experience with AAV vectors in human clinical trials will be described below.

**Retroviruses** are a large and diverse family of enveloped RNA viruses. They are broadly categorized into simple and complex, which are further divided into seven classes [47]. A few viruses in this group, such as moloney murine leukemia virus (MoMLV) and lentivirus have been developed as gene transfer vectors. Retroviral vectors have been extensively used in the laboratory for stable expression of transgene cDNAs. Compared to Ad and AAV vectors, retroviral vectors can integrate their transgene into the chromosomes of target cells which may lead to long-term expression. They do not

transfer virus-derived coding sequence, avoiding the recognition and destruction of transduced cells by vector-specific cytotoxic T lymphocytes [48]. Furthermore, LV vectors (LV) can transduce dividing and nondividing cells (other retroviruses, such as MoMLV can only transduce dividing cells); this is due to mitosis-independent nuclear import of the preintegration complex [49]. Studies on human immunodeficiency virus 1 (HIV-1) derived lentiviral vectors to date show no evidence of cellular immune response at the sites of administration in vivo [50]. These characteristics of LV make them one of the most promising viral vectors in human gene therapy. The best known and most actively studied LV are HIV-1 and 2, simian immunodeficiency viruses (SIV), feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). Integration of retroviral DNA can occur at many sites on target DNA, although it is not entirely random, which may activate or inactivate nearby genes (insertional mutagenesis) [51]. This is a major concern in the use of retroviral vectors. Attempts to manipulate the site of integration have achieved moderate success but further studies are necessary [52]. Knowledge of the roles of *cis* and *trans*-acting elements of HIV-1 led to the first generation of HIV-1 based LV [53], in which the packaging signal was deleted and the 3' long terminal repeats (LTR) were replaced with the polyadenylation (polyA) sequence from simian virus 40 (SV40). Naldini *et al* described a similar vector system which expressed the core proteins, enzymes and accessory factors from heterologous transcriptional signals and the envelope from the vesicular stomatitis virus G-glycoprotein (VSV-G) [54]. VSV-G pseudotyped LV vectors have a broad cell tropism and the advantage of stability, so higher titers of vectors can be produced by ultracentrifugation. "First generation" LV vectors have been used to transduce neurons in

vivo and several months of transgene expression (more than 5 months in liver and more than 8 weeks in muscle) have been achieved [55] without an observed immune response. In "second generation" LV vector systems, all HIV-1 accessory genes (*vif, vpr, vpu* and *nef)* were deleted, while the packaging component was reduced to the *gag, pol, tat* and *rev* genes [56]. These modifications had no effect on vector titer and provided a significant increase in biosafety. Finally, a "third generation" of LV vector has been developed by the deletion of 3'-LTR in the U3 region to create a so-called self-inactivating (SIN) vector [57]. This vector system only comprises three of the nine genes of HIV-1: *gag*, coding for the virion main structural proteins; *pol*, coding for the enzymes; and *rev*, responsible for a post-transcriptional regulator necessary for efficient *gag* and *pol* expression [58]. These modifications result in transcriptional inactivity and minimize the risk of emergence of replication competent lentivirus (RCL). It may also reduce the risk of tumorigenesis via promoter insertion. Although many *in vitro* and *in vivo* studies have been done by using LV on animal models, no clinical trials have been undertaken to date. Due to the absence or low numbers of VSV-G receptors on the apical membrane of the airway epithelium (VSV-G receptors are located on the basolateral surface), HIV-1 derived vectors cannot transduce fully differentiated airway epithelium unless it is first injured by the calcium chelator ethyleneglycol-tetraacetic acid (EGTA) or after inhalation exposure to sulfur dioxide ($SO_2$) [59, 60]. In another study, pretreatment of murine airways with lysophosphatidylcholine (LPC) led to sustained gene expression of up to 92 days [61]. Because the cell turnover time of rodent airway epithelium is thought to be about 3 months, this result suggested that HIV integration into stem/progenitor cells in the airways was likely. It has been shown that LV pseudotyped with envelope

glycoproteins from the filoviruses Ebola or Marburg can transfect airway epithelia cells via the apical membrane [62]. However, third generation VSV-G pseudotyped LV vectors have demonstrated highly efficient transduction of murine airway epithelium due to the insertion of cPPT (the polypurine tract in the central position) and WPRE (post-transcriptional regulatory element of the woodchuck gene), which are additional *cis*-acting regulatory sequences [63]. With regard to the functional study of CFTR gene transfer, the only study that has evaluated LV- mediated, therapeutic gene transfer in a murine disease model of CF is that by Limberis *et al.* [64]. In this study, an HIV-1 derived LV vector formulated with LPC for the transduction of CFTR gene resulted in partial recovery of the chloride transport defect which was evaluated by transepithelial potential difference in the nasal airway epithelium of CF knockout mice for at least 110 days. Goldman *et al.* showed that a first generation VSV-G pseudotyped HIV-1 derived vector was able to transduce CFTR gene into poorly differentiated human bronchial xenografts and the expression normalized both the chloride transport defect and reverted the bacterial killing activity [65]. From available experimental data, LV-vector mediated gene transfer to airway epithelium has been shown to be limited by the paucity of viral receptors on the apical surface, although this limitation has been largely overcome by either pseudotyping with heterologous envelopes or modification of paracellular permeability.

## 3. Gene therapy for CF:

Standard, current treatment for CF is palliative. It improves the quality of life and extends lifespan to a degree, but cannot offer a cure. With the development of gene

transfer techniques and the identification of the CFTR gene in 1989, the theoretical paradigm of cure through CFTR gene replacement becomes possible. Gene therapy is particularly attractive not only for CF, but for a substantial list of monogenic disorders for which satisfactory treatment options are non-existent. These diseases include metabolic disorders such as glycogen, sphingolipid and mucopolysaccheride storage diseases, neuromuscular dystrophies, and immunologic/hematologic disorders such as severe combine immunodeficiency syndrome and the thalassemias [66].

In CF patients, respiratory infection is a common complication and the lung disease is the major cause of morbidity and mortality. Gene therapy for CF may be the ultimate curative treatment based on the hypothesis that transfer and expression of the CFTR cDNA in the airway epithelium may result in the restoration of CFTR function and improvement of the respiratory manifestations of the disease [67]. Since the CFTR gene was sequenced in 1989, there has been a sustained experimental interest in gene therapy for CF. Almost immediately, in vitro transfer of CFTR to human respiratory epithelium by retroviral vector was established [68]. Shortly thereafter, adenovirus-mediated gene transfer was carried out to the airway epithelium of animals [69, 70].

The first clinical trials in CF patients were carried out in 1993, and to date there have been 29 published Phase 1 and 2 clinical trials of gene therapy for CF. The majority of these trials have involved the instillation of large volumes of vector-containing fluid into lung via nasal or oral inhalation of aerosolized vectors. Replication-deficient adenovirus (Ad), cationic liposomes and adeno-associated virus (AAV) have been used to deliver CFTR cDNA to CF patients [71, 72]. Both viral and cationic liposome transgene delivery in the nose can produce some gene transfer and some degree of correction of chloride

transport. In the early days of CF gene therapy, Ad vectors were widely used, however gene transfer and transgene expression was inefficient and of short duration (< 30 days), following initial transfection, and thereafter could not be achieved beyond a third administration of vector due to the formation of specific antibodies [73]. Cationic liposomes have been used to treat CF patients in 8 clinical trials; two involving nebulisation of the lipoplexes into the lower airways [72, 74]. Gene transfer to nasal and respiratory epithelial cells with cationic liposome has resulted in demonstrable CFTR cDNA and correction of the chloride defect lasting up to 3 weeks, without an apparent inflammatory reaction. However, mild flu-like symptoms were observed following aerosolisation of liposome-DNA complexes. The correction of the chloride permeability might not specifically target CFTR-expressing cells[75]. Of all vector systems studied to date in human gene therapy for CF, AAV has held the greatest promise on the basis of the duration of transgene expression (up to 10 weeks), its biosafety profile and low immuno-reaction compared with other viral vectors [76, 77]. Studies of AAV-2 mediated CFTR gene transfer, have demonstrated the persistence of vector DNA in maxillary sinus epithelium for up to 10 weeks [77] and for between 14 to 30 days in the large airways [78]. AAV is a nonpathogenic parvovirus, and has been isolated from the respiratory and GI tracts of humans. Despite these favorable characteristics, the ability of AAV to transduce with repeated administration has not yet been established. A phase I trial was conducted in 2001, in which aerosolized AAV2-CFTR was administered into CF patients with mild to moderate lung disease. The vector was detected in the proximal airways, while vector-specific mRNA was not found [78]. A phase II trial of maxillary sinus instillation of AAV-2 CFTR in CF patients with mild to moderate disease demonstrated

significant improvement in the primary end points of time to sinusitis relapse, histopathology and interleukin-8 (IL-8) levels in treated patients compared to controls. On the other hand, transduction efficiency was likely lower in patients with moderate to severe disease as a result of the increased inflammation and sputum barrier[79]. The small packaging capacity of AAV (<5kb) also limits the application of this vector to transfer of larger genes; although it is large enough for CFTR cDNA (the open-reading frame alone spans 4443 bp), there is insufficient space to include promoter or enhancer elements. New techniques including trans-splicing and homologous recombination have been developed to overcome the AAV packaging problem [46, 80], and have shown that AAV2/6 recombination-dependent vector transduced lung airway cells in mice almost as efficiently as intact vector, with 10% of airway epithelial cells being positive. It has been postulated that AAV may not infect dendritic cells and may thereby avoid activation of the host immune system. In general, AAV vectors hold promise for human gene therapy as they appear to be safe and produce stable transgene expression. These Ad, AAV and cationic liposome vectors are the only ones that have been applied to clinical trials in patients with CF, others which include murine leukemia retroviruses and LV vectors, herpes-viruses, DNA viral vectors based on SV40, and those based on Epstein-Barr virus, papilloma virus and poxviruses are under study and development. In summary, in the 15 years following the cloning of the CFTR gene, considerable progress towards gene replacement for CF has been made, yet, sodium hyperabsorption has not been consistently altered in clinical trials. Gene transfer agents (GTAs) used so far have achieved limited success and the efficiency of in vivo gene transfer with currently available vectors needs to be improved [81]. Although most human trials of gene therapy

for CF demonstrate "proof of principle" transfer of gene to respiratory epithelium, it seems unlikely that human gene therapy as practised currently, will have a significant impact on outcome from CF.

## 4. Fetal gene therapy:

The concept of fetal gene replacement for genetic disorders depends on the ability to make an accurate genetic diagnosis in a fetus, and evidence to justify fetal intervention from an ethical viewpoint that recognizes the natural tension that exists between potential fetal benefit and maternal harm. CF is an example of a genetic condition associated with fetal pathology that can present in the newborn in the form of neonatal bowel obstruction from inspissated meconium called meconium ileus, which often requires immediate surgical intervention. One issue of controversy regarding the role of gene therapy in treating CF is understanding the continuum between genetic disease and end organ disease (lung, liver or pancreas), and understanding at which point treatment aimed at defective gene replacement becomes inefficacious in treating genetic disease. Pivotal to these discussions is the concept of the so-called "point of no return", when this occurs, and what window of therapeutic opportunity exists during which the genetic disease can be cured before organ disease develops [82]. Based on these considerations, one can reasonably justify a treatment strategy of fetal treatment for CF provided the likelihood of maternal harm or altered maternal reproduction approaches zero. In diseases which manifest in the fetal period, postnatal gene transfer may not be able to prevent irreversible tissue damage. Modern techniques of genetic diagnosis enable screening for genetic diseases, such as CF, based on the family history, abnormalities detected on

maternal serum screening and fetal features seen by ultrasound. The CF gene mutations can be detected in fetal cells sampled by amniocentesis, or other specific techniques of fetal tissue sampling including chorionic villus sampling (CVS), percutaneous uterine blood sampling (PUBS) or direct sampling during fetoscopy. Since tissue-specific gene expression has also been defined, it becomes possible to target therapeutic gene expression to the desired cell types, (such as airway epithelial cells), at an early stage of organ development. The major theoretical advantages of fetal compared to postnatal gene therapy are: (1) restitution of gene expression may avoid irreversible tissue injury; (2) transfection efficiencies associated with lower doses of vector needed to infect a fetus compared to an adult; (3) fetal cells are proliferating rapidly, and may be more susceptible to genetic integration of delivered genes, hence the accessibility barrier that results from the basolateral location of viral receptors in differentiated adult respiratory epithelium may be overcome by infecting undifferentiated fetal respiratory cells. (4) the relative abundance of stem cells and rapid proliferation of progenitor cells in the fetus which make this environment uniquely suitable for gene therapy; (5) the immaturity of the fetal immune system which diminishes the likelihood that viral vectors, transgene or gene product will be perceived as foreign by the host; (6) the fetus breathes and swallows amniotic fluid (AF), which means that standard techniques of amniotic cavity access (amniocentesis) should allow transgene delivery to target sinus, respiratory and gastrointestinal epithelium.

## 5. Animal Models Used in Experimental Fetal Gene Therapy:

5.1 Fetal Rabbit Model: The fetal rabbit has been used to develop disease and fetal treatment models for a number of conditions including gastroschisis [84], intrauterine

growth restriction [85], meningomyelocele [86], craniofacial anomalies [87], fetal uropathy [88], and congenital diaphragmatic hernia [89]. The advantages of fetal rabbit model compared to other fetal models (primate, sheep) include its short gestation period (term of 31d), fetal tolerance of surgical intervention, and the number of fetuses available for study within a single gestation (conveniently separated into 2 uterine horns). The reported experience with gene transfer in rabbits is limited to AAV vector, with only a single report of *in vivo* fetal rabbit gene transfer. In one study of neonatal gene therapy, Zeitlin PL *et al.* demonstrated that AAV mediated gene transfer into newborn rabbits following intra-tracheal instillation as assayed by LacZ transgene expression occurred preferentially in the alveolar epithelial progenitor cells, type II cells and in the large airway tracheal-bronchial basal and ciliated cells [95]. In 1997, Rubenstein RC *et al.* from the same laboratory instilled AAV vector with CFTR transgene into the tracheas of day 3 neonatal rabbits and showed that transgene expression lasted up to 6 weeks throughout the alveolar phase of lung development [96]. To date, only one group has described in utero gene transfer to rabbit pulmonary epithelium [97]. They demonstrated that intra-amniotic injection of a AAV-LacZ vector to fetal rabbits at 24-25 days gestation resulted in transgene expression in amniotic membranes, trachea and pulmonary epithelium. Transgene expression in lung peaked at 10 days after vector delivery, was decreased by day 17, and was no longer detectable after 24 days. Transgene expression was not detected in fetal rabbit intestines, skin or liver, nor in maternal ovaries or liver.

5.2 Fetal Mouse Model: In comparison to rabbits, mice models used for fetal gene transfer have been extensively studied, but to date, none have specially investigated the

ability of LV vectors to infect fetal respiratory epithelium in *vivo*. Ad, AAV, and LV have been used in a variety of in vivo models of fetal gene therapy.

Gene transfer to airways of fetal mice by intra-amniotic delivery was first reported by Holzinger *et al.* [100]. In this study, Ad carrying the LacZ gene was instilled into the amniotic cavity of fetal mice at 0.8 term gestation. Tissues were harvested after 3 days. Airways of fetal mice were infected but at very low efficiency. However, McCray *et al.* reported that Ad mediated gene transfer could not be achieved on pulmonary epithelium of gestational 15 fetal mice through amniotic injection [101]. Positive results were achieved on gestational day 15 fetal mice by amniotic injection of Ad[102, 103]. Douar *et al.* also found that no expression was detected in the tracheobronchial airways, the alveoli or the gastro-intestinal tract when injection was performed in fetuses younger than day 15 (day 13-14) or day 16. Lack of transfection in day 13 and 14 fetuses was attributed to minimal fetal breathing prior to gestational day 15 which impedes delivery of virus into the lower airways against the general outward flow of the fluid from the lungs. The reason for lack of infection of fetuses on day 16 was unclear. Contradicting results were reported by Masato et al. [104], who found that Ad vector coded LacZ gene injected into amniotic fluid on gestational day 14 mice, resulted in transgene expression in the lung and epithelium of the digestive tract within 3 days. These inconsistent results likely reflect the general limitations of Ad used for gene transfer, such as the cellular and humoral immune responses to viral gene products which limit the duration of transgene expression and prevented the re-administration of the same vector [101, 105-107].

In contrast, AAV vector transfection has proved far more successful in murine models. Successful transgene expression by AAV was achieved in both lungs and intestines on gestational day 15-16 mice through intra-amniotic injection [108].

## Hypothesis and Specific Objectives:

We hypothesized that short term, LV vector mediated gene transfer could be successfully performed in small animal fetal models (rabbit and mouse). The first objective of our rabbit studies was to create a whole fetal rabbit tracheal culture system to accomplish LV-mediated reporter gene transfer to respiratory epithelium *in vitro*. This would enable determination of the time required for transduction and transgene expression and could be used to target a specific gestational age at which in vivo transfection (with transgene expression at term) could be attempted. In the second part of our rabbit experiments, we sought to evaluate two techniques of in vivo transfection: direct tracheal injection and direct amniotic injection; both are techniques that could be theoretically applied to human fetuses.

The objective of the mouse experiments was to build upon a "proof of principle" established in the fetal rabbit model, and determine the postnatal persistence of transgene expression in fetuses transfected by amniotic injection.

# CHAPTER 2

# MATERIAL AND METHODS

## A. Lentiviral Vector Preparation and Purification

### A. 1 Subculturing of 293T Cell Line:

293 cell line is a human embryonic renal epithelial cell line which is transformed by adenovirus E1A gene product. 293T is a highly transfectable derivative of the 293 cell line into which the temperature sensitive gene for SV40 T antigen has been inserted. It allows episomal replication of plasmids containing the SV40 origin and early promoter region. It has the property of being highly transfectable by calcium phosphate transfection method. Up to 50% efficiency is attainable. The 293T cell line used in this study was kindly provided by Dr. William Jia (Brain Research Centre, University of British Columbia).

293T cells were grown in commercially-made Dulbecco's Modified Eagle's medium (DMEM; HyClone, Logan, Utah) containing 4.00mM L-glutamine, 4.5g/L glucose and supplemented with 10% heat-inactivated bovine growth serum (BGS)(HyClone; Logan, Utah) and 1% antibiotic-antimycotic (containing 10,000units/mL penicillin G sodium, 10,000µg/mL streptomycin sulfate, 25µg/mL amphotericin B in 0.85% saline)(GIBCO™ Invitrogen CO. Burlingto, Ontario) at 37°C in a 5% $CO_2$ water jacketed incubator (model 3110) (ThermoForma Series II; Marietta, Ohio). Adherent 293T cells (See Figure 1), were grown in 100mm tissue culture dishes (nunc™; Roskilde, Danmark).

Subculturing normally was performed every 3 days in a Class II A/B3 biological safety cabinet (NUAIR™; Plymouth, MN) to maintain the cell line. In brief, the culture supernatant was discarded and the cell monolayer was rinsed with 1x PBS to remove all traces of serum that contains trypsin inhibitor. 1mL of Trypsin-EDTA solution was added dropwise to the plate which was put back to the 37°C incubator for 2-4 minutes to disperse the cells without agitation. 5mL of complete growth medium was added to neutralize the Trypsin-EDTA and cells were aspirated by gently pipetting to make a single cell suspension. A subcultivation ratio of 1:4 was used to aliquot the cell suspension to new culture plates which were returned to a 37°C incubator.

## A. 2 Preparation of VSV-G Pseudotyped Lentiviral Vector:

Lentiviral vector (LV) used in this study was first generation vector, kindly provided by Dr. William Jia (Department of Surgery, Brain Research Centre, UBC). It comprises the transgene cassette flanked by cis-acting elements necessary for its encapsidation, reverse transcription and integration. The HIV-1 envelope gene is deleted in the packaging plasmid, so the tropism to T lymphocytes and macrophages is defective. This minimizes the risk of emergence of replication competent recombinants (RCR). It was constructed from three plasmids (See Figure 1):

i) pHR'CMV-eGFP: this transfer plasmid contains the reporter transgene green fluorescent protein (GFP) under the control of human cytomegalovirus (CMV) promoter and the minimal cis-acting elements of HIV. GFP expression makes its target cells fluorescent (microscopy, flow cytometry).

ii) pCMVΔR8.2: this packaging plasmid contains all HIV-1 core and enzymatic components of the virion except the envelope gene.

iii) pMD.G: this envelope plasmid is derived from vesicular stomatitis virus (VSV-G). Its G protein has high stability and broad tropism.

## A. 2.1 Plasmid DNA Transformation and Purification:

DH5α competent cells (Invitrogen; Burlington, Ontario) were slowly thawed on ice, and 100uL of cells was dispensed immediately into a pre-chilled 1.7mL eppendorf tube containing 10ng plasmid DNA. The tube was gently agitated to mix and then was placed on ice for 30 minutes. The tube was then placed in a 37°C water bath for 45 seconds (heat-shock) and then was put back in ice for 2 minutes. 9 volumes SOC media were added to the tube and then the tube was put into an Incubator- Shaker for 45 minutes at 37°C, 230rpm. Cells were spun down at 5000rpm for 1 minute, media was aspirated and 200uL of media was left in each tube for resuspending the pellet. Aliquots of transformation culture were spread on LB/Ampicillin plates, which were incubated for 12 to 16 hrs at 37°C incubator.

The next day, a single colony of cells was taken up into a 14mL polystyrene tube which contained 5mL LB with 0.1% Ampicillin. The tube was agitated by the Incubator-Shaker for 8 hrs at speed of 250rpm at 37°C. After that, the media in the tube was poured into a 1000mL flask containing 300mL LB with 0.1% ampicillin, which was agitated overnight at a speed of 250rpm at 37°C.

A QIA filter Plasmid Midi and Maxi Kit (QIAGEN Inc.; Mississauga, Ontario) was used to purify plasmid DNA according to the protocol in the handbook offered by

the company. In brief, the cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C. The pellet was resuspened in 10mL Buffer P1 (300mL cells/10mL P1). 10mL P2 was added to lyse the cells which were incubated at room temperature for 5 minutes. 10mL pre-chilled P3 was added to the lysate and the lysate was poured into the barrel of the QIAfilter Cartridge. The lysate was incubated at room temperature for 10 minutes. During this period, a QIAGEN-tip was equilibrated by applying 10mL QBT buffer. Then the cell lysate was filtered into the previously equilibrated QIAGEN-tip by inserting the plunger into the QIAfilter Cartridge. QIAGEN-tip was washed with 2 x 30mL buffer QC. DNA was eluted with 15mL buffer QF followed by adding 10.5mL isopropanol to the eluted DNA for precipitation, and the sample was centrifuged immediately at 15,000 x g for 30 minutes at 4°C. The DNA pellet was washed again with 5mL 70% ethanol and centrifuged at 15,000 x g for 10 minutes. After the pellet was air-dried for 10 minutes, the DNA was dissolved in 200uL TE buffer (PH 8.0) (from 300mL of cells). Finally, the DNA concentration was determined by UV spectrophotometery. The plasmid DNA was kept in -20°C.

## A. 2.2 LV Vector Production, Concentration and Titration:

Calcium phosphate co-transfection method (ProFection Mammalian Transfection system; Promega Corporation; Madison, USA), was used for lentiviral vector production. The day before co-transfection, 293T cells were seeded in 100mm culture dishes at a density of 2 x $10^6$ cells per dish and grown in 10ml DMEM containing 10% BGS and 1% antibiotics, so the cells would be 50% confluent on the day of transfection. The following day, three hours prior to transfection, medium was replaced with fresh pre-warmed

DMEM containing 5% BGS growth medium. All kit components were thawed and warmed to room temperature. Working in a Class II A/B3 tissue culture hood (NUAIR™; Plymouth, MN), the transfection mixture for one 10cm plate was prepared by mixing 8μg pHR'CMV-eGFP, 8μg pCMVΔR8.2 and 4μg pMD.G with sterile, deionized water. Then the 2M $CaCl_2$ 62μl was added and mixed again. 2X HBS 500μl was added to the second polystyrene tube. During gentle vortexing, the DNA solution was added dropwise, and the mixture was incubated at room temperature for 30 minutes, followed by a second vortexing. This solution was added dropwise to each plate. The precipitate was distributed evenly over the cells by swirling the plate. The plates were returned to the incubator. Fourteen to 16 hours later, the medium was changed with pre-warmed 10ml DMEM containing 5% BGS. The plates were put back to the incubator. The next day, the plates were checked under the fluorescent microscope for the transfection efficacy (Fig. 2). The supernatant was harvested from day2 through day4 and was replaced with 10ml medium. The supernatant was kept in the 4°C fridge. Concentrated LV was made by ultracentrifugation. First, culture supernatant was spun down at 2000 RPM for 5 minutes and was filtered through 0.45μm low protein binding, MCE sterile filter (Fisher Scientific; Ottawa, ON) to remove cell debris. We used 30ml Beckman Polyallomer Centrifuge tubes (Beckman Coulter Canada Inc.; Mississauga, ON) and samples were centrifuged at 27.8k RPM for 2h at 4°C using a Beckman SW-28 swinging bucket rotor. The supernatant was gently discarded by inversion. The pellet was resuspened with 100μl sterile 1x PBS. LV stock was aliquoted and stored at -80°C until use (See Fig. 3).

The infectious titer of LV stock was determined by incubating 0.1 x $10^6$ 293T cells seeded in 24-well plate coating with Poly-Lysine Hydrobromide (Sigma; St. Louis, MO)

the day before with limiting dilutions of the viral stock (1/100, 1/1000, etc.). The plates were put back into the incubator for 1hr; and were shaken every 10 minutes. After 1hr, 0.5ml fresh medium was added directly to each well. After 48hr, the number of plaque formed units (pfu) was determined by counting the number of GFP-positive cells per well under the inverted fluorescent microscope (Axiovert 200) equipped with Northern Eclipse (Carl Zeiss Inc.; Thornwood, NY) and the titers of the LV stock were expressed as pfu/ml.

# B. Rabbit Studies:

## B. 1 Bioavailability of LV within Amniotic Fluid:

These experiments were performed to determine if amniotic fluid (AF) had an inhibitory effect on *in vitro* LV vector infection. AF was harvested from a pregnant NZW rabbit on gestational day 24 and kept in -20°C fridge until use. A 24-well plate was coated with Poly-Lysine before seeding with 0.1 x $10^6$ 293T cells per well. The next day, the medium was changed and different concentrations of AF mixed with the certain amount of LV stock solution were added. 1 x $10^7$pfu/ml LV stock was used in this experiment. 10μL of diluted LV stock containing about 200pfu in DMEM was mixed with different concentration of AF: 100% AF, 100% DMEM, 50% AF, 25% AF, and 12.5% AF. A total volume of 200ul LV in DMEM and AF was added to each well, and these experiments were performed in duplicate. After 1hr of incubation in 37°C incubator, 0.5ml of growth medium was added into each well and the plate put back to incubator.

48hr later, GFP-positive cells were counted in each well under the fluorescent microscope.

## B. 2 General Surgical Protocol Used in Rabbit Experiments:

The surgical protocol for all rabbit experiments was approved by the UBC Committee for Animal Care, and all experiments were performed in the Research Animal Facility in the Vancouver Hospital (UBC site). Time-mated NZW rabbits (3.5-4.5kg) were purchased from Charles River Laboratories in Montreal between days 19 to 22 of their normal 31 gestational day period. The pregnant does were singly housed and acclimatized to their cages for 1-5 days prior to use, and were provided with rabbit chow and water ad libitum. 75,000 IU procaine penicillin G (for infection prophylaxis) and 3mg/kg medroxyprogesterone acetate (Depo-Provera®) (Phamacia Canada Inc.; Mississauga, ON, Canada) (for post-operative tocolysis) were administered by intramuscular injection. Anesthesia was induced by an initial dose of atropine (0.04 mg/kg) intramuscularly. The rabbit was placed in a supine position with the limbs restrained over a warming blanket and orotracheal intubation was performed by a qualified veterinarian. Isoflurane anesthesia (0.5-1.5% in oxygen) by mask inhalation was administered with ECG, temperature and blood pressure monitoring. Arterial blood gases were periodically sampled to avoid maternal hypoxia and acidosis. Intravenous access was obtained through the marginal auricular vein. The abdomen was shaved and all surgical procedures were performed under sterile conditions, through an infraumbilical maternal laparotomy.

## B. 3 *In vitro* Transfection Studies: fetal tracheal transgene analysis and determination of time required for successful transfection

Gravid does underwent surgery on gestational day 24 to 26 for the purpose of *in vitro* transfection studies. Under the conditions described above, a maternal laparotomy was performed. The bicornuate uterus was delivered and individual fetuses were delivered and killed by intracardiac injection of air. Intact fetal tracheas were harvested immediately, rinsed twice with Dulbecco's phosphate-buffered saline (PBS) (GIBCO™ Invitrogen Co.; Burlington, ON) and placed in DMEM medium on ice for transport back to the laboratory. Working in the Class II A/B3 tissue culture hood, tracheas were rinsed again in Dulbecco's PBS and placed in a 12-well tissue culture plate containing 2mL of DMEM with 10% heat-inactivated fetal bovine serum (FBS) (HyClone; Logan, Utah) and 1% antibiotic-antimycotic (GIBCO) in each well and were incubated in 5% $CO_2$ at 37°C for 16 hours as a screening procedure to exclude any fetal rabbit tracheas contaminated by bacteria or fungus. The next day, non-infected fetal tracheas were transferred into 96-well culture dish and 50uL DMEM with 10% FBS containing 1 x $10^6$ LV particles was added into each trachea. Control tracheas were put in another plate without addition of LV. The plates were then returned to the incubator for 4 hours of transfection (day0). 4 hours later, the medium was changed with tracheal organ culture medium as described elsewhere[83]. This medium was composed of 3:1 mix of DMEM and F-12 nutrient mixture (GIBCO™; Burlington, ON) supplemented by 2% FBS (HyClone); 100IU/mL penicillin 0.1mg/mL streptomycin (GIBCO); 7.25mmol/L L-glutamine (GIBCO); 2.25mmol/L Hepes (Sigma-Aldrich Co.; St Louis, MO); 10ng/mL epidermal growth factor (Sigma); 0.18mmol/L adenine (Sigma); 1mmol/L strontium

chloride (Sigma); 1.88mmol/L calcium chloride (Sigma); 0.65mmol/L choline chloride (Sigma); 1mmol/L L-serine (Sigma); 53nmol/L selenic acid (Sigma); 0.1mmol/L ethanolamine (Sigma); 0.1mmol/L o-phosphorylethanolamine (Sigma); 5μg/mL insulin (Sigma); 5μg/mL transferrin (Sigma); 20pmol/L triiodothyronine (Sigma); 0.4μg/mL hydrocortisone (Sigma); and 10nmol/L progesterone (Sigma) at pH of 7.5. The fetal tracheal organ culture medium was changed every day and the tracheas were examined daily for evidence of reporter gene GFP expression by whole-mount fluorescent microscopy. Tracheas were harvested at different time-points (day4, day6, day7 and day8) for the detection of transgene GFP DNA by polymerase chain reaction (PCR) and the confirmation of reporter gene expression by immunohistochemistry (IHC).

## B. 4 *In vivo* Transfection Studies:

Two techniques of *in vivo* transfection were evaluated:

### B. 4.1 Direct Fetal Tracheal Injection of LV-GFP:

The direct fetal tracheal injections were technically feasible in fetuses of gestation day 26 or older, because of fetal size. In these fetal and maternal survival experiments, maternal intravenous antibiotic was administered for infection prophylaxis (75,000 IU procaine penicillin G) and intramuscular tocolytic (3mg/kg medroxyprogesterone acetate) was given for prevention of premature labor. Maternal anesthesia was performed as above with monitoring and maintenance of maternal temperature (heating blanket). Maternal blood pressure and blood gases were monitored to ensure adequate uterine perfusion. Following maternal laparotomy, the right uterine horn (designated the "experimental" horn), was exposed and maintained moist and warm with frequent

applications of warm, sterile normal saline. Each fetus in the horn was identified by palpation, and individual hysterotomies were made over each fetal head to allow controlled delivery of the fetal head and neck. With the neck extended, an incision was made in the midline from the larynx to sternum. Using 2.5X magnification, the trachea (which measures 2.5mm in the 23day gestation and 3.5mm in the 27day gestation fetus) was carefully dissected from the surrounding thymus and overlying tissues, and encircled with a 5/0 Prolene suture at the level of the larynx, which was tied, occluding the lumen and preventing subsequent egress of injected viral particles. Using a 30-gauge needle and micro-syringe (BD™; Franklin Lakes, NJ), 50μL LV solution, (containing about 5 x $10^6$ pfu viral stock mixed with PBS) was injected directly into the fetal rabbit tracheal lumen below the occluding ligature. The neck incision was closed with 5/0 Prolene suture and then the fetus was gently returned to its amniotic sac. The uterine wall was closed with 4/0 Vicryl suture. This procedure was repeated on all fetuses in that uterine horn. Control fetuses in the opposite side (left) horn underwent an identical procedure but received injections of 0.9NS rather than LV. The maternal laparotomy incision was then closed and the doe was placed in an incubator until fully recovered. During this time, IV hydration was continued and buprenorphine (0.02-0.05mg/kg) was given subcutaneously every 6 hours for pain control.

## B. 4.2 Direct Amniotic Injection of LV-GFP:

Following exposure of the right uterine horn as described above, individual fetuses were identified by palpation. Using a 25-gauge angiocatheter, the individual amniotic sacs were punctured, taking care not to insert the needle into either the placenta or fetus.

50μL of lentiviral solution containing 5 x $10^6$ pfu viral stock was injected directly into the amniotic fluid through the angiocatheter. After withdrawal of the angiocatheter, the uterine wall was carefully observed for signs of leakage of amniotic fluid, and if seen, the puncture site was oversewn with 4/0 Vicryl suture. The left uterine horn served as a control and amniotic sacs were injected with 0.9NS in an identical manner (Fig.8.A).

Following both injection methods, the maternal incision was closed and the doe was placed in an incubator to keep her warm until fully recovered. During this period, IV hydration was continued and buprenorphine (0.02-0.05mg/kg) was given subcutaneously every 6 hours for post operative pain.

## B. 4.3 Procurement of Fetal, Maternal Tissues following in vivo Transfection:

On gestation day 30, the maternal rabbit was re-anesthetized and all pups along with their control littermates were delivered via cesarean section and killed. Fetal tissues (trachea, lung, intestine, liver, kidney, placenta, amniotic membrane and gonad) were harvested for the confirmation of gene transfer by the following techniques: 1) marker gene GFP localization in cryostat sections by fluorescent microscopy, 2) detection of GFP DNA by PCR, and 3) GFP localization by Immunohistochemistry (IHC). Following euthanasia with sodium pentobarbital, maternal tissues were also harvested (trachea, lung, liver, ovary, gut) for maternal biosafety studies (PCR detection of marker gene GFP).

## C. Mouse Studies:

Time-mated CD-1 pregnant mice (term = 21d) were used for the second part of our project. They were purchased from the UBC (South Campus) Animal Facility. The experimental protocol was approved by the UBC Committee for Animal Care.

### C. 1 *In vivo* Assessment of Gene Transfer by Amniotic Injection:

Time-mated CD-1 mice were purchased between days 12 to 14 of gestation for 1 to 5 days acclimation at the RAF, where they were fed mouse chow and allowed water ad libitum. On gestational day 15 to 17, they underwent LV mediated gene transfer by amniotic injection. Maternal anesthesia was induced with 2% isoflurane in oxygen and maintained with a snout mask (1-1.5% isoflurane in oxygen), and a heating-lamp was used to keep the mouse warm. The abdomen was shaved, and a midline laparotomy was performed under sterile conditions. One uterine horn was carefully delivered with a pair of cotton-tipped applicators, and during the surgical procedure, pre-warmed normal saline was added dropwise to the surface of the uterus to keep it moist.

Through a 28-gauge 0.5mL Insulin syringe (Becton Dickinson and Company; Franklin Lakes, NJ, USA), about 1 x $10^6$ lentiviral particles in 20μL stock were injected into each of the fetal amniotic sacs in the right uterine horn. An identical procedure using sterile saline was performed on fetuses in the left uterine horn, which served as controls. The abdomen was closed by 4-0 Vicryl suture after injecting 10 mL of pre-warmed 0.9NS into the peritoneal cavity. The mouse was recovered and then euthanized on gestational day 20 or 21 (term=21 d), when fetal tissues were harvested for transgene analysis by fluorescent microscopy, PCR and IHC.

## C. 2 Assessment of Durability of Gene Transfer in Survived Pups:

In order to find out the durability of gene transfer in this model, selected animals (gestational day 16) underwent injection of all amniotic sacs (both horns) with either LV particles or saline (controls), and were allowed to deliver spontaneously. The survived pups were euthanized at various time points postnatally for to assess the duration of GFP expression by Quantitative Real-Time PCR (RT-PCR).

## D. Analytical Methods used in Tissue Analysis:

### D. 1 GFP Localization by Fluorescent Microscopy:

Following dissection, fetal tissues were rinsed twice in ice cold PBS. The tissues were infused with 20% sucrose infusion for 1 hour. The tissues were washed again with PBS and then imbedded in O.C.T. (optimal cutting temperature) compound (Tissue-Tek®; Torrance, USA).. The mould was wrapped with parafilm and foil, and placed in a -80°C freezer until sectioning.Tissue blocks were cut using a LEICA Cryostat (CM 3050 S) (Meyer Instruments Inc.; Houston, TX, USA) at 5μm thickness and sections were read directly under the fluorescent microscope (Axiovert 200) and pictures taken.

### D. 2 GFP Localization by IHC:

Washed, fresh tissues were fixed in 4% paraformaldehyde for 1 hour in room temperature (RT), and then rinsed twice in PBS. Tissues were infused with 20% sucrose for 2 hours. Following the procedure of cryosectioning described above, sections were

air-dried overnight (O/N) at room temperature (R/T). The next day, slides were rinsed with PBS and incubated in 3% $H_2O_2$ (Sigma) in PBS for 15 minutes at RT to block endogenous horseradish peroxidase. The slides were washed 3 times in PBS and the sections were outlined on the glass slides with a Pap pen (Vector Laboratory; Burlingame, CA, USA). The slides were put in a moist chamber and 10% goat serum (CHEMICON International; Temecula, CA, USA) was added to block non-specific binding from 2$^{nd}$ antibody, and incubated for 1 hour at RT or 4°C O/N. The first antibody (mouse anti-GFP monoclonal antibody, CHEMICON), at a working dilution at 1:300 in 10% goat serum was applied and incubated O/N at 4°C. The next day, the slides were washed with 0.1% PBS/Tween-20 for 5 minutes x 5 times. The 2$^{nd}$ antibody, which was goat anti-mouse IgG (H & L) peroxidase conjugated antibody at 1:500 dilution in 10% goat serum was applied for 30 minutes at RT. The slides were washed with 0.1% PBS/Tween-20 for 5 times (5min/wash). A DAB substrate kit (VECTOR) was used to react with peroxidase: 2.5mL $dH_2O$, 1 drop buffer stock solution, 2 drops DAB stock solution and 1 drop $H_2O_2$ were mixed and added to each section, incubated at RT for 2 to 8 minutes. The slides were washed with running water, counterstained with hematoxylin, washed with running water and then dehydrated sequentially by 70%, 80%, 95% and 100% ethanol (5min/each). Xylene was applied for 10 minutes, and the slides were mounted with Permount (FisherScientific), and examined under a light microscope. Tissues from a GFP-transgenic mouse were used for positive control, while uninfected tissues as well as tissues parallel processed without primary antibody served as negative controls.

## D. 3 GFP DNA Detection by PCR:

PCR was performed on fetal and maternal tissues to detect transgene GFP DNA. Tissues were washed and digested with 0.5 ml of Digestion Buffer, containing 5mL 1M Tris-Cl (Sigma), 25mL 0.2M EDTA (Sigma), 25mL 1M NaCl (Sigma), 12.5mL 20% SDS (Bio-Rad Laboratories; Hercules, CA), and autoclaved distilled $H_2O$ to a final volume of 500mL; and 10 ul of Proteinase K, containing 100mg Proteinase K (Sigma) in 10mL of 10mM Tris-Cl (PH 7.5) (Sigma), 20mM $CaCl_2$ (Sigma), and 50% Glycerol (Sigma), at -20°C. After digestion in a 55°C incubator for 6 to 14 hours, DNA was extracted by adding 0.4mL of Phenol/Chloroform (1:1 mixture) (Sigma). Samples were shaken vigorously by hand for 20 seconds and then centrifuged at 12,000 x g for 5 minutes. Following centrifugation, the mixture separated into an upper aqueous phase containing separated DNA, an interphase containing protein and a lower phenol-chloroform phase. Aqueous phase was transferred into a fresh eppendorf tube without disturbing the interphase. DNA was precipitated from the aqueous phase by mixing with 1mL 95% ethanol/3M NaOAc (20:1 mixture), shaked well and incubated for 1 to 2 hours at RT. The samples were centrifuged at 12,000 x g for 10 minutes; the supernatant was discarded, and the DNA-containing pellet was air-dried for 10 minutes. The pellet was dissolved in 400uL RNase/DNase free distilled water (GIBCO). Samples were kept in a 4°C fridge.

Reporter gene GFP DNA was amplified using a Platinum *Taq* DNA Polymerase kit (Invitrogen). Briefly, PCR was carried out using a forward primer (5'-ATGGTGAGCAAGGGCGAGGAG-3') and a reverse primer (5'-TTACTGTACAGCTCGTCCATGCCG-3 of eGFP (PROLIGO Primers & Probes;

Boulder, CO, USA). In all amplifications, 25μL of reaction mixture containing 1.0μL of genomic DNA, 2.5μL 10 x PCR buffer, 0.75μL 50mM $MgCl_2$, 0.2μL 25mM dNTPs, 0.5μL of each of the primers, 0.2μL of platinum *Taq* DNA polymerase and 19.35μL RNase/DNase free water were used. The samples were run in the Biometra T-Gradient Thermoblock (Montreal Biotech Inc.; Kirkland, PQ, Canada). The program was set up as follow: 95°C for 5 minutes for DNA denaturation and enzyme activation, 35 amplification cycles were performed consisting of denaturation for 1 minute at 95°C, annealing for 1 minute at 62°C, and extension for 1.5 minutes at 72°C. A 750bp eGFP product was generated from the amplification. 1% agarose gel containing 0.5μg/mL ethidium bromide was used to check the absence of eGFP DNA band. Gel was run in a horizontal gel electrophoresis apparatus (Horizon®58) (GIBCO BRL) at 100 Voltage for 20 minutes. The gel was photographed by a Kodak EDAS 290 camera with a UV transilluminator (SYNGENE; Frederick, MD, USA).

## D. 4 GFP mRNA Detection by Quantitative Real Time QRT-PCR:

Survived mouse pups were sacrificed at various post-natal days and tracheas and lungs were harvested to assess the durability of gene transfer (GFP-DNA) and gene expression (GFP-mRNA). Transgene GFP mRNA in fetal tracheas and lungs was quantified by RTPCR.

RNA Isolation: trachea and one lobe of lung were dissected from a pup and put in 1.7mL micro-centrifuge tubes, washed in ice cold PBS and then homogenized in 1mL of TRIZOL Reagent (Invitrogen) using a power homogenizer. The samples were incubated for 5 minutes at RT to completely dissociate nucleoprotein complexes. After shaking the

samples and leaving them to stand for 2 minutes at RT, they were centrifuged at 12,000 x *g* for 15 minutes at 4°C. Following centrifugation, the dissociated RNA remained in the aqueous phase, which was transferred into a fresh tube, and precipitated by mixing with 0.5mL isopropyl alcohol. The samples were incubated at RT for 10 minutes and centrifuged again at 12,000 x *g* for 10 minutes at 4°C. The supernatant was discarded, and the RNA-containing pellet washed with 1mL of 75% ethanol and centrifuged at 7,500 x *g* for 5 minutes at 4°C. The resulting RNA pellet was air-dried for 5 minutes, dissolved in 20μL (tracheas) or 80μL (lung) of RNase/DNase free water (GIBCO). and the samples were stored in a -70°C freezer.

RNA concentration measurement: 50 ul of a 1:50 dilution of total RNA in RNase free water was placed in a 384 well micro UV protective plate (Corning; NY, USA), and the optical density (O.D). at 260/280nm was read using a UV spectrophotometer.

DNA digestion: all contaminating DNA was digested using DNase I (MBI Fermentas Inc.; Burlington, ON, Canada). To each 1 ug sample of RNA was added: 1μL 10x DNase I Reaction Buffer (MBI Fermentas Inc.), 1μL DNase I and RNase/DNase free water to a final volume of 10μL in a 0.5mL RNase-free micro-centrifuge tube. The tubes were incubated for 15 minutes at RT and then the DNase I was inactivated by addition of 1μL of 25mM EDTA (MBI Fermentas Inc.).. The samples were heated for 10 minutes at 65°C.

Reverse Transcription: First-Strand cDNA was synthesized by using SuperScript™ II Reverse Transcriptase (Invitrogen). Following DNA digestion, 1μL each of Oligo$(dT)_{12-18}$Primer (Invitrogen) and 10mM dNTP mix (Invitrogen) were added to each tube, which was heated 65°C for 5 minutes, then put on ice. To each tube, the following were added:

4μL of 5x first-Strand Buffer (Invitrogen), 2μL of 0.1M DTT, and 1μL of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen), and the tubes were incubated at 42°C for 2 minutes. Then 1μL of SuperScript™ II was added and the tubes were incubated 42 °C for 50 minutes. The reaction was stopped by heating at 70 °C for 15 minutes.

Confirmation of the Reverse Transcriptase by conventional PCR: This step was optional. It was designed to check if the cDNA was successfully synthesized. β-actin was amplified by conventional PCR in each sample. Briefly, to each PCR tube, the following were added: 1μL of 10x PCR buffer, 25mM $MgCl_2$ 0.6μL, 1μL sample cDNA, 0.4μL of mixture of 10μM forward actin primer (5'-ACGAGGCCCAGAGCAAGAG-3') and reverse primer (5'-TCCTCCATGTCGTCCCAGTTG-3')(Invitrogen), 0.2μL dNTP mix, 0.1μL platinum *Taq* DNA polymerase and 6.7μL RNase-free water. The samples were run in the Biometra T-Gradient Thermoblock (Montreal Biotech Inc.;) at 94 °C for 5 minutes. 35 amplification circles were performed consisting of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 58 °C, and extension for 1 minute at 72 °C. A 100bp β-actin product was generated from the amplification step and re-dissolved in 2% agarose for to check for absence of the band.

Real-Time PCR: A pair of PCR primers were designed to amplify a portion of the marker gene GFP and β-actin primers were used to normalize the cDNA quantity of β-actin and GFP between tissues from the experimental and control groups. The sequences of the GFP primers were 5'-CTGCTGCCCGACAACCA-3'(Forward) and 5'-TGTGATCGCGCTTCTCGTT-3'(Reverse) (Invitrogen). SYBR Green Max Mix (Applied Biosystems; Foster City, CA, USA) was used to bind to the amplified cDNA. All samples were tested for both GFP gene and β-actin and both were tested in ½, 1/10,

and 1/20 dilutions. Working in a cell culture hood, all samples dilutions were loaded into a MicroAmp® optical 96-well reaction plate (Applied Biosystems). Each dilution was performed in duplicate. A 2μL sample of cDNA, 12.5μL SYBR Green, 0.75μL GFP primer or β-actin primer and 9.75μL RNase/DNase free water were added to each well. All samples were run in a ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The program was set as follow: 50°C for 2 minutes for 1 cycle, followed by 95°C for 5 minutes for DNA denaturation and activation of the enzyme; 40 amplification and quantification cycles were then performed consisting of denaturation for 15 seconds at 95°C, annealing for 1 minute at 60°C, and 72°C for 60 seconds with a single fluorescence measurement, and finally a cooling step to 40°C. The results were analyzed with the SDS 7000 software. Log2-Fold change was derived from the normalization of β-actin and GFP cDNA copy numbers between tissues from control and experimental groups, and a change of greater than 1.5, was taken to represent GFP transgene expression.

# CHAPTER 3

# Rabbit Studies: Results and Discussion

## A. Results:

### 1. Bioavailability of LV within Amniotic Fluid:

Because the ultimate goal of this project was to develop an in vivo system of gene transfer via amniotic fluid (AF), this experiment was performed to determine whether AF had an inhibitory effect on LV infection in vitro. Green fluorescent protein (GFP) positive 293T cells were counted in each well following transfection by equivalent numbers of LV particles within varying concentrations of AF (ranging from 0% to 50%) under the fluorescent microscope. These data confirmed that LV-GFP produced similar rates of transfection of 293T cells in vitro, regardless of the concentration of AF (Figure 4), and therefore, AF would not be expected to pose a barrier to infection in an *in vivo* transamniotic transfection system.

### 2. In vitro Fetal Rabbit Tracheal Transfection:

### 2. 1 Whole-mount Direct Fluorescent Microscopy:

Fetal tracheas from gestational days 24 to 26 were dissected, and maintained in tracheal organ culture medium for up to 10 days without significant contamination. Figure 5 shows a series of fluorescent microscopy images of gestational day25 fetal whole mount tracheas in culture medium from post-transfection day 1 to day 7. GFP

marker gene fluorescence is first visible on day 1 post-transfection, and is present in increased numbers of cells by post-transfection day 7 (Fig.5.1-5). Similar results were seen in all three gestational groups (images of G24 and 26 not shown).

## 2. 2 LV-GFP DNA Transfer:

PCR analysis for the presence of marker gene GFP DNA in G25 cultured fetal tracheas is shown in Figure 6-1. Fetal tracheas transfected in vitro on day 0 demonstrated transgene GFP DNA by day 4 following transfection, which persisted up to day 8. Similar results were obtained with *in vitro* transfection of G24 and 26 fetal tracheas (see Fig.6.2 and Fig.6.3). All control tracheas (from three gestational groups) were negative for GFP DNA by PCR (data not shown).

## 2. 3 Tracheal LV-GFP Protein Expression:

Following *in vitro* transfection, LV transgene product, GFP was detected in trachea sections by immunohistochemistry (IHC). As shown in Figure 7, GFP was localized to the luminal surface of respiratory epithelium only (brown stain).

## 3. In vivo Transfection of Fetal Rabbits by Tracheal Injection (TI):

### 3. 1 Determining the Therapeutic Window for Gene Transfer via TI:

Pilot studies with our model were performed to determine the feasibility of in vivo transfection by TI. Considering issues of fetal tracheal size (intraluminal diameter increases from 2 mm to 3.5 mm between gestational days 23 and 26), and procedural fetal mortality with tracheal injection (which requires hysterotomy and partial delivery of

the fetus), we determined that in vivo transfection by direct tracheal injection was feasible if performed on or after gestational day 26. Our in vitro data suggested that transgene introduced on day 26, should be expressed by day 30 (term 31d), and so we determined that transfection by TI would be most feasible if perfomed on gestational day 26.

## 3. 2 LV Transgene GFP DNA Fetal Tissue Distribution by PCR:

In order to assess transgene GFP tissue distribution following TI, fetal tissues were harvested on gestational day 30. Total genomic DNA was extracted from fetal trachea, lung, liver, kidney, small intestine, gonad and placenta. Figure 9 shows that transgene GFP DNA was detectable in trachea and lung, but not in liver, kidney, intestine, skin, gonad or amniotic membrane.

## 3. 3 LV Transgene GFP Expression by IHC:

Fetuses, which underwent TI on gestational day 26, were sacrificed on gestational day 30 along with control littermates. Selected fetal tissues were analyzed for GFP expression and localization by IHC. Figures 10.1-2 show the transgene GFP protein is localized to the luminal surface of fetal tracheal epithelium (brown stain). In these two sections, we see that only a fraction of the tracheal epithelial cells are infected. This may be due to the localization of the LV-GFP to the sites of tracheal injection. Figure 10.4-5 confirms GFP expression in some alveolar cells of fetal lung.

## 4. In vivo Transfection of Fetal Rabbits by Amniotic Injection (AI):

Compare to TI, AI is a simpler way to deliver viral vectors to fetal pulmonary airways through fetal breathing movements [98, 99]. It is less invasive, and based on our demonstration of LV bioavailability in AF, we felt that this delivery system would adequately target fetal tracheobronchial epithelium through fetal breathing.

### 4. 1 LV Transgene GFP Expression by Direct Fluorescence Microscopy:

Amniotic injection was carried out on gestational day 24 and 25 time-mated rabbits. On gestational day 30, fetal tissues were harvested. Figures 11.1-2 show that GFP positive cells were present along the luminal surface of tracheal epithelium pulmonary. These images suggest that LV mediated gene transfer reached the pulmonary epithelium in amniotic fluid through fetal breathing movements. Similar results were achieved for gestational day 24 and 25 mice.

### 4. 2 LV Transgene GFP Expression by IHC:

Having detected GFP fluorescence in epithelial cells in fetal tracheas and lungs, we performed IHC analysis using anti-GFP antibody to further corroborate these results. The results show strong expression of GFP protein in the surface epithelium of tracheas, (but not lung) of both gestational day 24 and day 25 fetal rabbits (Fig. 12).

### 4. 3 LV Transgene GFP Fetal Tissue Distribution by PCR:

To evaluate the tissue distribution of LV transgene GFP following AI, fetal tissues were harvested on gestational day 30, and total genomic DNA was extracted and

examined for the presence of GFP DNA by PCR analysis. Figure 13 shows that GFP DNA was detectable in trachea, lung, small intestine, liver, kidney, skin, amniotic membrane, and placenta.

### 5. *In vivo* LV Transfection in Rabbits: Maternal Assessments

Following in vivo gene transfer, all does were monitored by daily assessment of health, and following maternal sacrifice, for evidence of LV infection in tissues by PCR analysis. There was no maternal mortality and no sign of maternal illness up to the date of sacrifice on gestational day 30. Maternal tissues, trachea, lung, liver, ovary and gut were harvested and genomic DNA was extracted for transgene GFP PCR analysis. Maternal tissues from the AI group were all negative for transgene GFP. We observed the presence of transgene GFP DNA in the lung of one doe whose fetuses underwent direct tracheal injection (Fig.14), although IHC performed on the same lung did not demonstrate GFP.

## B. Discussion (Rabbit Model):

The purpose of our in vitro study was to create an experimental model of LV-mediated reporter gene transfer to respiratory epithelium of intact fetal rabbit tracheas, as a first step towards in vivo gene transfer. We felt that an in vitro system that simulated gene transfer through AF would enable us to determine i) the susceptibility of tracheal epithelium to LV infection via its luminal epithelial surface, ii) the time required to

demonstrated reporter gene GFP expression after vector exposure, and iii) the overall feasibility of an in vivo system of LV-mediated gene transfer in the fetal rabbit.

Our demonstration of LV vector bioavailability in AF supports the concept that with normal fetal breathing and swallowing, a transamniotic delivery system should ensure contact between vector and target respiratory and foregut epithelium. Our in vitro transfection experiments show that there was no difference in the efficiency of gene transfer to luminal surface respiratory epithelium among gestation day 24, 25 and 26. Transgene GFP can be detected in fetal tracheal tissues by direct fluorescent microscopy, confirmed by IHC, and PCR. These findings strongly support the hypothesis that in vivo gene delivery by about gestation day 24 to 26 should allow detection of transgene in fetal tissues near term, in the fetal rabbit model. The results of this study using whole fetal tracheal cultures suggest that LV allows effective transduction of surface exposed cells through amniotic fluid. The time required to demonstrate both transgene presence and efficient expression is well within a time period during which the fetal respiratory epithelium should be accessible via a transamniotic delivery system in an intact fetomaternal unit.

Based on the results of studies of in vitro transfection of whole fetal tracheas using LV mediated gene transfer, in which we observed that 3 to 5 days post transfection was necessary to achieve transgene expression, we concluded that our in vivo model should attempt transfection no later than gestational day 26 to ensure gene expression by term. We tested two in vivo techniques that would target transfection of respiratory epithelium: direct tracheal injection as well as the less invasive technique of amniotic injection, which relies on fetal breathing and swallowing to deliver the vector to its

cellular targets. Although both techniques produced transfection of respiratory epithelium, direct tracheal injection was associated with a prohibitive fetal mortality rate (75%). We also observed that, in comparison to AI, TI caused significant transgene contamination of control fetuses, with both gene and gene product detectable in lung, liver, and kidney of control littermates, and even in the lung of one doe, where transgene, but not gene product was detected. These observations led us to hypothesize that LV vector contamination of the fetomaternal circulation may have occurred during the more invasive fetal tracheal injection procedure. In addition to the transfection of respiratory epithelial cells and other tissues (placenta and amniotic membrane) in direct contact with amniotic fluid, AI also produced transfection of tissues not in contact with amniotic fluid (fetal liver and kidney) suggesting that some LV vector must gain access to the fetal circulation through either the lungs or gut. Hence in this AI delivery model, vector access to fetal tissues for transfection appears to be by both the intraluminal and hematogenous route.

The observation of selective and efficacious short term LV vector mediated gene transfer to airway epithelium and the low fetal mortality associated with amniotic injection (33%) support the use of the rabbit model in these "proof of principle" experiments of fetal gene therapy for CF. Further studies are required to evaluate the duration of gene expression postnatally (discussed further in mouse section), and to consider other biosafety issues including transgene site insertion and long term genome stability.

# CHAPTER 4

# Mouse Studies: Results and Discussion

## A. Results

### 1. In vivo Transfection of Fetal Mice:

Proof of principle experiments in the rabbit model confirmed that amniotic injection (AI) permitted gene transfer to the fetus within the injected amniotic sac. Subsequent experiments were performed in the less expensive mouse model. The principle objective of experiments using our mouse model was to assess the short term persistence of LV mediated gene transfer (three weeks after LV injection) by amniotic injection in mice survived after birth.

Pilot experiments confirmed that mouse tracheas transfected in vitro demonstrated transgene presence and expression within time periods comparable to fetal rabbit tracheas (data not shown), and so we knew it was necessary to target in vivo transfection 4 or more days before term (term =21 d in mice). CD-1 time-mated pregnant mice underwent AI of LV-GFP on gestational days 15-17. On gestation day 20, fetal mice were sacrificed; fetal tracheas and amniotic membrane were harvested for the evaluation of LV-mediated gene transfer and expression by direct fluorescence microscopy and IHC. Having proved by term sacrifice that transgene GFP was present in fetal tracheas following AI, dams, whose fetuses had all undergone transfection by AI were allowed to deliver, and fetal tracheas and lungs were harvested at various time point over a three week period to test the persistence of transgene GFP expression by Quantitative Real-Time PCR.

## 1.1 Transgene Expression Following Amniotic Injection: Direct Fluorescence

Following LV mediated transfection by AI on gestational days 15, 16 and 17 (term = 21 days), injected fetuses were euthanized on gestational day 20, and their tracheas and amniotic membrane were harvested and analyzed for GFP expression. Figure 14 shows that following AI, amniotic membrane and fetal tracheal epithelial cells were successfully transfected by LV. GFP-positive cells were found within the epithelium of fetal tracheal sections and amniotic membrane at all 3 gestational time points (Fig.15.1-3, 5). Tissues from control fetuses whose amniotic sacs were injected with 0.9NS saline, were negative (Fig. 15.4, 15.6).

## 1. 2 Transgene Expression by IHC:

Reporter gene GFP expression was confirmed by IHC. GFP positive staining was located on the luminal surface of tracheal epithelium and amniotic membrane at all three gestational time points (Fig.16.1-3, 5).

## 2. The Durability of LV-Mediated Gene Transfer by AI in Tracheas and Lungs of Survived Mice by QRT-PCR:

Gestational day 16 mice were used for our durability assessment of LV-mediated gene transfer. After AI of LV-GFP, a total of 15 delivered fetuses were kept alive, and the tracheas and lungs from survived pups were harvested at various post-natal time-points: days 5, 9, 14, 19 and 21. Total mRNA was isolated, and copied to cDNA by

reverse transcriptase. The quantitative difference in GFP mRNA was measured by SYBR green. Beta actin served as an internal standard to normalize all the data since the amount of beta actin (copy number) does not change significantly in expression in different tissues or cells. The ratio of GFP mRNA (cDNA) in our experimental versus control fetal tracheas and lungs was expressed by Log2-fold change (value greater than 1.5 represents gene expression) which is summarized for the different postnatal time points in Figure 17.1. One survived mouse showed that the transgene GFP mRNA was present in lung 21 days after birth; while neither GFP mRNA nor DNA could be detected in trachea at this time-point (Fig.17.3). These results suggest that transgene GFP DNA in the trachea of this mouse had been lost by this time-point. Lungs from 4 of 15 survived pops were negative by quantitative real-time PCR (no GFP mRNA), but GFP DNA by DNA PCR was still positive (Fig.17.2). This result suggests that although GFP DNA was present, its transcription had been significantly reduced or "shut off". Shutdown of the CMV promoter or DNA methylation may play an important role in turning off the transgene GFP.

## 3. Fetal Mortality Rate:

We found that there were two critical factors which determined fetal mortality (stillbirth rate) in mice, in which amniotic injection was the only surgical technique used. The first was the anesthetic technique used. Initially, we used ketamine for our mouse model, but encountered an extremely high fetal mortality rate (87%). Then we tried isoflurane (see Methods), and the mortality rate decreased to 67%.

Another critical technical factor is to keep the mice well hydrated and warm during the surgical procedure, which we accomplished with the use of a heat lamp, regular

moistening of the uterus with warmed sterile saline, and the injection of 15 to 20mL warm saline into the maternal abdomen at the time of abdominal closure. This simple technical adjustment further reduced the mortality rate to 26% (see Table 1).

## B. Discussion: Mouse Model

Mouse models have been widely used in medical research. Many disease models are now available with the development of gene knockout techniques, including a CFTR knockout mouse. In order to develop a clinically relevant technique of fetal gene transfer through amniotic fluid, we assessed the efficacy of direct AI in gestation 15 to 17 CD-1 pregnant mice as well as the short term persistence of transgene and its transcription in gestational day 16 mice.

Studies in murine fetal lung ontogeny demonstrate that during gestational 15 to 17 days in mice, fetal airways are lined with undifferentiated multipotential stem cells, and the lung is in its canalicular period, which is the ideal time for gene delivery (corresponds to 12-20 weeks in human) [109-112]. This may be the ideal therapeutic window for targeted fetal gene transfer, since CF can be diagnosed, using current techniques of antenatal diagnosis, as early as 9 weeks after conception by chorionic villus sampling (CVS) or amniocentesis at about 15 weeks [113].

Our experiments of LV gene transfer on gestation day 15 to 17 mice suggested that 1 x $10^6$ viral particles introduced into the amniotic cavity of the fetus is sufficient to produce transfection in vivo. This model demonstrated that abundant GFP expression was present in the amniotic membrane and tracheal epithelium. Vector GFP DNA was detectable in fetal intestine, liver and kidney. The detection of transgene GFP DNA in

fetal liver and kidney suggests that some LV vector must gain access to the fetal circulation through either the lungs or gut. Although transgene GFP DNA was detectable in fetal trachea and lung, we could only detect transgene product (by IHC and fluorescent microscopy) in tracheal epithelium. Our interpretation of this finding is that vector "washout" may occur by non-specific transfection of fetal skin and amniotic membrane, which effectively reduces the transduction efficiency of cells within pulmonary alveoli. Another interpretation might that although transgene was inserted into fetal alveolar cells (and was therefore detectable by PCR), it may not have been expressed.

In our short term study of the durability of LV mediated gene transfer in the mouse model, we observed transgene GFP expression in tracheas and lungs for up to 21 days after birth. We noticed that transgene GFP expression was shutdown in 4 of 15 survived pups by quantitative real-time PCR. CMV promoters are used extensively in viral vector engineering due to their activity in many cell types, and therefore often result in high levels of transgene expression. However, the use of this promoter in amniotic injection for gene therapy for CF may produce undesirably high levels of transgene GFP expression in cells other than the desired airway epithelium and digestive tract targets (such as amniotic membrane, skin, liver or kidney), leading to reduced transfection of the intended targets. Furthermore, the CMV promoter is particularly prone to "switching off" expression [114], which may explain why 4 of 15 pups were negative by quantitative real-time PCR, while DNA PCR was positive. So CMV promoter replacement with a tissue-specific mammalian promoter may further increase the titer of viral vector for transfecting target tissues and may also increase the persistence of transgene expression [115]. Ostrowski *et al.* have developed a ciliated cell-specific promoter for CF therapy.

In this model, a human FOXJ1 promoter region was inserted into an eGFP expression cassette, and resulted in strong eGFP expression in the ciliated cells of airway epithelium [116].

We found that fetal mortality rate was closely related with the anesthetic method and surgical technique. In our study, ketamine (the preferred anesthetic for large animals) resulted in a very high fetal mortality rate. Ketamine becomes concentrated in the fetus and inhibits fetal movements [117]. It also takes 1 to 1.5 hours for animals to recover. Isoflurane, is a short term volatile anesthetic and mice recover from it just within 5 minutes. In addition, isoflurane relaxes the muscles of the uterus that then contract upon removal of the anesthesia and may aid in forcing the viral vector rapidly into the lungs and intestines. All survived pups were healthy compared to those controls injected with saline. This suggests that the mortality rate is somehow related to factors (surgical anesthetic) other than the viral vector itself.

To date, our study is the first to report short term lentiviral vector-mediated reporter gene transfer in vivo, to fetal respiratory epithelium in intact, survived animals. Using a novel human fetal tracheal culture system, Lim et al used a VSVG-pseudotyped lentiviral vector carrying the reporter transgene LacZ, to successfully transfect human fetal tracheas "in vivo", in severe combined immunoincompetent (SCID) murine hosts which have had subcutaneous pouches engrafted with human fetal tracheas which are then injected intraluminally with LV-marker gene [124]. Although marker gene transfer was highly efficient (80% of cells transduced), and durable (expression persisted for 9 months following transfection), this system of gene delivery and the host's immune capacity do not simulate in vivo gene transfer in an intact fetomaternal unit. Other studies have

reported reporter gene transfer to fetal respiratory epithelium through amniotic fluid using Ad with variable expression [101, 102, 104]. Similar studies in fetal small animal models using AAV have demonstrated pulmonary epithelial GFP within 72 hours post-amniotic injection (mice), with persistence of up to 9 months in the lungs of rats [108], however the utility of continued animal experiments of AAV-mediated fetal gene therapy (for eventual application to CF) is of questionable value, given the transgene packaging limitations associated with this vector

LV appears to be an ideal candidate for fetal gene therapy. Its ability to integrate transgene into the host genome and infect both dividing and nondividing cells, the lack of host immune response, and the large transgene capacity, make it a unique and desirable gene transfer agent.

# CHAPTER 5

# CONCLUSIONS

In this study, we evaluated LV mediated gene transfer to fetal respiratory epithelium in two animal models. Two gene transfer techniques were applied to fetal rabbits, which were direct tracheal injection and amniotic injection. Given that 3 to 4 days were necessary for successful transgene expression based on *in vitro* fetal rabbit experiments, LV mediated gene transfer to the fetal respiratory epithelium was achieved by term, by both direct tracheal injection (G26) and amniotic injection (G24 to 26). Amniotic injection was used in our mouse experiments. Successful gene transfer was achieved via amniotic injection on G15 to 17 CD-1 mice. In animals sacrificed at term, we observed preferential transgene expression in tracheas compared to lung, suggesting either reduced transduction efficiency of pulmonary epithelium resulting from vector "washout" through non-specific infection of irrelevant tissues (skin, amniotic membrane), or due to "switched off" gene expression in pulmonary alveolar cells compared to tracheal epithelium. In animals survived after fetal gene transfer, we observed the persistence of transgene expression (mRNA transcripts) for up to 21 days after birth in fetal lungs, but not trachea. We also saw the persistence of transgene by DNA PCR despite the absence of GFP mRNA in 4 of 15 pups, suggesting that gene expression had been "switched off" in these tissues.

Maternal biosafety is always an important concern in any fetal therapy model. We did not observe any maternal mortality, or treatment-specific maternal morbidity. We did however note gene transfer to maternal lung in one of our tracheal injection

rabbit experiments. The other important aspect of maternal safety not assessed in these experiments was the effect of fetal therapy on future reproduction.

Future studies in the mouse model of LV-mediated gene transfer should include an elucidation of the reason for reduced transgene expression after birth. The use of tissue specific mammalian promoters may allow preferential targeting of respiratory epithelium, and avoid a potential "washout effect" of non-specific gene transfer to tissues like skin and amniotic membrane. Finally this experimental system should be used to assess the efficacy of CFTR gene transfer in a disease (CFTR-knockout) model.

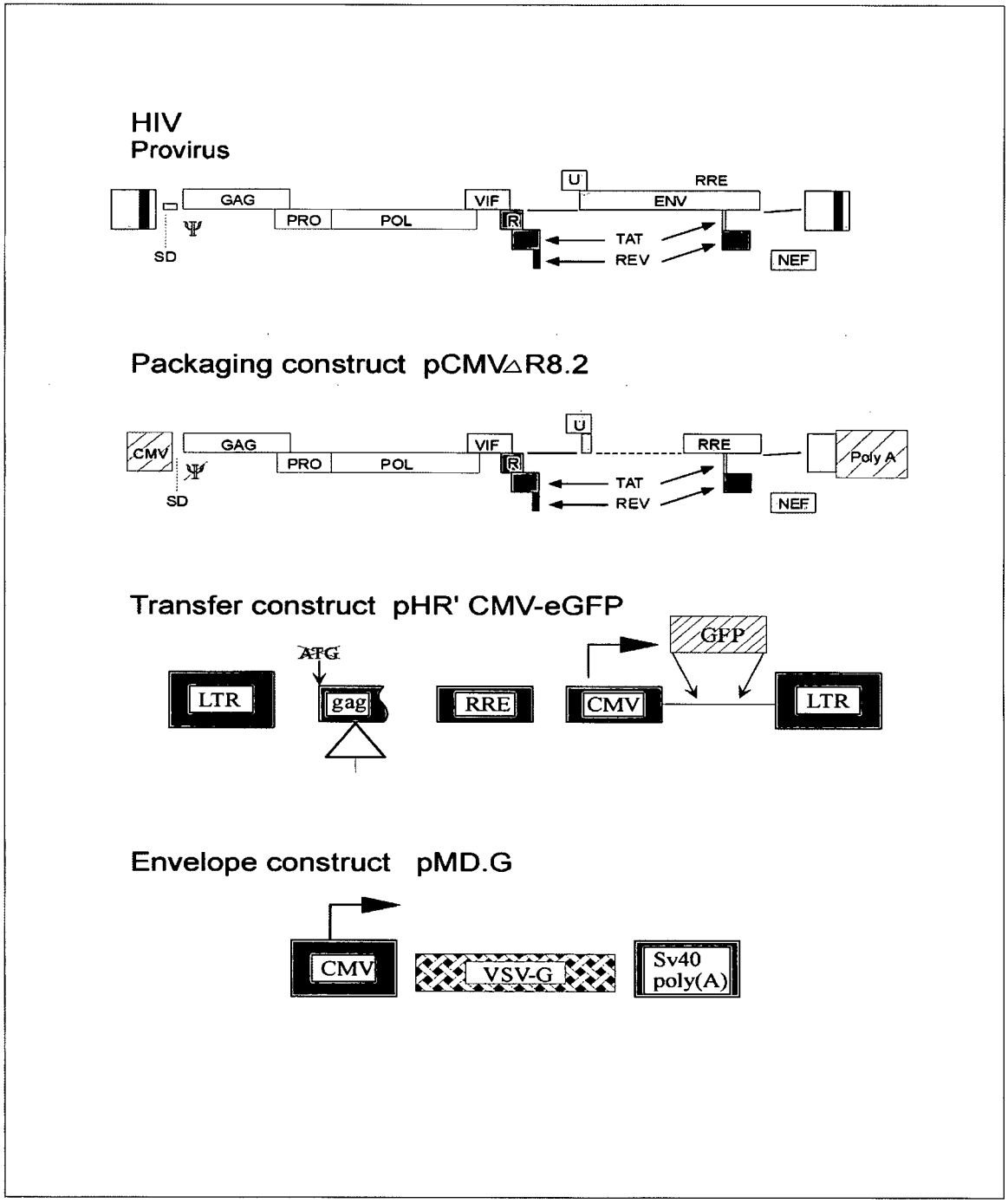



**Figure 1: The HIV-1 based lentiviral vector system**. The packaging construct, pCMVΔR8.2, contains all HIV-1 core and enzymatic components of the virion except the envelope gene. The transfer construct, pHR'CMV-eGFP, contains the reporter transgene GFP under the control of human cytomegalovirus (CMV) promoter and the minimal *cis*-acting elements of HIV. The envelope construct, pMD.G, is derived from vesicular stomatitis virus (VSV-G). Its G protein has high stability and broad tropism.

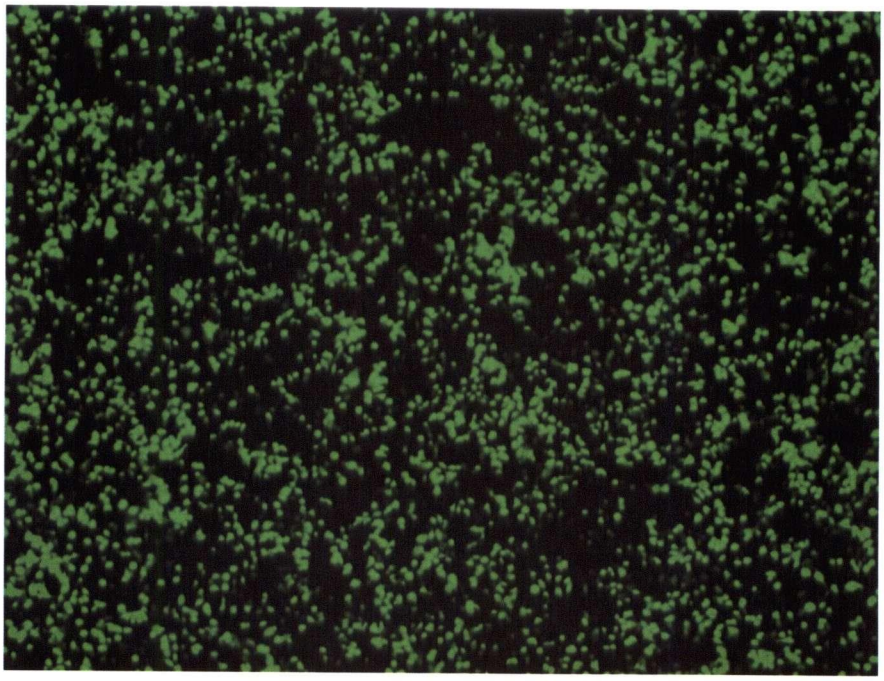


**Figure 2:** GFP-positive 293T cells under the fluorescent microscope one day after co-transfection (magnification x 100)

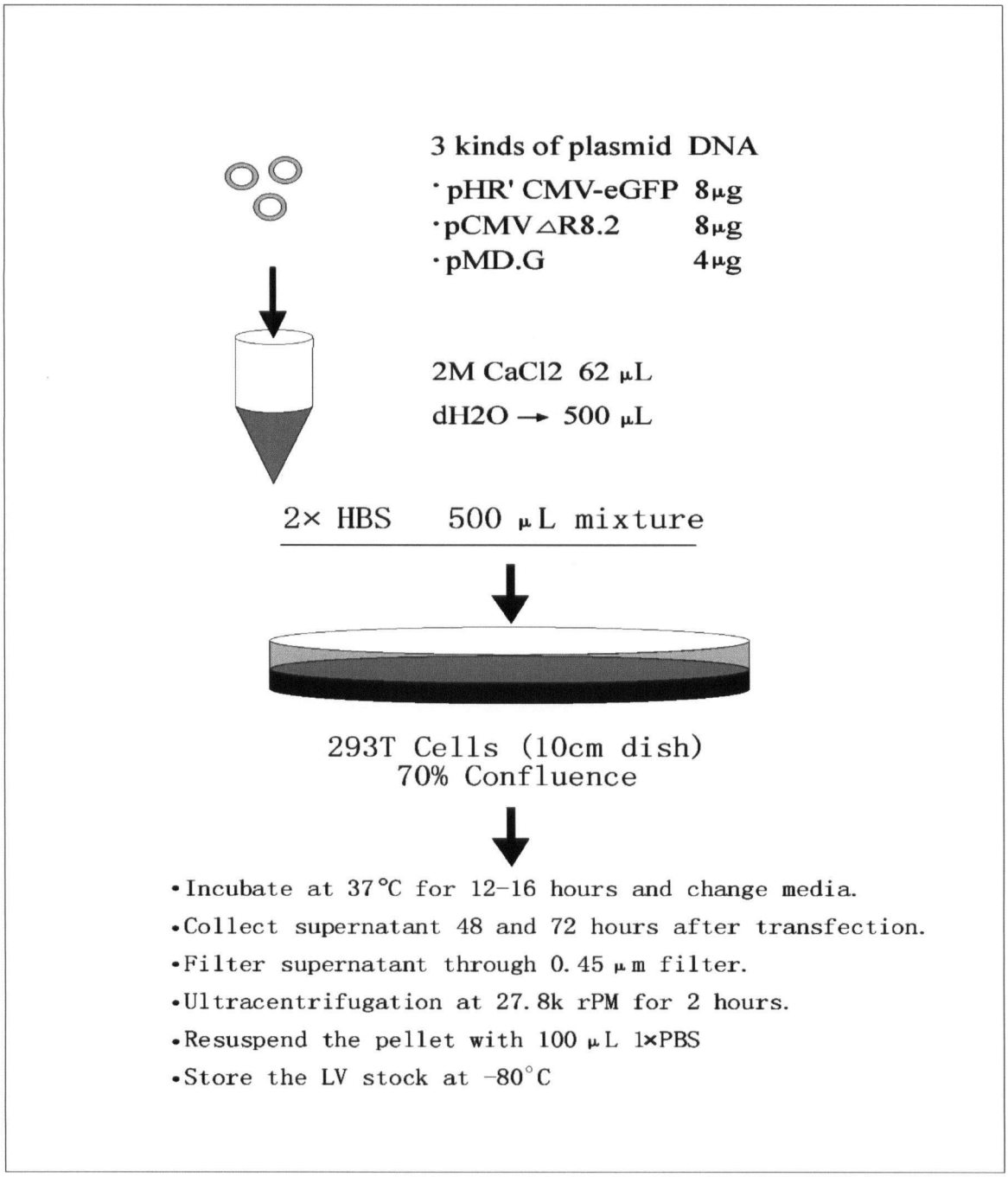



**Figure 3: Schematic of LV vector production**. The LV vector was constructed by calcium phosphate transfection to 293T cells with 3 plasmids; the supernatant was collected from day 2 through day 4 post-transfection and high-titer virus stock was generated by ultracentrifugation at 27.8k rpm for 2 hours.

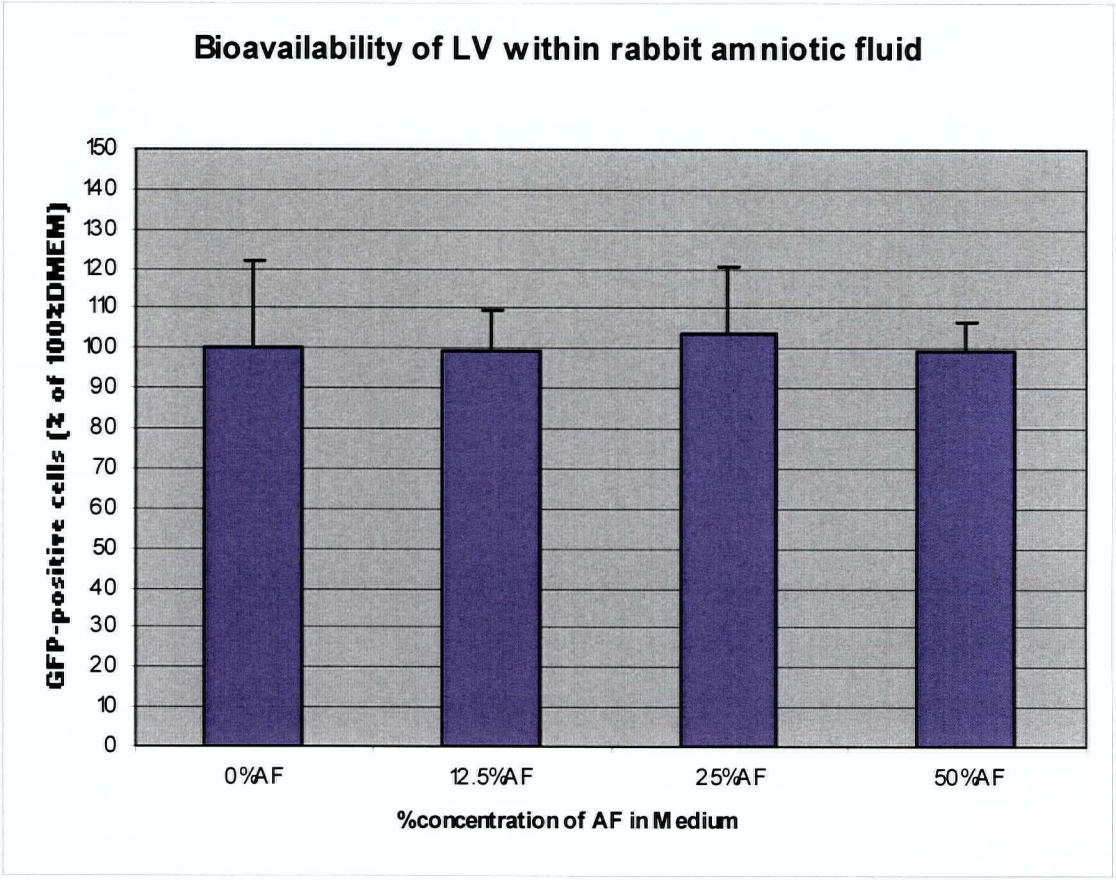



**Figure 4: Bioavailability of LV within rabbit amniotic fluid**. 10μL of diluted LV stock in DMEM containing equivalent numbers of LV particles was mixed with various concentration of AF and total 200μL of solution was added into a 24-well plate. After 1hr of incubation, 0.5mL of growth medium was added into each well. 48hrs later, GFP-positive cells were counted under the fluorescent microscope

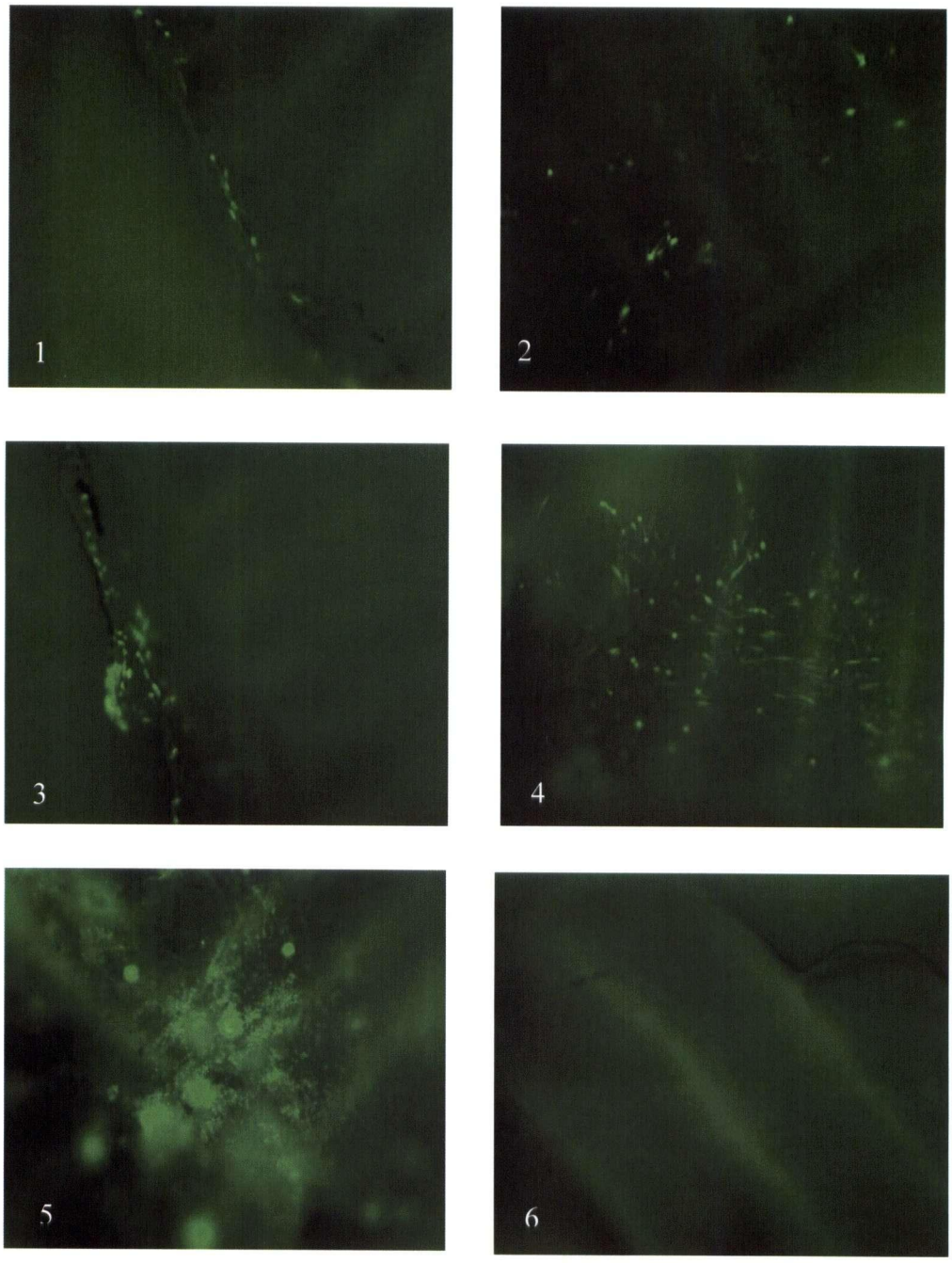
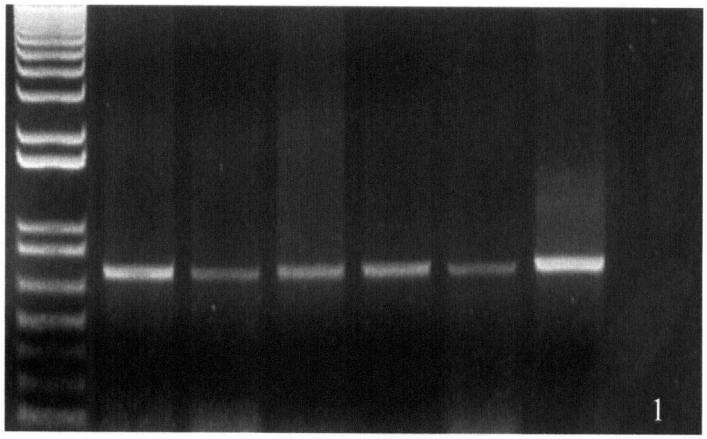
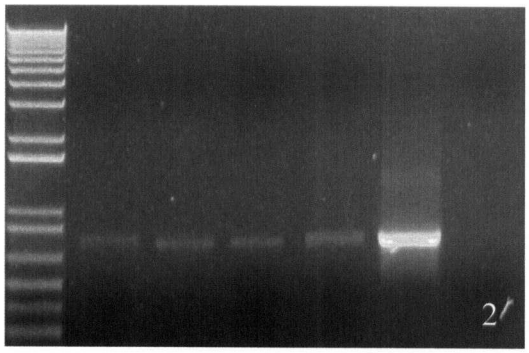


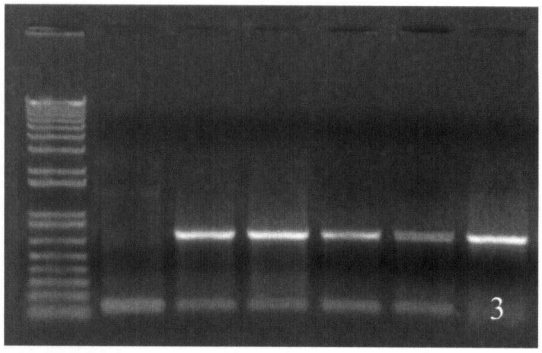

**Figure 5: In vitro fetal rabbit trachea transfection:** Determination of time required for infection. Fetal tracheas from G24 to 26 rabbits were put in organ culture medium and infected with 1x $10^6$ LV particles. The fetal tracheas were examined daily for evidence of GFP expression by whole-mount fluorescent microscopy. Images of day1, 2, 3, 5 and day 7 post transfection tracheas; (Fig.5.1-5). Fig.5.6 is negative control (x100)




1kb$^{+}$ladder D4 D5 D6 D7 D8 (+)ve (-)ve




D4 D6 D7 D8 (+)ve (-)ve




(-)ve D4 D6 D7 D8 (+)ve

**Figure 6: DNA PCR of Fetal Rabbit Tracheas following in vitro Transfection.** GFP was positive in fetal tracheas from day 4 to day 8 post-transfection. Specific PCR product is 700 bp. **Fig 6.1:** G24 fetal tracheas; **Fig. 6.2** G25 fetal tracheas; **Fig. 6.3** G26 fetal tracheas.

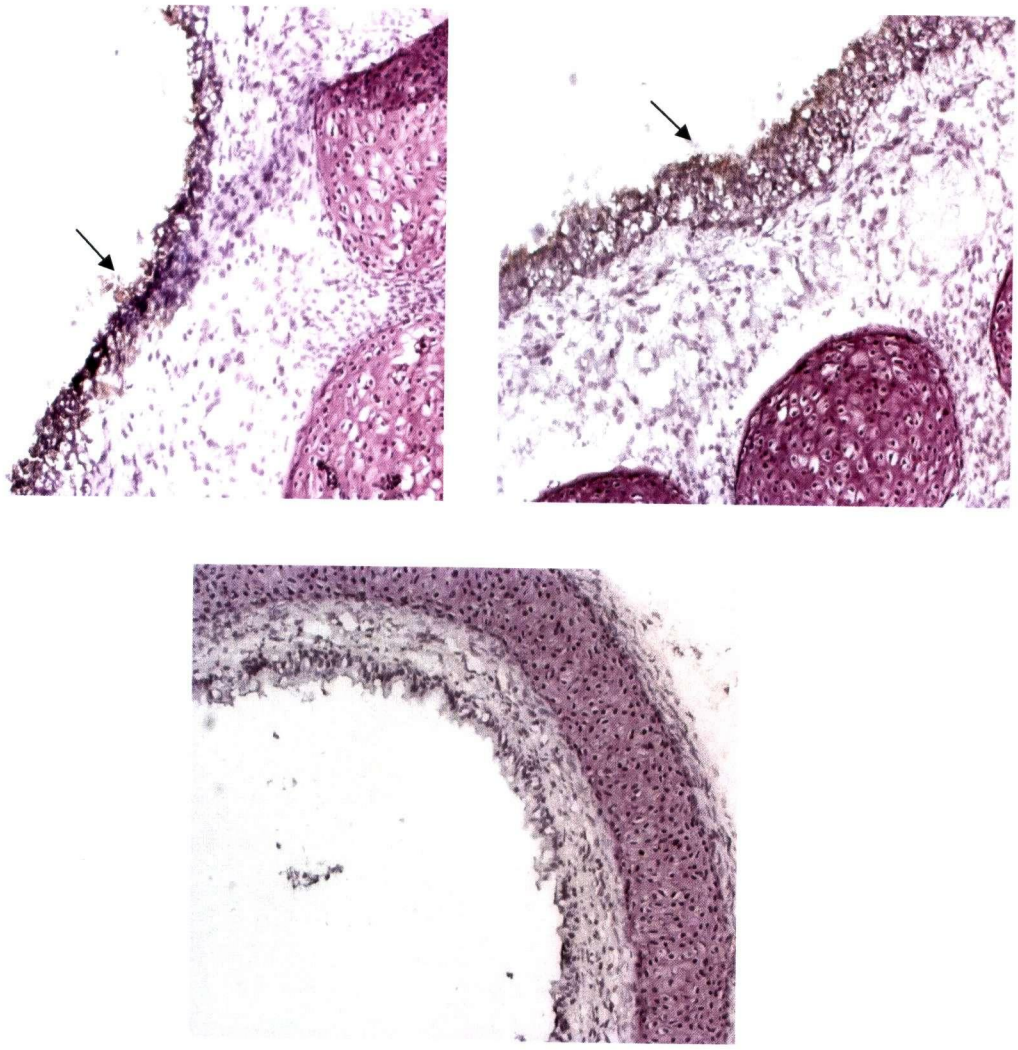


**Figure 7: Localization of GFP expression by IHC following in vitro transfection of fetal rabbit tracheas.** GFP positive cells were located on the luminal surface of tracheal epithelium (brown stain, arrows, x100)

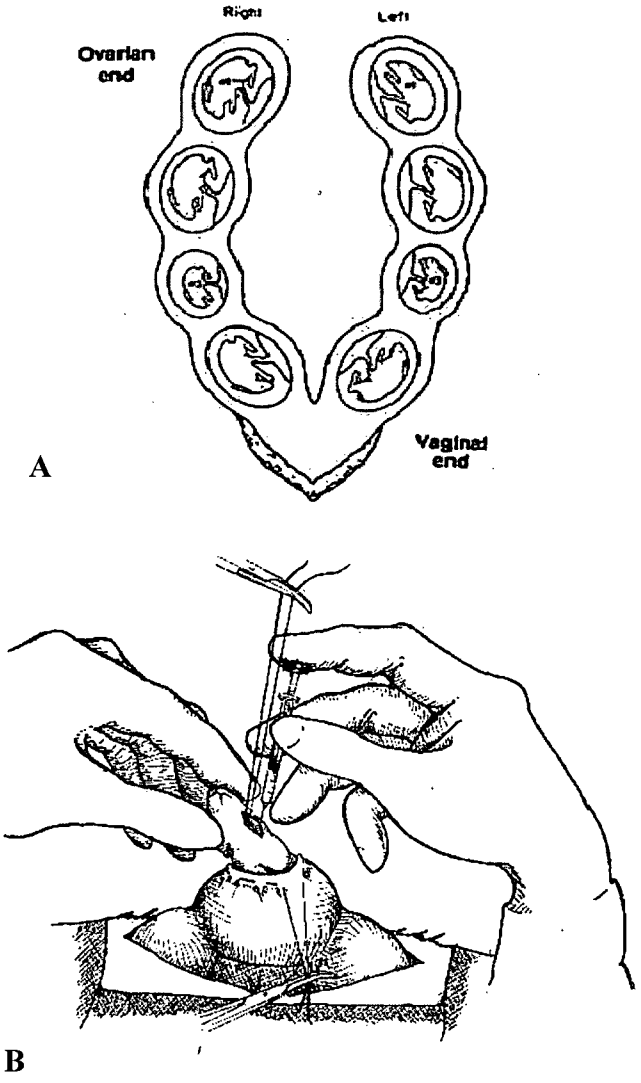
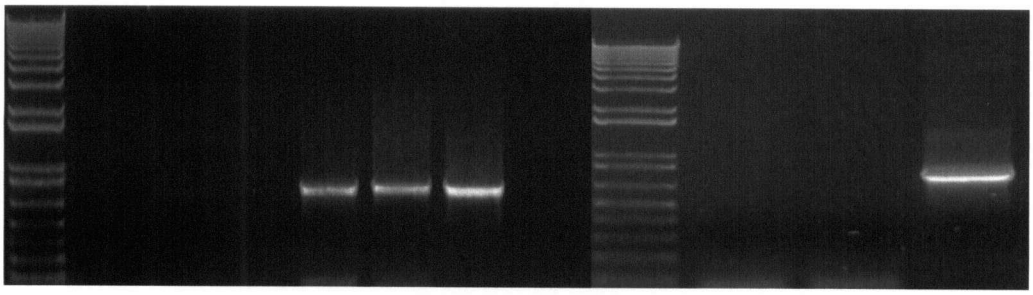



**Figure 8: *In vivo* Transfection Techniques**
A: Schematic of Rabbit Bicornuate Uterus (AI)
B: Illustration of tracheal injection (TI)



1 2 3 4 5 6 7 8 9 10 11 12

**Figure 9: Fetal rabbit tissue distribution of transgene GFP DNA after in vivo tracheal injection.** GFP DNA was present in fetal trachea and lung (lane5,6); fetal liver, kidney, small intestine, placenta and gonad were negative (lane 2, 3, 4, 10 and 11); lane 1 and 9 were $1kb^{+}$ DNA ladder; lane 7 and 12 were positive control (pHR'-CMV-eGFP plasmid DNA); lane 8 was negative control.

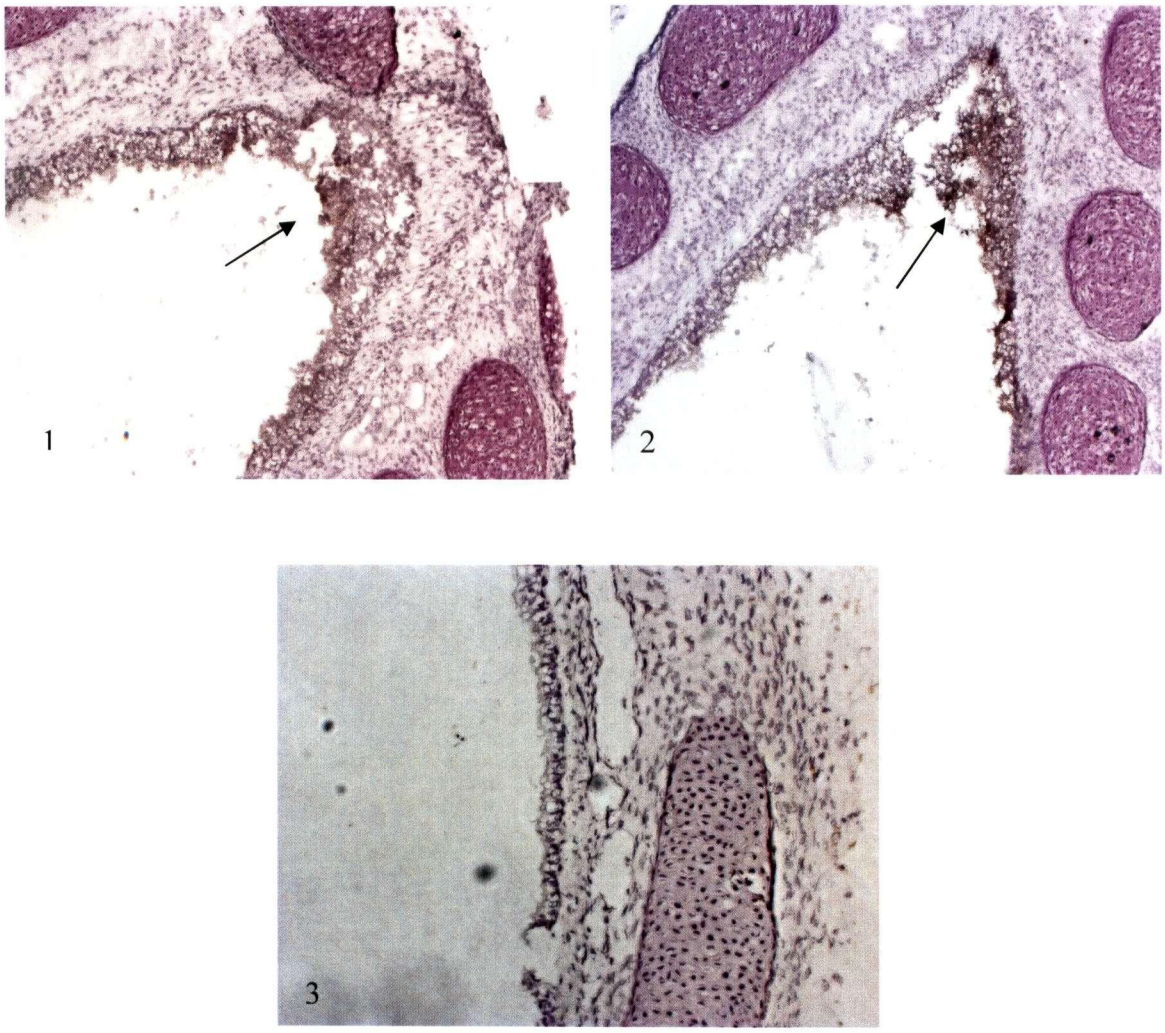



**Figure 10.1-3: Rabbit tracheal transgene GFP localization after TI (G26) by IHC.** Fig. 10.1- 2 show GFP expression was present on the luminal surface of fetal tracheal epithelium (brown staining, arrows); Fig. 10.3 is control (x 200)

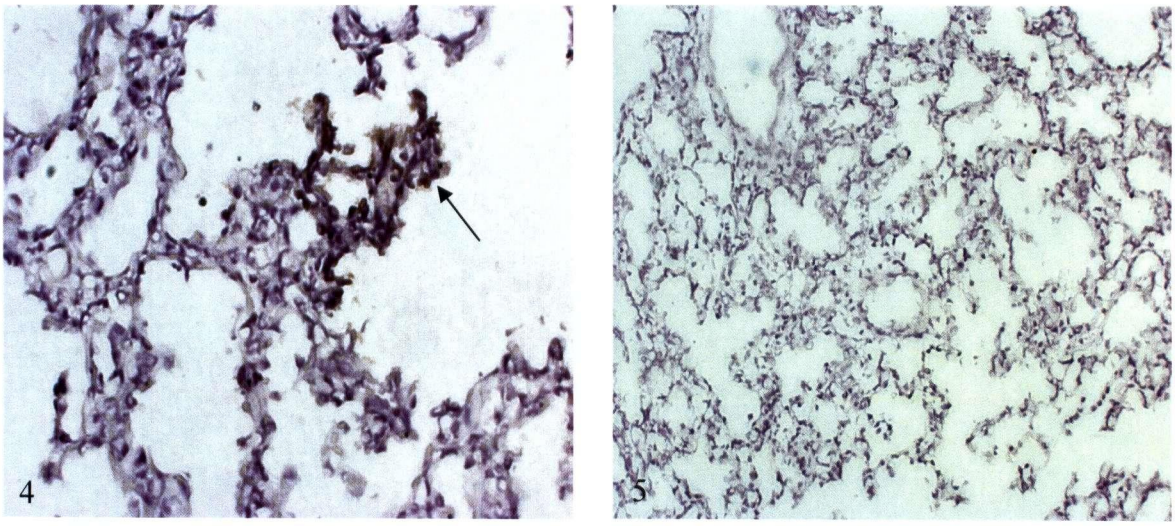



**Figure 10.4-5: Rabbit lung GFP localization after TI (G26) by IHC.** Fig. 11.4 shows that GFP expression (brown stain, arrow) was present in lung after direct tracheal injection (x200). Fig. 11.5 is control (x100)

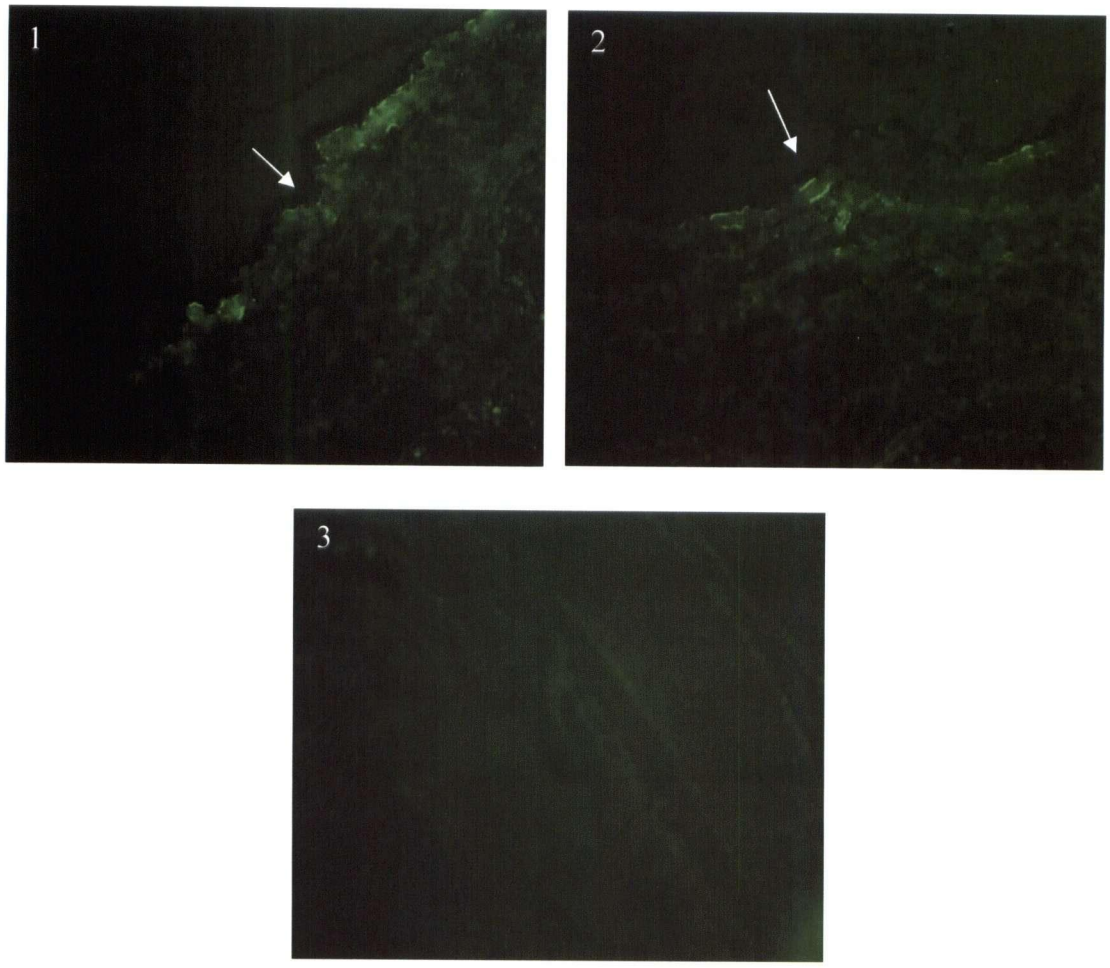



**Figure 11: Rabbit tracheal GFP expression by direct fluorescence microscopy:** fetal rabbit tracheas were harvested on G30 after amniotic injection on G24 (Fig. 11.1) and 25 (Fig 11.2). GFP positive cells were located on the surface of fetal tracheal epithelium. Fig. 11.3 is control (x 200)

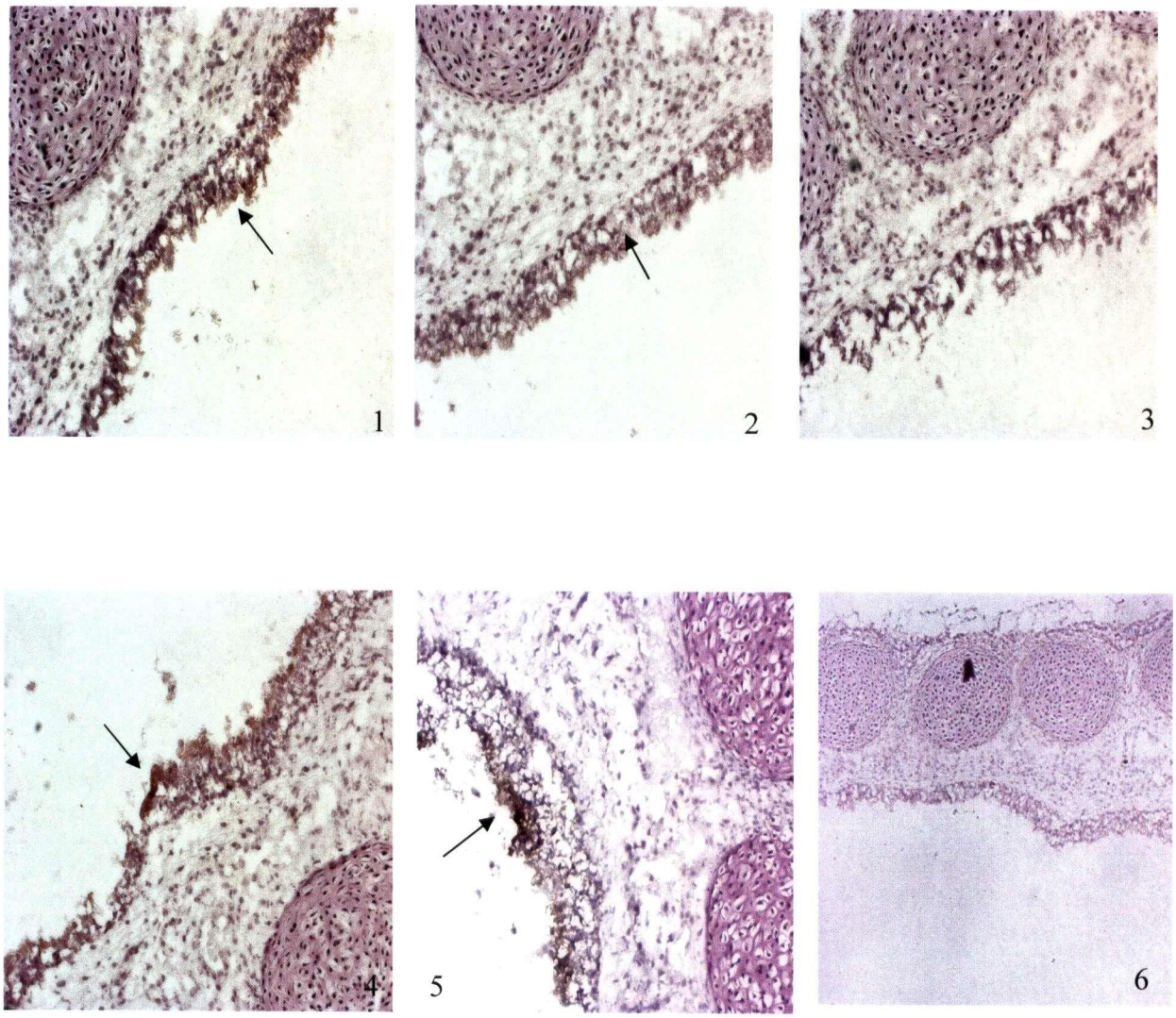
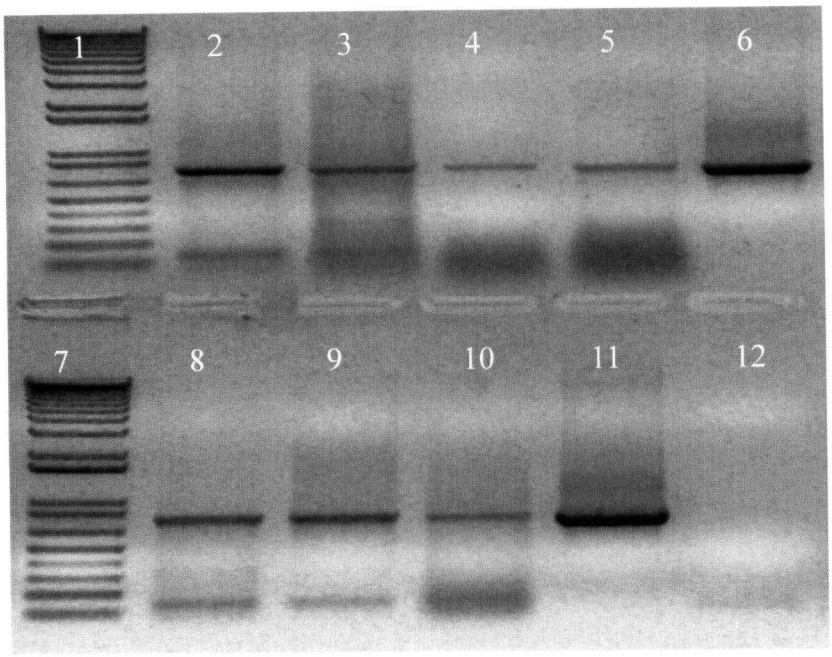



**Figure 12: Rabbit tracheal GFP localization by IHC.** GFP expression was detected on the luminal surface of fetal rabbit tracheal epithelium (brown stain, arrows) of both G24 (Fig. 12.1-2) and G25 (Fig. 12.4-5) fetal rabbits . Fig.12.3, 12.6 are controls (x100)




**Lane 1, 7: 1kb$^{+}$ DNA ladder**
**Lane 2: Trachea**
**Lane 3: Lung**
**Lane 4: Liver**
**Lane 5: Kidney**
**Lane 6, 11: Positive control: GFP plasmid DNA**
**Lane 8: Placenta**
**Lane 9: Small intestine**
**Lane 10: Amniotic membrane**
**Lane 12: Negative control**

**Figure 13: Distribution of transgene GFP DNA in fetal rabbit tissues at term following in vivo amniotic injection.** Transgene GFP DNA was detectable in fetal trachea, lung, liver, kidney, small intestine, placenta, amniotic membrane (and skin, not shown) of G25 fetal rabbit after amniotic injection. Same results were achieved on G24 fetal rabbits (data not shown).

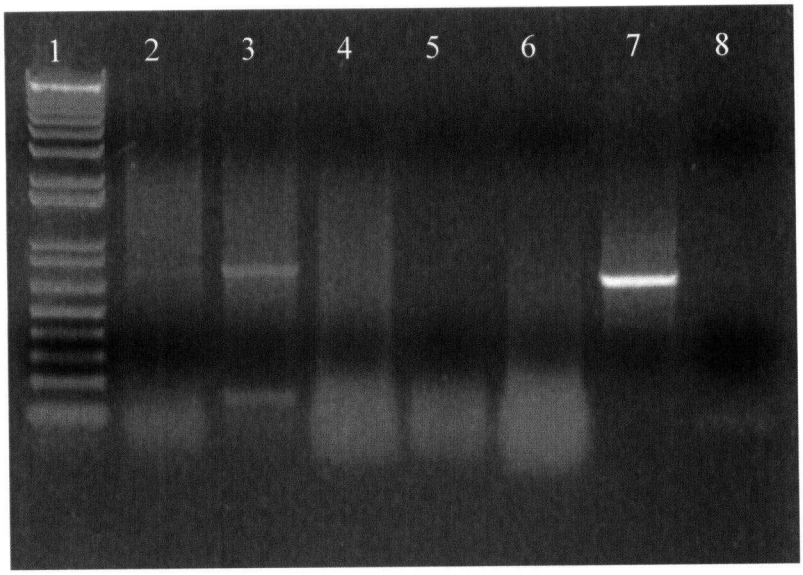



**Figure 14: Transgene detection in maternal rabbit.** Fig. 14 shows that GFP DNA was detected in lung of one doe whose fetuses underwent direct tracheal injection of LV-GFP (lane 3). Lane 1: $1kb^{+}$DNA ladder; lane 2: trachea; lane 4: liver; lane 5: ovary; lane 6: intestine; lane 7: pCMV-GFP plasmid DNA; lane 8: negative control.

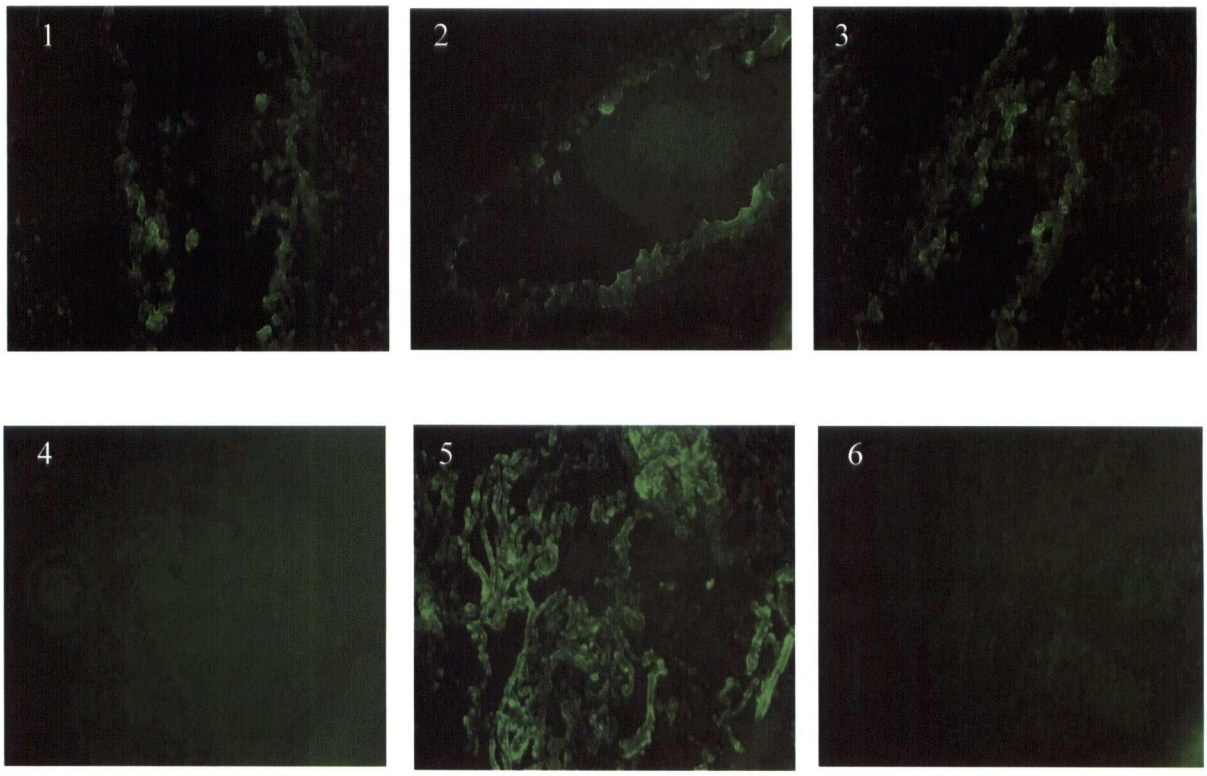



**Figure 15: Fetal Mouse fluorescent micrographs following amniotic injection on G15, 16, 17.** GFP positive cells were located on the tracheal epithelium of mice from 3 gestation time points (Fig. 15.1-2 and 15.3 show the tracheal cryo-sections of G15, 16 and 17, respectively). GFP positive cells were also present on the amniotic membrane (Fig. 15.5). Fig. 15.4, 15.6 show the trachea and amniotic membrane from control fetuses (x200)

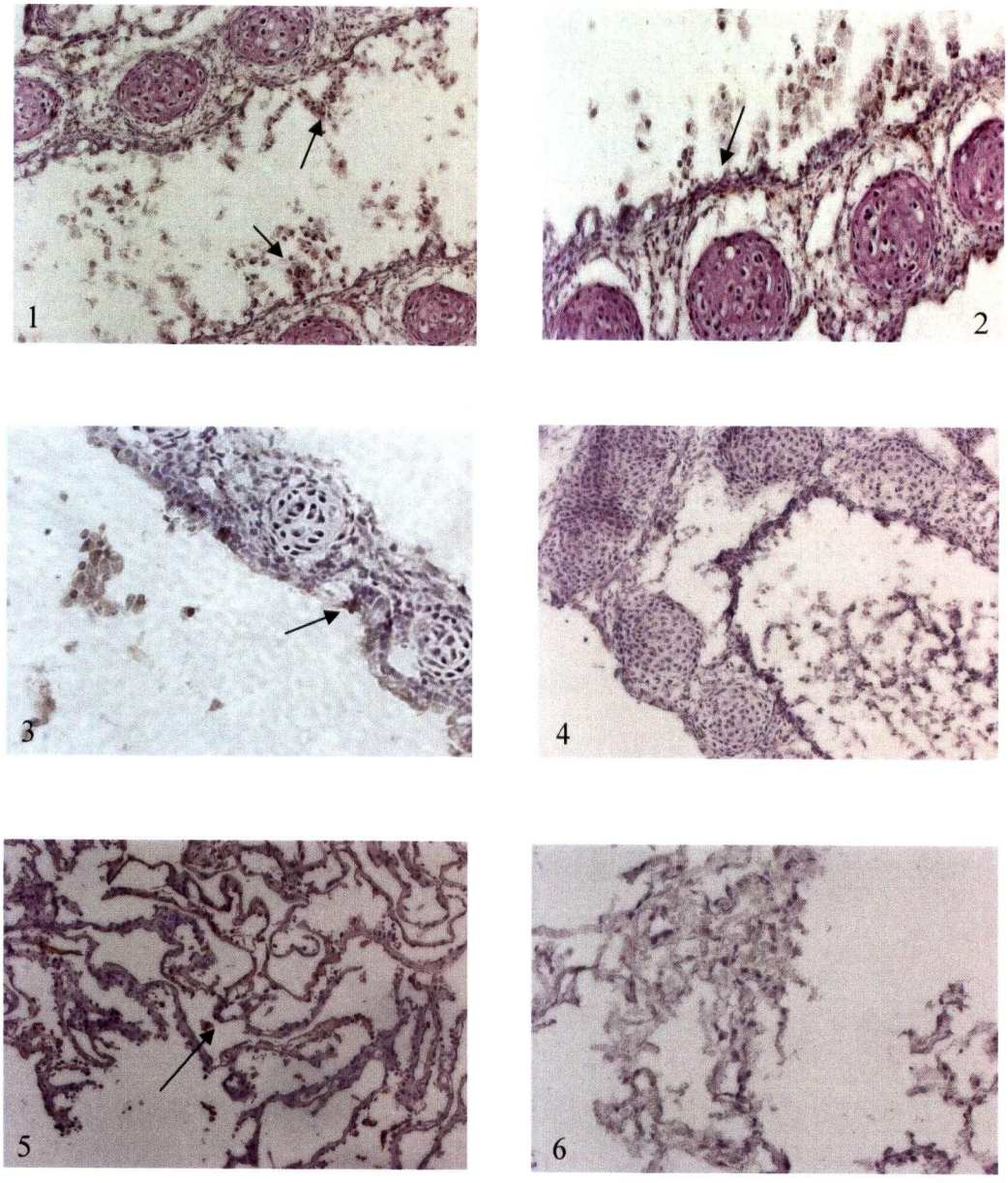



**Figure 16: GFP localization by IHC following AI in fetal mice.** Fig. 16.1-3 demonstrate that GFP expression were localized to tracheal epithelium of G15, 16 and 17 fetal mice (arrows). GFP expression was also detected on the amniotic membrane (Fig. 16.5). Fig. 16.4, 16.6 are control trachea and amniotic membrane (x200)

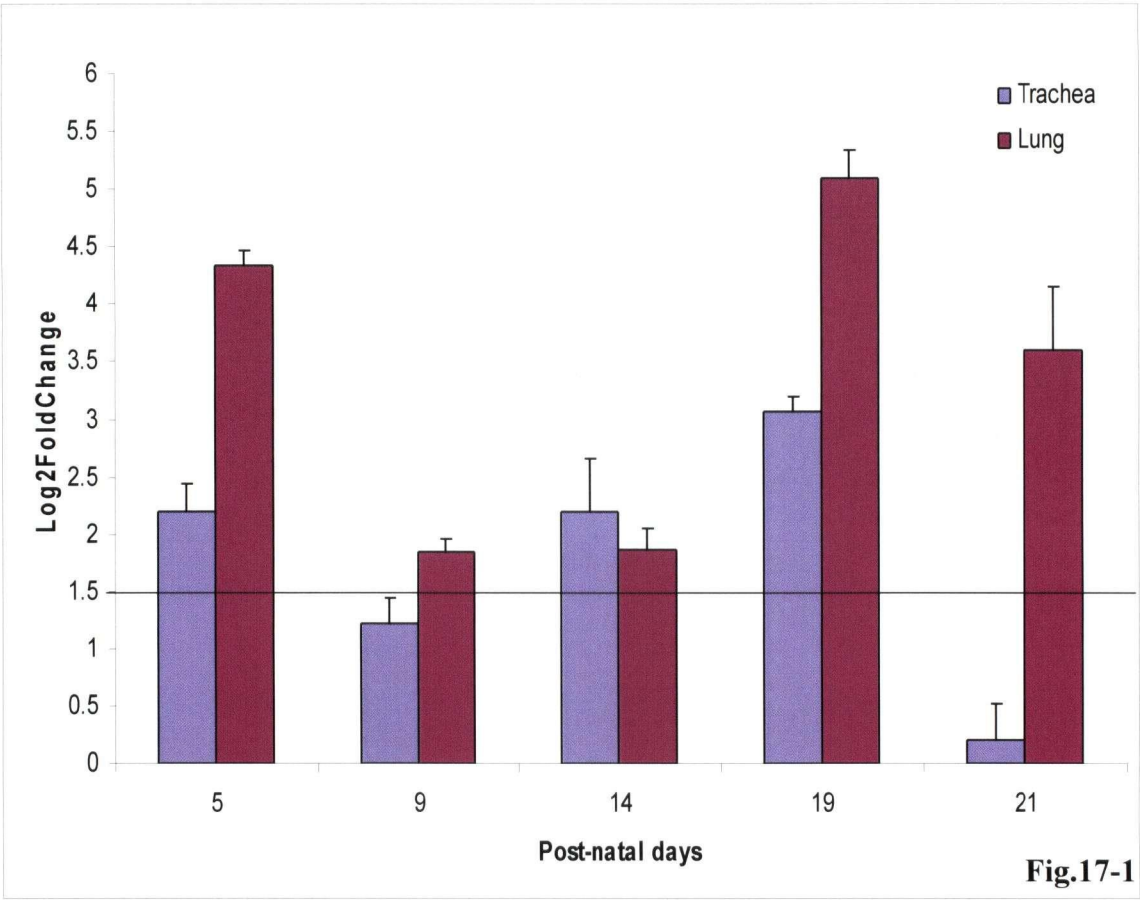



Fig.17-1

| Fig. 17-2 | Experiment 1 | | | Experiment 2 | | |
|---|---|---|---|---|---|---|
| | No. pups | Q-RT PCR | PCR | No. pups | Q-RT PCR | PCR |
| D5 | 2 | + | + | 2 | + | + |
| D9 | 2 | + | + | 2 | -- | + |
| D14 | 2 | + | + | 1 | + | + |
| D19 | 1 | + | + | 1 | -- | + |
| D21 | 1 | -- | + | 1* | + | Tra(-) |

Fig. 17-3

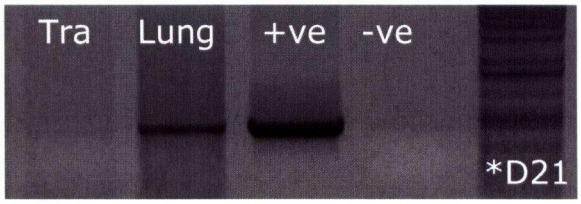



**Figure 17: GFP transgene persistence after birth by QRT-PCR (mRNA) and PCR**
Fig.17.1: Duration of transgene expression by QRT-PCR following AI of G16 mice. $Log_2$ Fold Change (derived from the normalization of actin and GFP cDNA between tissues from control and injected groups) > 1.5 means transgene is being expressed (mRNA produced). Fig.17.2: Concordance between transgene presence (PCR) and expression (QRT-PCR). Fig. 17.3: PCR gel from D21 postnatal mouse (asterisk). Note lung contains transgene DNA which is expressed (QRT-PCR+), while trachea does not (negative by both PCR and QRT-PCR).

## Table 1: Fetal Mortality: Effects of Anesthetic Agent and Fetal Hydration

| | Total does | Total fetuses | Alive fetuses | Mortality rate |
|---|---|---|---|---|
| Ketamine | 3 | 30 | 4 | 87% |
| Isoflurane | 4 | 36 | 12 | 67% |
| Isoflurane+ maternal injection of saline | 5 | 35 | 26 | 26% |

# References:


1. Kerem, B. and E. Kerem, *The molecular basis for disease variability in cystic fibrosis.* Eur J Hum Genet, 1996. 4(2): p. 65-73.
2. *Population variation of common cystic fibrosis mutations. The Cystic Fibrosis Genetic Analysis Consortium.* Hum Mutat, 1994. 4(3): p. 167-77.
3. Riordan, J.R., et al., *Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA.* Science, 1989. 245(4922): p. 1066-73.
4. Wine, J.J., *The genesis of cystic fibrosis lung disease.* J Clin Invest, 1999. 103(3): p. 309-12.
5. Lambert, E.J., S.A. Peters, and C.J. Rolles, *Cystic fibrosis; current survival and population estimates to the year 2000.* Thorax, 1992. 47(5): p. 400.
6. Buscher, R., et al., *beta2 adrenoceptor gene polymorphisms in cystic fibrosis lung disease.* Pharmacogenetics, 2002. 12(5): p. 347-53.
7. Stutts, M.J., B.C. Rossier, and R.C. Boucher, *Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics.* J Biol Chem, 1997. 272(22): p. 14037-40.
8. Donaldson, S.H., E.G. Poligone, and M.J. Stutts, *CFTR regulation of ENaC.* Methods Mol Med, 2002. 70: p. 343-64.
9. Gabriel, S.E., et al., *CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship.* Nature, 1993. 363(6426): p. 263-8.
10. Schwiebert, E.M., et al., *Chloride channel and chloride conductance regulator domains of CFTR, the cystic fibrosis transmembrane conductance regulator.* Proc Natl Acad Sci U S A, 1998. 95(5): p. 2674-9.
11. Cahill, P., et al., *Identification of the cystic fibrosis transmembrane conductance regulator domains that are important for interactions with ROMK2.* J Biol Chem, 2000. 275(22): p. 16697-701.

12. Kunzelmann, K., et al., *The cystic fibrosis transmembrane conductance regulator attenuates the endogenous Ca2+ activated Cl- conductance of Xenopus oocytes.* Pflugers Arch, 1997. 435(1): p. 178-81.

13. Bradbury, N.A., et al., *Regulation of plasma membrane recycling by CFTR.* Science, 1992. 256(5056): p. 530-2.

14. Larson, J.E., et al., *CFTR modulates lung secretory cell proliferation and differentiation.* Am J Physiol Lung Cell Mol Physiol, 2000. 279(2): p. L333-41.

15. Tsui, L.C. and P. Durie, *Genotype and phenotype in cystic fibrosis.* Hosp Pract (Off Ed), 1997. 32(6): p. 115-8, 123-9, 134, passim.

16. Grody, W.W., et al., *Laboratory standards and guidelines for population-based cystic fibrosis carrier screening*, in *Genet Med.* 2001. p. 149-54.

17. Zeitlin, P.L., *Novel pharmacologic therapies for cystic fibrosis.* J Clin Invest, 1999. 103(4): p. 447-52.

18. Vankeerberghen, A., H. Cuppens, and J.J. Cassiman, *The cystic fibrosis transmembrane conductance regulator: an intriguing protein with pleiotropic functions.* J Cyst Fibros, 2002. 1(1): p. 13-29.

19. *Correlation between genotype and phenotype in patients with cystic fibrosis. The Cystic Fibrosis Genotype-Phenotype Consortium.* N Engl J Med, 1993. 329(18): p. 1308-13.

20. Wilson, R.D., et al., *Cystic fibrosis carrier testing in pregnancy in Canada.* J Obstet Gynaecol Can, 2002. 24(8): p. 644-51.

21. Grody, W.W., et al., *Laboratory standards and guidelines for population-based cystic fibrosis carrier screening.* Genet Med, 2001. 3(2): p. 149-54.

22. Friedmann, T., *The maturation of human gene therapy.* Acta Paediatr, 1996. 85(11): p. 1261-5.

23. Morgan, J.R., et al., *Expression of an exogenous growth hormone gene by transplantable human epidermal cells.* Science, 1987. 237(4821): p. 1476-9.

24. Wilson, J.M., et al., *Implantation of vascular grafts lined with genetically modified endothelial cells.* Science, 1989. 244(4910): p. 1344-6.

25. Garver, R.I., Jr., et al., *Clonal gene therapy: transplanted mouse fibroblast clones express human alpha 1-antitrypsin gene in vivo.* Science, 1987. 237(4816): p. 762-4.

26. Palmer, T.D., et al., *Efficient retrovirus-mediated transfer and expression of a human adenosine deaminase gene in diploid skin fibroblasts from an adenosine deaminase-deficient human.* Proc Natl Acad Sci U S A, 1987. 84(4): p. 1055-9.

27. Blaese, R.M., et al., *T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years.* Science, 1995. 270(5235): p. 475-80.

28. Lim, B., et al., *Long-term expression of human adenosine deaminase in mice transplanted with retrovirus-infected hematopoietic stem cells.* Proc Natl Acad Sci U S A, 1989. 86(22): p. 8892-6.

29. Friedmann, T., *Overcoming the obstacles to gene therapy.* Sci Am, 1997. 276(6): p. 96-101.

30. Levy, R.J., S.A. Goldstein, and J. Bonadio, *Gene therapy for tissue repair and regeneration.* Adv Drug Deliv Rev, 1998. 33(1-2): p. 53-69.

31. Bergelson, J.M., et al., *Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5.* Science, 1997. 275(5304): p. 1320-3.

32. Wickham, T.J., et al., *Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization.* J Cell Biol, 1994. 127(1): p. 257-64.

33. Greber, U.F., et al., *Stepwise dismantling of adenovirus 2 during entry into cells.* Cell, 1993. 75(3): p. 477-86.

34. Nicklin, S.A. and A.H. Baker, *Tropism-modified adenoviral and adeno-associated viral vectors for gene therapy.* Curr Gene Ther, 2002. 2(3): p. 273-93.

35. Lanciotti, J., et al., *Targeting adenoviral vectors using heterofunctional polyethylene glycol FGF2 conjugates.* Mol Ther, 2003. 8(1): p. 99-107.

36. Nettelbeck, D.M., et al., *Retargeting of adenoviral infection to melanoma: combining genetic ablation of native tropism with a recombinant bispecific single-chain diabody (scDb) adapter that binds to fiber knob and HMWMAA.* Int J Cancer, 2004. 108(1): p. 136-45.

37. St George, J.A., *Gene therapy progress and prospects: adenoviral vectors.* Gene Ther, 2003. 10(14): p. 1135-41.

38. Liu, Q., et al., *The role of capsid-endothelial interactions in the innate immune response to adenovirus vectors.* Hum Gene Ther, 2003. 14(7): p. 627-43.

39. Schiedner, G., et al., *Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors.* Mol Ther, 2003. 7(1): p. 35-43.

40. Schiedner, G., et al., *A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells.* Hum Gene Ther, 2003. 14(17): p. 1631-41.

41. Raper, S.E., et al., *Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer.* Mol Genet Metab, 2003. 80(1-2): p. 148-58.

42. Buning, H., et al., *AAV-based gene transfer.* Curr Opin Mol Ther, 2003. 5(4): p. 367-75.

43. Flotte, T.R., *Recombinant adeno-associated virus vectors for cystic fibrosis gene therapy.* Curr Opin Mol Ther, 2001. 3(5): p. 497-502.

44. Summerford, C. and R.J. Samulski, *Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions.* J Virol, 1998. 72(2): p. 1438-45.

45. Halbert, C.L., J.M. Allen, and A.D. Miller, *Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors.* J Virol, 2001. 75(14): p. 6615-24.

46. Halbert, C.L., J.M. Allen, and A.D. Miller, *Efficient mouse airway transduction following recombination between AAV vectors carrying parts of a larger gene.* Nat Biotechnol, 2002. 20(7): p. 697-701.

47. Johnson, L.G., *Retroviral approaches to gene therapy of cystic fibrosis.* Ann N Y Acad Sci, 2001. 953: p. 43-52.

48. Trono, D., *Lentiviral vectors: turning a deadly foe into a therapeutic agent.* Gene Ther, 2000. 7(1): p. 20-3.

49. Bukrinsky, M.I., et al., *A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells.* Nature, 1993. 365(6447): p. 666-9.

50. Kafri, T., et al., *Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors.* Nat Genet, 1997. 17(3): p. 314-7.

51. Withers-Ward, E.S., et al., *Distribution of targets for avian retrovirus DNA integration in vivo.* Genes Dev, 1994. 8(12): p. 1473-87.

52. Bushman, F.D. and M.D. Miller, *Tethering human immunodeficiency virus type 1 preintegration complexes to target DNA promotes integration at nearby sites.* J Virol, 1997. 71(1): p. 458-64.

53. Parolin, C., et al., *Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes.* J Virol, 1994. 68(6): p. 3888-95.

54. Naldini, L., et al., *In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector.* Science, 1996. 272(5259): p. 263-7.

55. Naldini, L., et al., *Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector.* Proc Natl Acad Sci U S A, 1996. 93(21): p. 11382-8.

56. Zufferey, R., et al., *Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo.* Nat Biotechnol, 1997. 15(9): p. 871-5.

57. Zufferey, R., et al., *Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery.* J Virol, 1998. 72(12): p. 9873-80.

58. Dull, T., et al., *A third-generation lentivirus vector with a conditional packaging system.* J Virol, 1998. 72(11): p. 8463-71.

59. Borok, Z., et al., *Vesicular stomatitis virus G-pseudotyped lentivirus vectors mediate efficient apical transduction of polarized quiescent primary alveolar epithelial cells.* J Virol, 2001. 75(23): p. 11747-54.

60. Johnson, L.G., et al., *Pseudotyped human lentiviral vector-mediated gene transfer to airway epithelia in vivo.* Gene Ther, 2000. 7(7): p. 568-74.

61. Borthwick, D.W., et al., *Evidence for stem-cell niches in the tracheal epithelium.* Am J Respir Cell Mol Biol, 2001. 24(6): p. 662-70.

62. Sinn, P.L., et al., *Lentivirus vectors pseudotyped with filoviral envelope glycoproteins transduce airway epithelia from the apical surface independently of folate receptor alpha.* J Virol, 2003. 77(10): p. 5902-10.

63. Follenzi, A., et al., *Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences.* Nat Genet, 2000. 25(2): p. 217-22.

64. Limberis, M., et al., *Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer.* Hum Gene Ther, 2002. 13(16): p. 1961-70.

65. Goldman, M.J., et al., *Lentiviral vectors for gene therapy of cystic fibrosis.* Hum Gene Ther, 1997. 8(18): p. 2261-8.

66. Chauhan, D.P., et al., *In utero gene therapy: prospect and future.* Curr Pharm Des, 2004. 10(29): p. 3663-72.

67. Flotte, T.R. and B.L. Laube, *Gene therapy in cystic fibrosis.* Chest, 2001. 120(3 Suppl): p. 124S-131S.

68. Drumm, M.L., et al., *Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer.* Cell, 1990. 62(6): p. 1227-33.

69. Rosenfeld, M.A., et al., *Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo.* Science, 1991. 252(5004): p. 431-4.

70. Rosenfeld, M.A., et al., *In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium.* Cell, 1992. 68(1): p. 143-55.

71. Knowles, M.R., et al., *A controlled study of adenoviral-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis.* N Engl J Med, 1995. 333(13): p. 823-31.

72. Alton, E.W., et al., *Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial.* Lancet, 1999. 353(9157): p. 947-54.

73. Zabner, J., et al., *Repeat administration of an adenovirus vector encoding cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis.* J Clin Invest, 1996. 97(6): p. 1504-11.

74. Ruiz, F.E., et al., *A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis.* Hum Gene Ther, 2001. 12(7): p. 751-61.

75. Caplen, N.J., et al., *Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis.* Nat Med, 1995. 1(1): p. 39-46.

76. Flotte, T., et al., *A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease.* Hum Gene Ther, 1996. 7(9): p. 1145-59.

77. Wagner, J.A., et al., *Efficient and persistent gene transfer of AAV-CFTR in maxillary sinus.* Lancet, 1998. 351(9117): p. 1702-3.

78. Aitken, M.L., et al., *A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease.* Hum Gene Ther, 2001. 12(15): p. 1907-16.

79. Griesenbach, U., D.M. Geddes, and E.W. Alton, *Advances in cystic fibrosis gene therapy.* Curr Opin Pulm Med, 2004. 10(6): p. 542-6.

80. Duan, D., Y. Yue, and J.F. Engelhardt, *Expanding AAV packaging capacity with trans-splicing or overlapping vectors: a quantitative comparison.* Mol Ther, 2001. 4(4): p. 383-91.

81. Ferrari, S., D.M. Geddes, and E.W. Alton, *Barriers to and new approaches for gene therapy and gene delivery in cystic fibrosis.* Adv Drug Deliv Rev, 2002. 54(11): p. 1373-93.

82. Drittanti, L., et al., *Cystic fibrosis: gene therapy or preventive gene transfer?* Gene Ther, 1997. 4(10): p. 1001-3.

83. Lim, F.Y., et al., *Adeno-associated virus (AAV)-mediated gene transfer in respiratory epithelium and submucosal gland cells in human fetal tracheal organ culture.* J Pediatr Surg, 2002. 37(7): p. 1051-7; discussion 1051-7.

84. Phillips, J.D., et al., *An improved model of experimental gastroschisis in fetal rabbits.* J Pediatr Surg, 1991. 26(7): p. 784-7.

85. Skarsgard, E.D., et al., *Fetal therapy with rhIGF-1 in a rabbit model of intrauterine growth retardation.* J Surg Res, 2001. 99(1): p. 142-6.

86. Housley, H.T., et al., *Creation of myelomeningocele in the fetal rabbit.* Fetal Diagn Ther, 2000. 15(5): p. 275-9.

87. Mommaerts, M.Y., et al., *Unicoronal suture immobilization in the fetal rabbit.* J Craniofac Surg, 2004. 15(2): p. 192-9.

88. Peters, C.A., *Animal models of fetal renal disease.* Prenat Diagn, 2001. 21(11): p. 917-23.

89. Roubliova, X.I., et al., *Effect of tracheal occlusion on peripheric pulmonary vessel muscularization in a fetal rabbit model for congenital diaphragmatic hernia.* Am J Obstet Gynecol, 2004. 191(3): p. 830-6.

90. Khan, T.Z., et al., *Early pulmonary inflammation in infants with cystic fibrosis.* Am J Respir Crit Care Med, 1995. 151(4): p. 1075-82.

91. Sturgess, J. and J. Imrie, *Quantitative evaluation of the development of tracheal submucosal glands in infants with cystic fibrosis and control infants.* Am J Pathol, 1982. 106(3): p. 303-11.

92. Ornoy, A., et al., *Pathological confirmation of cystic fibrosis in the fetus following prenatal diagnosis.* Am J Med Genet, 1987. 28(4): p. 935-47.

93. Nelson, J.M., et al., *Operative techniques in the fetal rabbit.* J Invest Surg, 1990. 3(4): p. 393-8.

94. Buchmiller, T.L., et al., *Transamniotic fetal feeding: enhancement of growth in a rabbit model of intrauterine growth retardation.* Surgery, 1994. 116(1): p. 36-41.

95. Zeitlin, P.L., et al., *Alveolar stem cell transduction by an adeno-associated viral vector.* Gene Ther, 1995. 2(9): p. 623-31.

96. Rubenstein, R.C., et al., *CFTR gene transduction in neonatal rabbits using an adeno-associated virus (AAV) vector.* Gene Ther, 1997. 4(5): p. 384-92.

97. Boyle, M.P., et al., *In utero AAV-mediated gene transfer to rabbit pulmonary epithelium.* Mol Ther, 2001. 4(2): p. 115-21.

98. Hasan, S.U. and A. Rigaux, *The effects of lung distension, oxygenation, and gestational age on fetal behavior and breathing movements in sheep.* Pediatr Res, 1991. 30(2): p. 193-201.

99. Kozuma, S., et al., *Maturational sequence of fetal breathing movements.* Biol Neonate, 1991. 60 Suppl 1: p. 36-40.

100. Holzinger, A., et al., *Intraamniotic administration of an adenoviral vector for gene transfer to fetal sheep and mouse tissues.* Pediatr Res, 1995. 38(6): p. 844-50.

101. McCray, P.B., Jr., et al., *Adenoviral-mediated gene transfer to fetal pulmonary epithelia in vitro and in vivo.* J Clin Invest, 1995. 95(6): p. 2620-32.

102. Douar, A.M., et al., *Foetal gene delivery in mice by intra-amniotic administration of retroviral producer cells and adenovirus.* Gene Ther, 1997. 4(9): p. 883-90.

103. Mitchell, M., et al., *Long-term gene transfer to mouse fetuses with recombinant adenovirus and adeno-associated virus (AAV) vectors.* Gene Ther, 2000. 7(23): p. 1986-92.

104. Senoo, M., et al., *Adenovirus-mediated in utero gene transfer in mice and guinea pigs: tissue distribution of recombinant adenovirus determined by quantitative TaqMan-polymerase chain reaction assay.* Mol Genet Metab, 2000. 69(4): p. 269-76.

105. Vincent, M.C., et al., *Adenovirus-mediated gene transfer to the respiratory tract of fetal sheep in utero.* Hum Gene Ther, 1995. 6(8): p. 1019-28.

106. Wang, G., et al., *Ultrasound-guided gene transfer to hepatocytes in utero.* Fetal Diagn Ther, 1998. 13(4): p. 197-205.

107. Yang, E.Y., et al., *Persistent postnatal transgene expression in both muscle and liver after fetal injection of recombinant adenovirus.* J Pediatr Surg, 1999. 34(5): p. 766-72; discussion 772-3.

108. Garrett, D.J., et al., *In utero recombinant adeno-associated virus gene transfer in mice, rats, and primates.* BMC Biotechnol, 2003. 3(1): p. 16.

109. Sekhon, H.S. and J.E. Larson, *In utero gene transfer into the pulmonary epithelium.* Nat Med, 1995. 1(11): p. 1201-3.

110. Cohen, J.C., et al., *Molecular pathophysiology of cystic fibrosis based on the rescued knockout mouse model.* Mol Genet Metab, 1998. 64(2): p. 108-18.

111. Morrow, S.L., et al., *Modification of development by the CFTR gene in utero.* Mol Genet Metab, 1998. 65(3): p. 203-12.

112. Ten Have-Opbroek, A.A. and C.G. Plopper, *Morphogenetic and functional activity of type II cells in early fetal rhesus monkey lungs. A comparison between primates and rodents.* Anat Rec, 1992. 234(1): p. 93-104.

113. Brock, D.J., *Prenatal screening for cystic fibrosis: 5 years' experience reviewed.* Lancet, 1996. 347(8995): p. 148-50.

114. Loser, P., et al., *Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NFkappaB.* J Virol, 1998. 72(1): p. 180-90.

115. Waddington, S.N., et al., *Long-term transgene expression by administration of a lentivirus-based vector to the fetal circulation of immuno-competent mice.* Gene Ther, 2003. 10(15): p. 1234-40.

116. Ostrowski, L.E., et al., *Targeting expression of a transgene to the airway surface epithelium using a ciliated cell-specific promoter.* Mol Ther, 2003. 8(4): p. 637-45.

117. Larson, J.E., et al., *Gene transfer into the fetal primate: evidence for the secretion of transgene product.* Mol Ther, 2000. 2(6): p. 631-9.

118. Flotte, T.R., et al., *Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study.* Hum Gene Ther, 2003. 14(11): p. 1079-88.

119. Hacein-Bey-Abina, S., et al., *LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1.* Science, 2003. 302(5644): p. 415-9.

120. Kohn, D.B., M. Sadelain, and J.C. Glorioso, *Occurrence of leukaemia following gene therapy of X-linked SCID.* Nat Rev Cancer, 2003. 3(7): p. 477-88.

121. Kumar, M., et al., *Systematic determination of the packaging limit of lentiviral vectors.* Hum Gene Ther, 2001. 12(15): p. 1893-905.

122. Miyoshi, H., et al., *Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector.* Proc Natl Acad Sci U S A, 1997. 94(19): p. 10319-23.

123. Vigna, E. and L. Naldini, *Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy.* J Gene Med, 2000. 2(5): p. 308-16.

124. Lim F-Y, Kobinger GP, Weiner DJ, Radu A, Wilson JM, Crombleholme TM. *Human fetal trachea-SCID mouse xenografts: efficacy of vesicular stomatitis virus-G pseudotyped lentiviral-mediated gene transfer.* J Pediatr Surg 2003. 38(6): p. 834-9.