Universidade do Minho
Escola de Ciências da Saúde

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Modulation of Lung Development by *In Utero* Gene Transfer

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Modulation of Lung Development by *In Utero* Gene Transfer

Tese de Doutoramento
Ciências da Saúde – Ciências Biológicas e Biomédicas

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DECLARAÇÃO

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Modulation of Lung Development by *In Utero* Gene Transfer.

Modulação do Desenvolvimento Pulmonar por Terapia Génica *In Utero*

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“A little knowledge that acts is worth infinitely more than much knowledge that is idle”.

Khalil Gibran (1883-1931)

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ABSTRACT

Advances in prenatal diagnosis of genetic and congenital disorders with progressively more sensitive techniques may increase opportunities for consideration of prenatal gene therapy. There are a number of genetic and acquired disorders with peri or postnatal pulmonary manifestations. These include monogenetic diseases like cystic fibrosis or surfactant protein B deficiency that would presumably require long-term expression of the deficient or defective gene. However, there are also abnormalities of lung growth, such as congenital diaphragmatic hernia, or lung maturation, such as respiratory distress syndrome of prematurity, that could potentially benefit from strategies that achieve transient gene expression in specific pulmonary distributions. Considered an attractive target organ for fetal gene transfer, the developing lung, poses also some obstacles that would only be overcomed with the development of a variety of gene transfer methodologies: different types of vector, optimal site, route and timing of gene delivery. The fundament of this dissertation was to modulate lung growth/maturation by in utero gene transfer, aiming to unveil underlying mechanisms of normal and abnormal lung development.

The first objective of this dissertation was to develop a new approach of gene transfer targeting the fetal lung in early stages of lung development. We developed a new method for direct injection of viral vectors into the rat fetal lung as early as the pseudoglandular phase of lung development using ultrasound guided microinjections. The pseudoglandular stage, characterized by intense branching morphogenesis, is the period of greatest overall growth of the airways and vasculature of the fetal lung and corresponds to a stage of immunologic immaturity. Therefore, gene transfer during this period has the potential to have major effects on key elements of lung growth with minimal potential for detrimental immune responses. We aimed to compare two distinct types of vectors: an adenoviral vector and a lentiviral vector (equine infectious anemia virus-based), both expressing the enhanced green fluorescent protein reporter gene. This study confirmed that adenoviral vectors are more suitable when rapid, high-level and transient expression of the transgene is required; whereas lentiviral vectors are more appropriate to induce sustained and long-term expression. One of the concerns in gene transfer protocols is to target a specific cellular compartment of a determined organ/system. Interestingly, interstitial compartment rather than epithelial cells were transduced in opposition to previous studies describing intrapulmonary, intraamniotic and intratracheal administrations of viral vectors. The observation of transduction of distinct cell populations within the lung with different routes of transduction raises the possibility of manipulating gene expression in

specific and separate cell populations within the developing lung. We then decided to use this model system to perform an *in vivo* study of dynamic lung morphogenesis, involving a major player in branching morphogenesis, fibroblast growth factor 10 (FGF10). We observed that FGF10 mesenchymal overexpression, on the fetal rat lung, resulted in the induction of consistent patterns of malformation, the appearance of which were developmental stage and location dependent. These malformations, in total, appear to closely recapitulate the morphology and histology of the entire spectrum of human Congenital Cystic Adenomatoid Malformation (CCAM).

RESUMO

Os avanços no diagnóstico pré-natal de patologias genéticas e congénitas devido a técnicas progressivamente mais sensíveis, aumenta as oportunidades de aplicação de terapias génicas pré-natais. Existem várias patologias pulmonares genéticas e adquiridas que se manifestam no período peri e pós-natal. Estas incluem doenças monogenéticas como a Fibrose Cística ou a Deficiência em Proteína Surfactante B, que requerem expressão a longo-termo do gene em causa. No entanto, existem também patologias relacionadas com crescimento pulmonar, como a Hérnia Diafragmática Congénita, ou maturação pulmonar, como o Síndrome Prematuro de Distress Respiratório, que podem beneficiar de estratégias de indução de expressão genica de forma transiente. Considerado um aliciante órgão-alvo para a terapia génica fetal, o pulmão fetal, coloca alguns obstáculos que só poderão ser ultrapassados com o desenvolvimento de várias metodologias: tipos de vectores, optimização de local, via e período de transferência génica. O fundamento desta dissertação foi modular crescimento/maturação pulmonar através de transferência génica *in utero*, pretendendo esclarecer os mecanismos moleculares reguladores no desenvolvimento pulmonar normal e anormal.

O primeiro objectivo desta dissertação foi desenvolver uma nova abordagem de transferência génica para o pulmão nas fases inicias do seu desenvolvimento. Desenvolveu-se um novo método de injecção directa de vectores víricos no pulmão fetal de rato, durante a fase pseudoglandular, utilizando microinjecções guiadas por ultrasonografia. A fase pseudoglandular, caracterizada por intensa ramificação e crescimento global das vias aéreas e vasculatura do pulmão fetal, corresponde a um estadio de imaturidade imunológica. Sendo assim, a transferência génica durante este período terá, um maior efeito em elementos fundamentais do crescimento pulmonar com menor hipótese de desencadear respostas imunes. Dois tipos distintos de vectores víricos foram utilizados: um vector adenovírico e um vector lentivírico (*Equine Infectious Anemia Virus),* ambos expressam *Enhanced Green Fluorescent Protein* como gene marcador. Este estudo confirmou que os vectores adenovíricos são mais adequados quando se pretende obter expressão rápida, elevada e transiente do transgene; enquanto que os vectores lentivíricos induzem uma expressão sustentada no tempo. Uma das preocupações nos protocolos de transferência génica é atingir especificamente um determinado compartimento celular de um determinado órgão ou sistema. É de salientar que, ambos os vectores, transfectaram células do compartimento interstitial em oposição ao epitelial, descrito em estudos anteriores em que as vias de administração foram a intrapulmonar, a intra-

amniótica e a intra-traqueal. A transdução de populações celulares distintas em estreita relação com o tipo de via de administração, aumenta as possibilidades de manipulação genética do pulmão em desenvolvimento. A etapa seguinte consiste em utilizar este modelo para efectuar um estudo *in vivo* da morfogénese pulmonar, manipulando um dos factores fundamentais no processo de ramificação, o *fibroblast growth factor* 10 (FGF10). A sobreexpressão mesenquimatosa de FGF10, no pulmão fetal de rato, resultou na indução de malformações císticas, cujo fenótipo era dependente do local e período em que se induzia essa mesma sobreexpressão. O fenótipo de todos os tipos de malformações induzidas, parece recapitular todo o espectro da Malformação Cística Adenomatoide Congénita do humano.

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Abbreviations

α-SMA α-smooth muscle actin
AAV adeno-associated virus
ALK activin receptor-like kinase
ASLV avian sarcoma leukosis viruses
BMP bone morphogenic protein
CAR coxsackie adenovirus receptor
CC10 clara cell marker 10
CCAM congenital cystic adenomatoid malformation
CCSP clara cell-secretory protein
CDH congenital diaphragmatic hernia
CF cystic fibrosis
CFTR cystic fibrosis transmembrane receptor
CHO chordin
COPD chronic obstructive pulmonary disease
DKK dickkopf
DNAI dynein axonemal intermediate chain
dpc days post-conception
EGF epidermal growth factor
EGFR epidermal growth factor receptor
EIAV equine infectious anemia virus
FGAd first generation adenoviral vector
FGF fibroblast growth factor)
FGFR fibroblast growth factor receptor
FIV feline immunodeficiency virus
FLK fetal liver kinase
FLT fetal liver tyrosinase
FN fibronectin
FOXA2 forkhead box A2
GATA A/TGAT/G –binding transcription factor
GFP green fluorescent protein
GLI glioblastoma transcription factor
GRE gremlin
HDAd helper-dependent adenoviral vector
HIF hypoxia inducible factor
HIP hedgehog interacting protein
HIV human immunodeficiency virus
HNF hepatocyte nuclear factor
IFN interferon
IL interleukin
ITR inverted terminal repeat
LEF lymphoid enhancer factor
LTR two long terminal repeat
MAPK mitogen activated protein kinase
MASH mammalian achaete-scute homologue
MLV murine leukemia virus
NF nuclear factor
NKX2.1 homeobox protein NK-2 homologue A
PDGF platelet-derived growth factor
PDGFR platelet-derived growth factor receptor
PITX paired-like homeodomain transcription factor
PTC patched
RA retinoic acid
RAR retinoic acid receptor
RAS resistance to audiogenic seizures
RRE Rev-responsive element
RTK receptor tyrosine kinases
SCID severe combined immunodeficiency
SHH sonic hedgehog
SIN self-inactivating vector
SIV simian immunodeficiency virus
SMC smooth muscle cells
SP surfactant protein
SPRY sprouty
TCF T cell factor
TGF transforming growth factor
TNF tumor necrosis factor
TTF thyroid transcription factor
UBM ultrasound biomicroscopy

VEGF vascular endothelial growth factor

VSVG vesicular stomatitis virus glycoprotein

WNT wingless

CHAPTER 1

GENERAL INTRODUCTION

Normal Lung Development

The bronchial tree of the human lung has more than 10^5 conducting and 10^7 respiratory airways arrayed in an intricate pattern crucial for oxygen flow [Weibel ER 1984; West GB, et al. 1997; Bejan A 2000; Mauroy B, et al. 2004]. Classical studies of lung structure raise the question of how the information required to generate a tree of such complexity is biologically encoded [Weibel ER and Gomez DM 1962; Metzger RJ and Krasnow MA 1999]. Lung development is a highly orchestrated process directed by mesenchymal-epithelial interactions which control and coordinate the temporal and spatial expression of multiple regulatory factors required for proper lung formation. Many endogenous and exogenous factors may disturb this delicate process leading to disorders of lung growth, maturation and function.

Mammalian lung development initiates with the emergence of a small diverticulum, the laryngotracheal groove, from the ventral foregut endoderm into the splanchnic mesoderm [Chuang PT and McMahon AP 2003]. In humans, lung development begins at 3-4 weeks of gestation, whereas in mice and rats at 10 and 11.5 days post-conception (dpc), respectively (term mice gestation ~19 dpc; term rat gestation ~22 dpc). Larynx and trachea originate from the proximal region of the laryngotracheal groove, whereas the distal portion gives rise to bronchial buds, which in turn originate left and right lobar branches of the bronchial tree [Warburton D, et al. 2005]. The primary buds grow ventrally and caudally producing secondary bronchi. Pathways that control left-right asymmetry determine the number of buds on the right and left sides, which is a species-specific feature. In mice and rats four lobes constitute the right lung and one lobe forms the left lung, whereas in humans the right lung is trilobed and the left is bilobed. As lung morphogenesis continues, each secondary bronchus undergoes dichotomous branching, a process responsible for the remarkable expansion of lung epithelium, until a functional lung with a particularly enhanced gas exchange surface area (0.1 micron thick by 70 square meters) is obtained [Chuang PT and McMahon AP 2003]. Three-dimensional branching pattern was carefully studied by Metzger et al [Metzger RJ, et al 2008]. They infer that the tree is generated by three geometrically simple local modes of branching: domain branching, planar bifurcation and orthogonal bifurcation, used in three different orders throughout the lung. Once left–right laterality of the lung is established, airway branching is driven by a ‘master’ branch generator, with three ‘slaves’ in the form of subroutines (series of discrete patterning events). Of these, one subroutine seems to instruct a periodicity clock, which times the appearance of subsequent branches; another determines the rotational orientation

of the branches around the axis of the parent airway; and the third mediates bifurcation. The molecular mechanism subjacent to this process was also investigated. The balance between fibroblast growth factor (FGF) expression, fibroblast growth factor receptor (FGFR) activation and Sprouty (Spry) 2-mediated inhibition of FGF signaling is possibly a central component, not only of the master branch generator, but also, of the periodicity-clock subroutine.

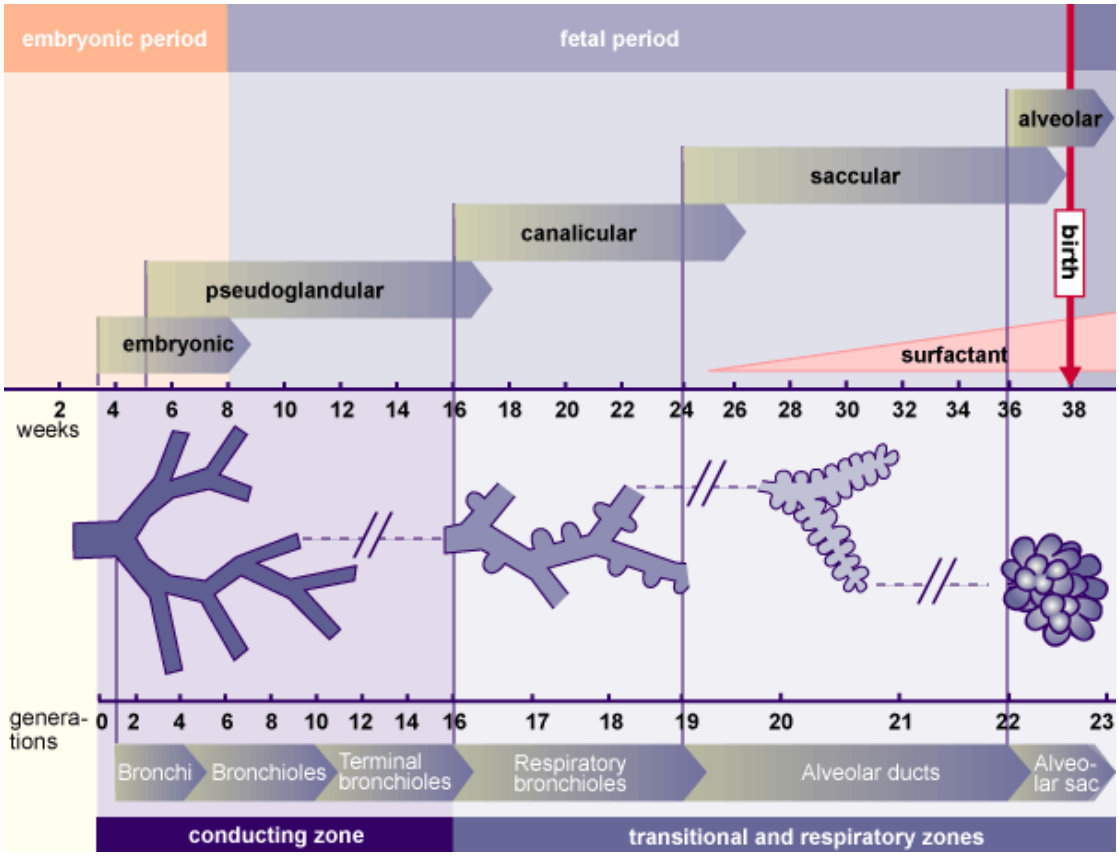

Figure 1. Overview of lung development: stages and structure.

Lung development comprises six different stages: the *embryonic stage* in which the lung primordium appears as a ventral diverticulum of the foregut, elongates caudally and branches for the first time giving rise to the main bronchi of the two lungs; the *pseudoglandular stage*, in which most of the branching morphogenesis takes place; the *canalicular stage* where the distal lung development and differentiat*ion* occurs concurrently with the pulmonary vascular network and surfactant synthesis; *the saccular stage*, when terminal sacs are formed in the peripheral airways with concomitant development of the capillary network and differentiation of type I and type II pneumocytes occurs; the *alveolarization stage*, the establishment of secondary septa results in alveolar formation; and the *microvascular maturation*, when double capillary layer of the immature alveolar septa is reduced to a single capillary layer [Perl AKT and Whitsett JA 1999; Roth-Kleiner M and Post M 2003]. To direct

this highly predetermined program, a multitude of controlling factors have been identified, namely transcription factors, growth factors and their receptors, extracellular matrix proteins and intercellular adhesion molecules. The role and the interplay between major molecular mediators will be reviewed in subsequent sections. Even if lung development is a continuum, in the following sections, molecular mediators and its interactions, structure and cell characterization, will be reviewed in close relation with each developmental stage.

Table 1 - Stages of lung development: characteristic events and major molecular mediators.

Stage	Duration	Characteristic events	Major molecular mediators
Embryonic	Human 4-7 weeks Mouse 9.5-14.2 dpc Rat 11-12.5 dpc	Outgrowth of trachea, right and left main bronchi and major airways	HNF3ß, TTF1, RA, RAR, Shh, Ptch, Gli2, Gli3, FGF8, FGF10, HNF4, N-cadherin, activin-ß, activin-ßR IIA, lefty1/2, nodal, Pitx2
Pseudoglandular	Human 5-17 weeks Mouse 14.2-16.6 dpc Rat 12.5-18.5 dpc	Formation of bronchial tree up to a preacinar level	GATA6, N-myc, PDGF, PDGFR, EGF, EGFR, FGF, TGF-ß, Shh, Ptc, VEGF, BMP4, RA, RAR, Ghrelin, IL-6
Canalicular	Human 16-26 weeks Mouse 17.5-18.5 dpc Rat 18.5-19.5 dpc	Formation of the pulmonary acinus and of the future air-blood barrier; increase of capillary bed; epithelial differentiation; first appearance of surfactant	GATA6, TTF1, HNF3ß, Mash1, VEGF
Saccular	Human 24-38 weeks Mouse 17.5-19 dpc to 5 days Rat 19.5-22 dpc to 7 days	Formation of transitory air spaces	HNF-3ß, TTF1, NF1, VEGF, VEGFR
Alveolar	Human 36 weeks to 2 years Mouse 5-30 days Rat 7-35 days	Alveolarization by formation of secondary septa	PDGF, PDGFR, FGF, FGFR, VEGF, VEGFR, angiopoietins, ephrins, RA, RAR
Microvascular maturation	Human birth to 2-3 years	Thinning of interalveolar walls; fusion of the capillary bilayer to a single layered network	VEGF, VEGFR, PDGF, PDGFR, angiopoietins, ephrins

EMBRYONIC STAGE

In the human, early lung formation lasts from day 26 to 7 weeks after conception. The trachea and lungs originate from the endoderm layer, like the thyroid, esophagus, stomach, liver, pancreas and the intestines. Lung formation starts with an outgrowth of the ventral foregut, which gives rise to two primordial lung buds on either side of the esophagus. These buds invade the surrounding mesenchyme by dividing dichotomously forming the left and the right primordial lung.

Early Lung Organogenesis

Primitive foregut endoderm is driven to differentiate into respiratory epithelium by interactions of hepatocyte nuclear factor 3ß (HNF-3ß or foxa2), thyroid transcription factor 1 (TTF1 or Nkx2.1) and GATA family transcription factors at the level of gene transcription regulation, thus mediating not only surfactant protein genes but also other transcription factors and including smooth muscle genes [Zhou L, et al. 1996b; Keijzer R, et al. 2001].

HNF-3ß is expressed in cells believed to be progenitors of respiratory epithelial cells. The genetic ablation of HNF-3ß in mice leads to embryonic death with absence of foregut and, therefore, absence of the lungs [Ang SL and Rossant J 1994]. Later in lung development, HNF-3ß-binding sites have been found in the promoter-enhancer elements of different genes expressed in differentiated respiratory epithelial cells, e.g. surfactant protein (SP) B, Clara cell-secretory protein (CCSP) and Clara cell marker 10 (CC10) [Stahlman MT, et al. 1998; Bingle CD and Gitlin JD 1993; Bohinski RJ, et al. 1994].

It was generally believed that TTF1 was important in endoderm specification because of colocalized expression with HNF3ß in the developing respiratory tract, HNF3ß regulation of TTF1 gene transcription and concerted transcriptional regulation via HNF3ß and TTF1. Mice lacking TTF1 exhibit tracheoesophageal fistula and have hypoplastic lungs resulting in neonatal lethal phenotype. Moreover, early epithelial cell lineage determination is abrogated in these mutants [Kimura S, et al. 1996]. Despite TTF1 proven roles in tracheoesophageal septation and lung development, no functional data is available for TTF1 role in endoderm specification [Zhou L, et al. 1996b; Keijzer R, et al. 2001].

GATA6, a member of the GATA family of zinc finger transcriptional factors, has been implicated in pulmonary endoderm specification and in epithelial and smooth muscle cell lineage diversity. *Gata6* is expressed in arterial smooth muscle, the fetal bronchi, urogenital ridge and bladder. GATA6 is

essential for the differentiation of visceral endoderm via regulation of HNF4 [Morrisey EE, et al. 1998].

Another crucial player in this early stage is retinoic acid (RA). It binds to its receptor that translocates to the nucleus and influences gene transcription in target cells. Deletion of both RA receptor-α and RA receptor-ß results in pulmonary agenesis, tracheoesophageal fistula and lobar agenesis [Mendelsohn C, et al. 1994].

Left-Right Asymmetry

Left-right asymmetries are an integral part of the body plan and necessary for normal formation and localization of intrathoracic and intra-abdominal organs. By the 5th week after conception, the human lung is divided into five lobes, two on the left and three on the right, whereas mice and rats possess four lobes in the right lung and one lobe in the left lung. Several distinct, though conserved, mechanisms have been proposed during the establishment of left-right axis in vertebrates: sonic hedgehog (Shh) [Meyers EN and Martin GR 1999], fibroblast growth factor-8 (FGF8) [Boettger T, et al. 1999], HNF4 [Chen J, et al. 1998], N-cadherin [García-Castro MI, et al. 2000], activin-ß and its receptor IIA [Oh SP and Li E 1997]. All the involved pathways seem to join into modulation of the expression of genes belonging to the transforming growth factor-ß (TGF- ß) family, named lefty1, lefty2 and nodal. These signalling molecules are expressed at the left side of the lung.

Epithelial-Mesenchymal Interactions

The importance of mesenchymal-epithelial tissue interactions for early lung development has been known for several decades [Wessels NK 1970]. Each tissue compartment produces unique sets of growth factors and other signaling molecules, which signal in a paracrine manner between the epithelium and the mesenchyme. Their expression must be well coordinated as at some sites they need to enhance cell proliferation (at the edge of the growing lung buds), while at other sites they have to inhibit cell division (at branching points). Some of these players have been identified, including fibroblast growth factors (especially FGF10 and FGF7), transforming growth factor-ßs (TGF-ß), Shh, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and bone morphogenic protein 4 (BMP-4), with their respective receptors and intracellular signaling molecules.

PSEUDOGLANDULAR STAGE

During this period from 5 to 17 weeks in the human, the hierarchical pattern of pre acinar airways and blood vessels develops. By dichotomous branching the whole bronchial tree is formed. Further steps in lung development are differentiation of epithelial cells in a centrifugal manner with appearance of ciliated cells, goblet and basal cells and production of cartilage, which can already be found around the main bronchi before 10 weeks after conception [Burri PH 1999].

Branching Morphogenesis

A highly ordered sequence of patterning events collectively referred to as branching morphogenesis generates the bronchial tree and defines the proximal-distal axis of the lung by the end of the pseudoglandular stage. This process results from reiterated combination of bud outgrowth, elongation and subdivision of terminal units [Hogan BLM 1999]. The initial branches are formed essentially by budding; in subsequent dichotomous subdivisions, extracellular matrix seems to play a role by accumulating at clefts, specific points that do not branch. The current model is depicted in Figure 2 and establishes that dynamic changing in relative activity of SHH, FGF10, and mSPRY2 in the branching process. SHH is high and FGF10 is correspondingly low where branching is not supposed to take place. In contrast, SHH is suppressed locally by patched (PTC) and hedgehog interacting protein (HIP), so that FGF10 is therefore high where a branch is supposed to occur. FGF10 in turn dynamically induces its inhibitor mSpry2 as branches lengthen. Thus, the net relative activities between SHH, FGF10, and mSPRY2 may determine FGF signal strength in the epithelium and hence the relative rate of bud outgrowth rate at a given point and hence interbranch length. As a bud begins to elongate toward a mesenchymal source of FGF10, mSpry2 begins to be expressed in the distal tip. During subsequent elongation, Fgf10 continues to be expressed in the distal mesenchyme and the level of mSpry2 gradually increases as the bud lengthens. When the bud finally approaches the pleura, the Fgf10 expression domain adjacent to the distal tip appears to thin out and some of it appears to be pushed laterally to lie between adjacent branch tips. At the time, mSpry2 expression in the distal tip is at its highest level, perhaps mediating bud outgrowth arrest. A tip-splitting event then occurs in which wingless (WNT) signaling drives fibronectin (FN) deposition between the branch tips, leading to epithelial cleft formation. Meanwhile, dickkopf-1 (DKK1) inhibits Wnt signaling away from the cleft, leading to lower levels of FN deposition where clefting does not occur. Of note is that mSpry2 expression is extinguished between the daughter bud tips, but continues to be expressed within the tips of the daughter bud epithelia. This cycle of interaction is

then repeated during subsequent branching events. Despite some controversy (see below), BMP4 is also an important factor that appears to enhance lung branch tip outgrowth according together with FGF10. FGF10 is shown stimulating BMP4 expression, whereas the ligand binding proteins gremlin (GRE) and chordin (CHO) exert negative modulation on BMP4. VEGF signaling appears to play necessary but complementary roles to accelerate this overall process [Warburton D, et al. 2005].

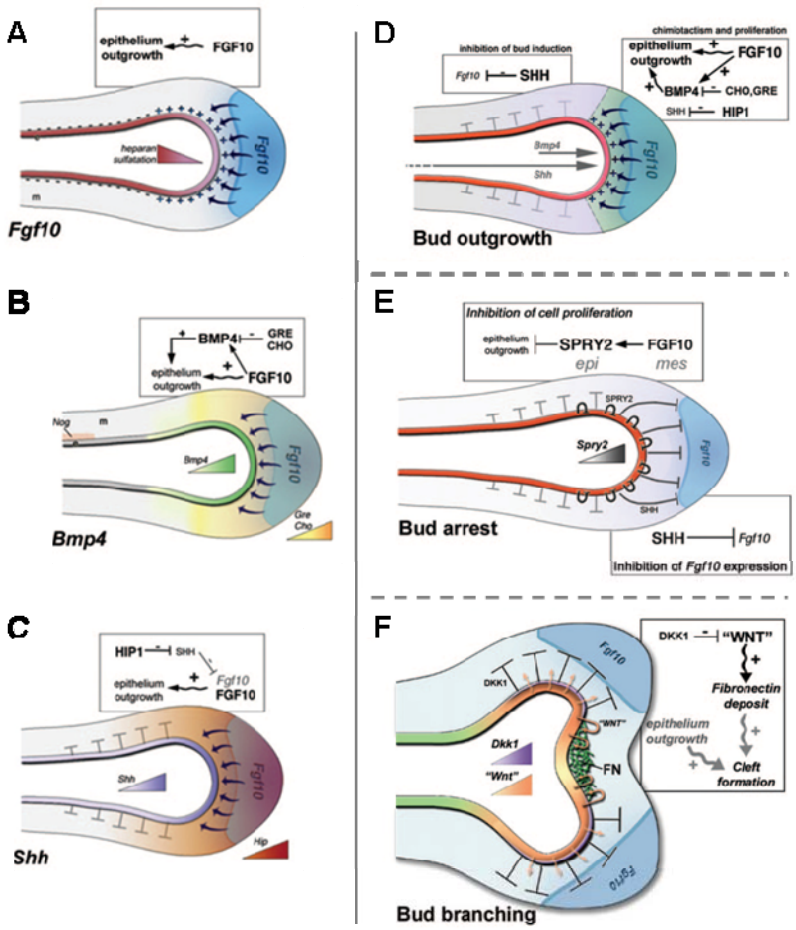

Figure 2. Current model for lung bud outgrowth, arrest and branching [from Warburton D, et al. 2005].

Growth Factors and Signal interactions in Branching Morphogenesis

In the following section transcription and growth factors with pivotal role in branching morphogenesis will be reviewed.

FGF family

There are 22 members of the fibroblast growth factor family in vertebrates, ranging in molecular mass from 17 to 34kDa and sharing 13-71% amino-acid identity [Ornitz DM and Itoh N 2001]. The defining features of the family are a high affinity for heparin and heparin-like glycosaminoglycans and a central core of 140 amino acids that is highly homologous between the family members.

Many FGF have been implicated in multiple aspects of vertebrate development. FGF 1, 2, 7, 9, 10, and 18 play overlapping, yet distinct roles in the lung. In particular, FGF10 has been associated with instructive mesenchymal-epithelial interactions, such as those that occur during branching morphogenesis, whereas FGF9 and 18 appear to play a role in the mesenchyme and FGF1 and 7 appear to play roles in mediating postnatal lung repair [Chailley-Heu B, et al. 2005].

FGF10. In the developing lung, Fgf10 is expressed in the distal mesenchyme at sites where prospective epithelial buds will appear. Moreover, its dynamic pattern of expression and its ability to induce epithelial expansion and budding in organ cultures have led to the hypothesis that FGF10 governs the directional outgrowth of lung buds during branching morphogenesis [Bellusci S, et al. 1997]. Furthermore, FGF10 was shown to induce chemotaxis of the distal lung epithelium [Weaver M, et al. 2000; Park WY, et al. 1998]. The chemotaxis response of the lung endoderm to FGF10 involves the coordinated movement of an entire epithelial tip, containing hundreds of cells, toward an FGF10 source. How this population of cells monitors the FGF gradient and which receptors trigger this effect remains unknown. Consistent with these observations, mice deficient for Fgf10 show multiple organ defects including lung agenesis [Min H, et al. 1998; Sekine K, et al. 1999; Ohuchi H, et al. 2000]. FGF10 also controls the differentiation of the epithelium by inducing SP-C expression and by up-regulating the expression of BMP4, a known regulator of lung epithelial differentiation [Weaver M, et al. 2000; Lebeche D, et al. 1999]. FGF10 is the main ligand for FGFR2b during the embryonic phase of development as evidenced by the remarkable similarity of phenotypes exhibited by embryos where these genes have been inactivated [Mailleux AA, et al. 2001; Sutherland D, et al. 1996]. Thus, the paradigm proposed so far is that FGF10 expressed by the mesenchyme acts on the epithelium (which expresses FGFR1b and 2b). The biologic activities mediated through these two epithelial receptors are likely to be different, as FGF7 (acting mostly through FGFR2b) exhibits a different activity compared with FGF10 [Bellusci S, et al. 1997].

Fgf7, or keratinocyte growth factor, is expressed by mesenchymal pulmonary cells. FGF7 mediates epithelial-mesenchymal interactions by binding to FGFR2b. Fgf7 overexpression in pulmonary epithelium results in abnormal morphogenesis similar to cytadenomatoid malformation [Simonet WS, et al. 1995]. In mesenchyme-free cultures of murine fetal lungs, FGF7 induced cysts and patchy SpC expression [Cardoso WV, et al. 1997]. However, when administrated with competence factors, which alone did not influence pulmonary epithelium, FGF7 was able to induce reprogramming of tracheal epithelium to an alveolar type II pneumocyte phenotype [Deterding RR, et al. 1996]. Hence, FGF7 is necessary but not fundamental for the induction of distal lung epithelial differentiation.

FGF9. During early pulmonary development, Fgf9 is expressed in endodermal epithelium and mesothelium, becoming later restricted to the mesothelium. Decreased branching and pulmonary hypoplasia result from targeting Fgf9, which originated diminished Fgf10 levels and consequently reduced mesenchymal proliferation [Colvin JS, et al. 1999; 2001]. It has been suggested that FGF9 inhibits SHH-induced differentiation of peripheral pulmonary mesenchymal cells, thus maintaining a FGF10 progenitor population [Weaver M, et al. 2003].

FGFR. The cloning of the signal-transducing receptors for FGFs has revealed a tyrosine kinase gene family with at least four members. These four cell surface FGFRs bind members of the FGF family with varying affinity [Ornitz DM and Itoh N 2001]. FGFRs contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. Following liagtion with FGFs, receptor tyrosine kinases (RTKs) activate the Ras/MAPK signalling pathway. Fgfr1, 2, and 3 encode two receptor isoforms (termed IIIb or IIIc) that are generated by alternative splicing, and each binds a specific repertoire of FGF ligands [Ornitz DM, et al. 1995]. FGFR2-IIIb (FGFR2b) is found mainly in epithelia and binds four known ligands (FGF1, FGF3, FGF7, and FGF10), which are primarily expressed in mesenchymal cells. Peters et al. [Peters K, et al. 1994] reported the first evidence of a key role for Fgfr2 during lung development. They showed that misexpression of a dominant negative form of Fgfr2 in the embryonic lung under the SP-C promoter led to a severe reduction in branching morphogenesis. Further evidence came from Fgfr2 inactivation in the embryo. Whereas mice null for the Fgfr2 gene die early during embryogenesis, those that are null for the Fgfr2b isoform, but retain Fgfr2c, survive to birth [De Moerlooze L, et al. 2000; Revest JM, et al. 2001]. Mice deficient for Fgfr2b show agenesis and dysgenesis of multiple organs, including the lungs, indicating that signaling through this receptor is critical for mesenchymal-epithelial interactions during early organogenesis. This idea is supported by the finding that prenatally induced misexpression of a dominant negative FGFR, to abrogate FGF signaling, results in a hypoplastic, emphysematous lung phenotype [Hokuto I, et al. 2003]. In contrast, induced abrogation of FGF signaling postnatally did not produce any recognizable phenotype.

FGF signaling pathway. Upon reception of the extracellular signals (FGFs), RTK activate the Ras/MAPK signalling pathway. Stimulation of FGFR not only results in formation of the FRS2/Grb2/Sos complex, but the binding of a positive tyrosine phosphatase regulator, Shp2, to FRS2, which is required for full potentiation of MAP-kinase activation [Hadari YR, et al. 1998]. Many of the molecular components, such as Ras, GTPase, Raf kinase and MAPK are shared among different RTKs. Ras directly interacts with and activates Raf, which in turn phosphorylates and activates MEK, which then phosphorylates and activates the MAP kinases, including ERK1 and ERK2. ERK is an extracellular-signal-regulated kinase that can enter the nucleus and phosphorylate certain transcription factors (like ELK-1), leading to phosphorylation of cytoplasmic proteins followed by cell growth and differentiation [Schaeffer HJ and Weber MJ 1999] (Figure3). Members of the Sprouty, Sef, and mitogen-activated protein kinase phosphatase families are negative modulators of FGF signaling. These molecules affect the FGF signaling cascade at different levels to regulate the final output of the pathway. This multilayered regulation suggests that precise adjustment of FGF signaling is critical in development [Tsang M and Dawid IB 2004].

Sprouty family

The first example of an FGF-inducible signaling antagonist arose from the discovery of the sprouty mutant during Drosophila trachea development, in which supernumerary tracheal sprouts arise. Spry is not only found downstream in the FGFR pathway, but also appears to be an inhibitor of other tyrosine kinase signaling pathways such as EGF and Torso [Tefft D, et al. 2002]. mSpry2 is localized to the distal tips of the embryonic lung epithelial branches and is down-regulated at sites of new bud formation [Sutherland D, et al. 1996]. On the other hand, mSpry4 is predominantly expressed throughout the distal mesenchyme of the embryonic lung. It has been suggested that both Spry2 and Spry4 share a common inhibitory mechanism. Both Sprouty translocate to membrane ruffles upon EGF stimulation. However, only SPRY2 was shown to associate with microtubules [Lim J, et al. 2000]. Abrogation of mSpry2 expression stimulates murine lung branching morphogenesis and increased expression of specific lung epithelial maturation/differentiation markers [Tefft JD, et al. 1999]. Conversely, overexpression of mSpry2 under the control of a SP-C promoter or by intratracheal microinjection of an adenovirus containing the mSpry2 cDNA, results in smaller lungs with a particular “moth-eaten” dysplastic appearance along the edges of the lobes, with decreased epithelial cell proliferation [Mailleux AA, et al. 2001]. Thus, not only is the function of Spry conserved during respiratory organogenesis, but also as seen by loss of function and gain of function studies, Spry plays a vital role in regulating lung branching morphogenesis. The investigation of mechanism

by which mSPRY2 negatively regulates FGF10 in mouse lung epithelial cells (MLE15), showed that mSPRY2 differentially binds to FGF downstream effector complexes [Tefft D, et al. 2002].

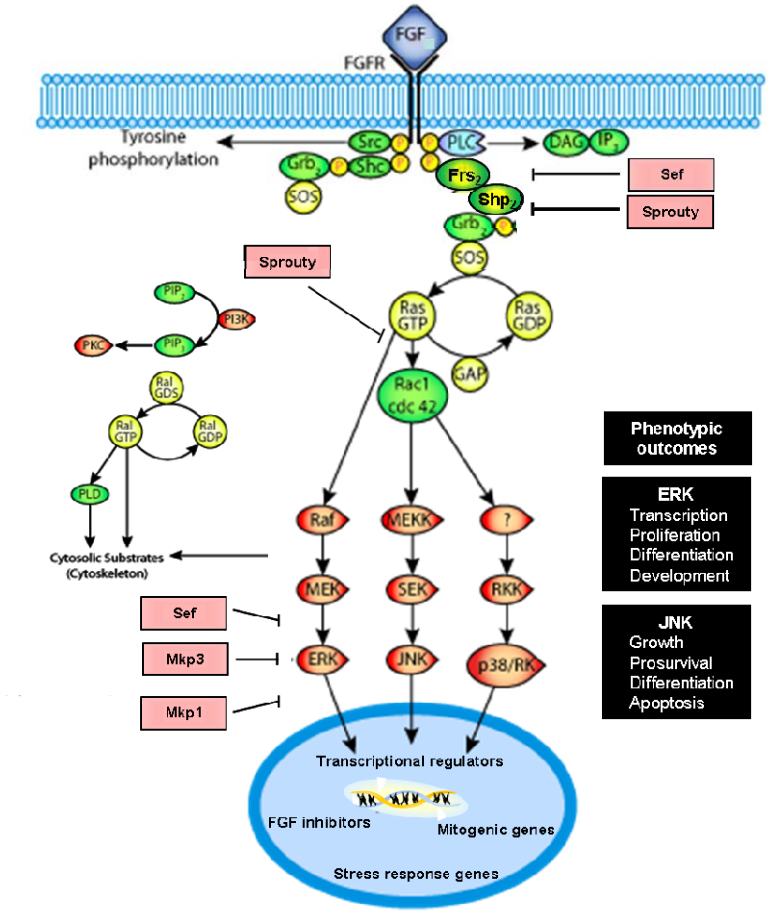

Figure 3. Schematic diagram of FGF signaling through Ras-MAPK pathway. The binding of the FGF to FGFR causes the autophosphorylation and activation of the receptor tyrosine kinases (RTK). The RTK binds to an adaptor protein Grb2 or Grb2-Sos complex via its docking protein Shp or FRS2. The Sos then activates the Ras, which in turn phosphorylates a series of MAP kinases (Raf, MEK, ERK). ERK enters nucleus phosphorylates and activates transcription factor like ELK-1, which regulates its targets. Sprouty, Sef, and mitogen-activated protein kinase phosphatase families are negative modulators of FGF signaling.

Regulation of FGF signaling by Sprouty

Early studies in chick limb bud demonstrated that overexpression of Spry resulted in a reduction in limb bud outgrowth, consistent with a decrease in FGF signaling [Minowada G, et al 1999]. This suggested a possible co-regulatory relationship between FGF signaling and Spry during development. In developing lung the native state mSPRY2 associates with Shp2 and Gap, which is a

GTPase-activating protein that hydrolyzes GTP to GDP. It is possible that in this state the binding of Shp2 to mSPRY2 regulates mSPRY2 activity. Upon FGFR activation, mSPRY2 disassociates from Shp2 and Gap and translocates to the plasma membrane, where it binds to both FRS2 and Grb2, thus blocking the formation of the FRS2/Grb2/Sos complex, resulting in a net reduction of MAP-kinase activation. Thus, Sprouty would inhibit the formation of specific signaling complexes downstream tyrosine-kinase receptors resulting in modulation and co-ordination of cell growth and development during organogenesis. It is also known that Spry4 inhibits branching of endothelial cells as well as sprouting of small vessels in cultured mouse embryos. Endothelial cell proliferation and differentiation in response to FGF and VEGF are also inhibited by mSpry4, which acts by repressing ERK activation. Thus, Spry4 may negatively regulate angiogenesis [Lee MK, et al. 1998].

Shh, Patched and Hip

Hedgehog signaling is essential for lung morphogenesis because Shh null produces profound hypoplasia of the lungs and failure of tracheoesophageal septation [Pepicelli CV, et al. 1998]. However, proximodistal differentiation of the endoderm is preserved in the Shh null mutant, at least in so far as expression of SP-C and CC10 genes are concerned. The expression of the SHH receptor, PTC, is also decreased in the absence of Shh as are glioblastoma transcription factors (Gli) 1 and 3. On the other hand, lung-specific misexpression of Shh results in severe alveolar hypoplasia and a significant increase in interstitial tissue [Bellusci S, et al. 1997]. Fgf10 expression, which is highly spatially restricted in wild type, is not spatially restricted and is widespread in the mesenchyme in contact with the epithelium of the Shh null mutant mouse lung. Conversely, local suppression of SHH signaling by the induction of Ptc and Hip at branch tips may serve to facilitate FGF signaling locally, where branch outgrowth is stereotypically programmed to take place [Chuang PT and McMahon AP 1999]. Thus, temporospatial restriction of Fgf10 expression by SHH appears to be essential to initiate and maintain branching of lung.

TGF-β family

The TGF-β superfamily can be divided into three subfamilies: TGF□, BMP and activin [Massague J 1998].

TGF-β. There are three TGF-β isoforms in mammals: TGF-β1, 2, and 3. All of them have been detected in murine embryonic lungs [Millan FA, et al. 1991; McLennan IS, et al. 2000]. In early mouse embryonic lungs (E11.5), TGF-β1 is expressed in the distal mesenchyme underlying branching points, whereas TGF-β2 is localized in distal epithelium, and TGF-β3 is expressed in proximal mesenchyme and mesothelium [Bragg AD, et al. 2001]. Mice lacking Tgfb1 develop normally but die within the first month or two of life of aggressive pulmonary inflammation. On the other hand, Tgfb2 null mutants die *in utero* of severe cardiac malformations, whereas Tgfb3 mutants die neonatally of lung dysplasia and cleft palate [Kaartinen V, et al. 1995; Buckley S, et al. 1996]. Embryonic lung organ and cell cultures reveal that TGF-β2 plays a key role in branching morphogenesis, whereas TGF-β3 plays a key role in regulating alveolar epithelial cell proliferation during the injury repair response [Zhou L, et al. 1996a; Zhao J, et al. 1999]. Thus, finely regulated and correct physiologic concentrations and temporospatial distribution of TGF-β1, 2, and 3 are essential for normal lung morphogenesis and defense against lung inflammation.

BMP. Several BMPs, including BMP3, 4, 5 and 7, are expressed during embryonic lung development. The expression of Bmp5 and Bmp7 has been detected in the mesenchyme and the endoderm of the developing embryonic lung respectively, while Bmp4 expression is restricted to the distal epithelial cells and the adjacent mesenchyme [King JA, et al. 1994; Bellusci S, et al. 1996; Takahashi H and Ikeda T 1996]. Most of the BMP signaling pathway components, such as BMP receptors: activin receptor-like kinase (ALK) 2, 3, and 6 and BMP specific receptor-regulated Smads (R-Smads), including Smad1, 5, and 8, are expressed in early mouse embryonic lung [Dewulf N, et al. 1995; Verschueren K, et al. 1995]. Overexpression of Bmp4, driven by the SP-C promoter in the distal endoderm of transgenic mice, causes abnormal lung morphogenesis, with cystic terminal sacs and inhibition of epithelial proliferation [Bellusci S, et al. 1996]. In contrast, SP-C promoter-driven overexpression of either the BMP antagonist Xnoggin or a dominant negative Alk6 BMP receptor to block BMP signaling, results in severely reduced distal epithelial cell phenotypes and increased proximal cell phenotypes in the lungs of transgenic mice [Weaver M, et al. 1999]. However, the exact roles of BMP4 in early mouse lung development remain controversial. In isolated E11.5 mouse lung endoderm cultured in Matrigel™ addition of exogenous BMP4 inhibited epithelial growth induced by the morphogen FGF10 [Weaver M, et al. 2000]. However, addition of BMP4 to intact embryonic lung explant culture stimulates lung branching morphogenesis [Shi W, et al. 2001]. Since conventional murine knockouts for BMP4 and BMP-specific Smads cause early embryonic lethality, their functions in lung development *in vivo* still need to be further defined. Interestingly, germ line mutations in BMP

type II receptors were found in familial primary pulmonary hypertension [Lane KB, et al. 2000]. Therefore, BMPs may play multiple roles in lung development.

Wnt growth factor family

Modulation of Wnt expression in embryonic and adult mouse lung suggests that Wnt pathways are important for cell fate decisions and differentiation of lung cell types. The Wnt growth factor family in the mouse is comprised of 19 different secreted ligands that interact with 10 known seven-span transmembrane receptors of the frizzled gene family and either one of two single-span transmembrane proteins, low-density-lipoprotein-receptor-related proteins 5 and 6 [Pinson KI, et al. 2000; Tamai K, et al. 2000; Wehrli M, et al. 2000]. Historically, Wnt proteins have been grouped into two classes, canonical and noncanonical. Canonical Wnt bind to frizzled receptors, inhibiting glycogensynthase kinase-3b mediated phosphorylation of -catenin. Hypophosphorylated b-catenin accumulates in the cytoplasm, after which it translocates to the nucleus, where it heterodimerizes with members of the T cell factor (TCF) / Lymphoid enhancer factor (LEF) transcription factor family to activate the transcription of TCF/LEF target genes. Non-canonical Wnt activate other Wnt signaling pathways, such as the planar-cell-polarity -like pathway that guides cell movements during gastrulation [Heisenberg CP, et al. 2000]. Between E10.5 and 17.5, -catenin is localized in the cytoplasm and often also in the nucleus of the pulmonary epithelium and adjacent mesenchyme [DasGupta R and Fuchs E 1999]. Wnt ligands, frizzled receptors, and the Tcf/Lef1 transcription factors are expressed during early lung development. Studies of the expression pattern of several Wnt proteins (Wnt7b, Wnt2a, Wnt5a, Tcf1, Lef1, sFrp1, sFrp2) suggest that Wnt signaling can originate from the epithelium and mesenchyme and can target both tissues in an autocrine and/or paracrine fashion. Recent studies have related particular Wnt production to specific lung cell types: Wnt2 has been mapped predominantly to the mesenchyme, while Wnt7b was exclusively expressed in the lung epithelium [Pongracz JE and Stockley RA 2006]. Wnt signalling has also been reported to be important in the regulation of spatial and distal branching of the lung. While the importance of canonical Wnt signalling in lung development is well established, the role of non-canonical Wnt signalling is less clear. Wnt5a knock-out studies have shown, however, that non-canonical Wnt signalling is also important. In Wnt5a-/- animals the lung is morphologically smaller than in the wild type and has thickened mesenchyme. Furthermore, alveolar development is delayed, although not prevented. Lungs of Wnt5a knock-out animals also have increased expression of FGF10, Shh and BMP4 [Li C, et al. 2002; 2005] suggesting that the morphological changes might be related to dysregulation of other signalling pathways modulated by Wnt signalling.

DKK. Dickkopf (DKK) reveals FN as an important matrix target of Wnt signaling in lung morphogenesis. Recent experiments show that early embryonic mouse lung organ cultures treated with DKK1, a potent and specific diffusible inhibitor of Wnt action that is also endogenously secreted by the distal lung epithelium, display impaired branching, characterized by failed cleft formation and enlarged terminal buds. The DKK1-treated lung explants show reduced α-smooth muscle actin (α-SMA) expression and defects in the formation of the pulmonary vascular network. These defects coincide with a pattern of decreased FN deposition and Pdgf-A expression. All of the DKK1-induced morphogenetic defects can be recapitulated by inhibition of FN with an antifibronectin antibody and conversely can be rescued by addition of exogenous FN [De Langhe S, et al. 2005]. This point out the importance of correct orientation of the extracellular matrix in response to growth factor signaling. It also suggests that fibronectin is a downstream target of Wnt signaling.

EGF

EGF is expressed in the distal epithelium and mesenchyme. Null mutation of Egfr results in a 50% reduction in branching and a neonatal lethal failure of lung maturation [Miettinen PJ, et al 1995; 1997]. EGF was the first growth factor to be shown to exert an inductive role on chick trachea to induce ectopic branching [Goldin GV and Opperman LA 1980], as well as to accelerate branching through activation of its cognate EGFR tyrosine kinase in mouse embryonic lung in culture [Warburton D, et al. 1992; Seth R, et al. 1993]. In addition, null mutation of tumor necrosis factor (TNF)-α converting enzyme, a cell surface protein sheddase that regulates release of active from latent forms of TNF-α, amphiregulin, neuregulin-1, HB-EGF, and HerbB4, also causes a hypoplastic phenotype of lung in the perinatal stage and lethality in new born mice [Zhao J, et al. 2001].

Proteoglycans

Proteoglycans are deposited within the extracellular matrix during early embryonic lung branching morphogenesis and inhibition of proteoglycan synthesis or treatment with heparitinase severely affects branching [Smith CI, et al. 1990; Toriyama K, et al. 1997]. Both heparan sulfate and chondroitin sulfate proteoglycans are required for lung branching and in fact mediate the inductive effects of FGF10 binding to the epithelium [Izvolsky KI, et al. 2003; Shannon JM, et al. 2003].

VEGF

Vascularization must perfectly match epithelial morphogenesis to ensure optimal gas exchange. Several VEGF isoforms are expressed in the developing epithelium, whereas their cognate receptors are expressed in and direct the emergence of developing vascular and lymphatic capillary networks within the mesenchyme. It is possible that VEGF signaling may lie downstream of FGF signaling, in as much as *in vivo* abrogation of FGF signaling severely affects both epithelial and endothelial morphogenesis. Vasculogenesis is initiated as soon as the lung evaginates from the foregut. The loss of even a single allele of Vegf leads to embryonic lethality between days E9.5 and E10.5 in the mouse [Miquerol L, et al. 1999]. VEGF is diffusely distributed in pulmonary epithelial and mesenchymal cells and is involved in controlling endothelial proliferation and the maintenance of vascular structure. VEGF is localized in the basement membrane of epithelial cells [Acarregui MJ, et al. 1999]. VEGF signals through the cognate receptors FLK-1 (fetal liver kinase- 1, or VEGFR2) and FLT-1 (fetal liver tyrosinase-1, or VEGFR1). VEGF signaling is responsible for the differentiation of embryonic mesenchymal cells into endothelial cells. In fact, epithelial cells of the airways are positive for VEGF, particularly at the budding regions of the distal airway [Brown KR, et al. 2001]. VEGF treated human lung explants show an increase of cellular proliferation in the distal airway epithelial cells with an upregulation of the mRNA expression of Sp-A and Sp-C but not Sp-B [Brown KR, et al. 2001].

Mechanical Forces

In situ several physical forces are exerted on the developing lung including (a) fetal breathing movements, (b) peristaltic airway contractions and (c) lung fluid. Fetal breathing movements resulting from episodic diaphragmatic contractions have been observed in the human fetus as early as the first trimester and their incidence increases throughout the fetal period [Harding R 1997]. If they are inhibited either mechanically by bilateral thoracoplasty [Liggins GC, et al. 1981], neurosurgically by transecting the cervical cord above the phrenic motoneurons [Wigglesworth JS and Desai R 1979] or genetically in the myogenin null mouse lacking normal skeletal muscle fibers [Tseng BS, et al. 2000], lung hypoplasia results. Spontaneous peristaltic airway contractions are rhythmic narrowings of the airways by phasic contractions of the surrounding smooth muscle cells, producing a pulsatile distal-driven movement of lung liquid which might cause an expansion of the end lung buds, thereby stretching the epithelial layer lining the buds [Schittny JC, et al. 2000]. If the physiological circulation of lung fluid filling the air spaces is disturbed, lung development is disturbed.

Chronic deflation of one lung by drainage of lung liquid in the fetal sheep results in significant hypoplasia, while chronic overexpansion of the other lung by retention of fetal lung fluid results in hyperplasia [Moessinger AC, et al. 1990]. Taken together, these observations demonstrate the importance of mechanical forces for proper lung development. Mechanotransduction, i.e. the conversion of mechanical forces into biochemical signals, appears to be related with stimulation of gene expression of growth factors and their receptors via stress-induced activation of protein tyrosine kinases [Liu M and Post M 2000].

Canalicular Stage

The period between 16 and 26 weeks is manifested by 'canalization' of the primitive interstitium by capillaries leaning against the epithelium, marking the beginning of the future blood-air interface. The second landmark of this stage is the differentiation of the pulmonary epithelium into type II cells, the producers of surfactant, and subsequently type I cells, which contribute to the formation of a thinned prospective air-blood barrier.

Epithelial Differentiation

With proceeding of branching, numerous cell phenotypes with different morphology and gene expressions are established. Several transcription factors play a specific part in these differentiation processes. Mash-1 (mammalian achaete-scute homologue 1) is important for the pulmonary neuroendocrine cells, and Foxa2 (forkhead box A2), GATA-6 and TTF1 for the epithelial cells (ciliated cell, Clara cell, goblet cell, type II and type I cells). The absence of the respective transcription factors, shown in genetically altered mice, goes along with absence of the corresponding cell types [Borges M, et al. 1997; Tichelaar JW, et al. 1999].

Saccular Stage

Entering this stage at about 24 weeks in human pregnancy is crucial for extrauterine survival. The widening of the peripheral air spaces distal to the terminal bronchioles at the expense of intervening mesenchyme will allow for sufficient gas exchange. The prospective lung parenchyma is gaining in size by dichotomously branching of the terminal generations of the airway tree. Furthermore, during

this period, preparation for the real alveolarization starts by deposition of elastic fibers at the localizations where future secondary septa will form [Wasowicz M, et al. 1996].

Surfactant

Surfactant is comprised of about 90% lipids, 10% proteins and a lesser amount of carbohydrates [Weaver TE and Whitsett JA 1991], which are synthesized, stored, secreted and recycled or catabolised by alveolar type II cells. Epithelial cells differentiate into their descendants, including type II cells, during the canalicular stage under the influence of HNF-3ß and TTF1. TTF1 is normally expressed in postnatal type II cells but not in type I cells. In its absence as a result of gene targeting, the phenotype exhibits tracheoesophageal fistula, severe lung hypoplasia and no expression of surfactant proteins in its epithelium [Kimura S, et al. 1996]. Other transcription factors such as members of the nuclear factor I family (NF) also seem to influence SP-C gene expression [Bachurski CJ, et al. 1997]. In addition, the alveolar microenvironment has an influence on surfactant protein gene expression via signals provided by cell-cell or cell-extracellular matrix interactions and growth factors. In culture, isolated type II cells rapidly decrease their expression of SP-A, SP-B and SP-C, whereas surrounded by fetal lung fibroblasts and collagen they sustain the mRNA expression of these proteins [Shannon JM, et al. 1992]. When primary type II cell cultures are supplemented with keratinocyte growth factor (KGF or FGF7), the mRNA levels of SP-A, SP-B, SP-D are increased [Sugahara K, et al. 1995; Xu X, et al. 1998].

Alveolar Stage

At around 36 weeks of human gestation the stage of alveolarization starts with appearance of low ridges along both sides of the saccular walls. By further growth perpendicularly into the air space, they subdivide the saccules incompletely into smaller units, the alveoli. The trans-section of these newly formed interalveolar walls, named secondary septa, demonstrates a doubled capillary layer separated by a sheet of connective tissue. This immature structure does not yet correspond to the adult morphology with its thin interalveolar septa, in which a capillary monolayer occupies almost the whole space of the septum. Therefore, these structures will undergo more restructuring, called microvascular maturation [Burri PH 1999]. Among the variety of factors that participate in the control of budding of secondary septa, elastin appears as playing an essentitial role. Elastin deposition in the thickness of primary septa appears to have a spatially instructive role inasmuch as the specific

sites of elastic fiber formation correspond precisely to the location of future buds. New septa then extend that are composed of a double capillary layer, and elastin localizes at their tip. Other factors involved are FGFs (FGF2, FGF3, FGF4 and FGF7), PDGF (see below), collagenases, FN, proteoglycans [Bourbon J, et al. 2005].

PDGF

One of the leading factors controlling alveolarization is PDGF-A and its receptor PDGFR-α. Mice lacking PDGF-A die either at early gestation or within the first weeks of life [Boström H, et al. 1996]. Animals, dying postnatally, have an emphysematous lung with areas of atelectasis without any signs of alveolarization [Boström H, et al. 2002]. They also lack alveolar smooth muscle cells, and as a consequence no elastin fibers can be demonstrated. Elastic fibers, however, are a prerequisite for secondary septa as mentioned above. Taken together, PDGF-A seems to prevent alveolarization by inhibition of alveolar smooth muscle cell formation. A double null mutant mouse has unveiled a couple of additional players influencing alveolarization, the FGFRs 3 and 4. If both receptors are unfunctional (FGFR-3–/– and FGFR-4–/–), the lungs show large dilated saccules, but no secondary septa and, therefore, no alveoli [Weinstein M, et al. 1998]. In contrast to the PDGF-A null mouse, alveolar smooth muscle cells are normally developed, suggesting another mechanism of action.

VEGF

VEGF has also been demonstrated to play a role in maintaining alveolar structure [Kasahara Y, et al. 2000]. Lungs from newborn mice treated with antibodies to FLT-1 are reduced in size and display significant immaturity with a less complex alveolar pattern [Gerber HP, et al. 1999]. In contrast, the accumulation of VEGF in the alveoli appears to make transgenic VEGF mice more resistant to injury by hyperoxia [Corne J, et al. 2002; Compernolle V, et al. 2002]. VEGF is a target of hypoxia inducible transcription factor-2a (HIF-2a). Hif-2a–deficient newborn mice die from respiratory distress syndrome [Compernolle V, et al. 2002]. In Hif-2a null mice the expression of VEGF is dramatically reduced in alveolar epithelial type 2 cells. Moreover, addition of VEGF to early mouse embryonic lung explants markedly stimulates epithelial as well as vascular morphogenesis, playing an important role in matching the epithelial-capillary interface during lung morphogenesis.

Stage of Microvascular Maturation

This final step in lung development takes place during the first 2–3 years after birth. The goal during this period is the restructuring of thick bilayered secondary septa to the mature interalveolar wall consisting of minimal interstitial tissue and a capillary monolayer. At the end of this developmental process, most of the alveolar capillary endothelium and flattened type I epithelium are in direct contact, which favors optimal gas exchange.

Vascularization

During the fetal period of lung development, important alterations of the three-dimensional structure of the capillary network lay the foundation for alveolarization. During the canalicular stage, there is a marked growth in capillary network which gets closer to the pulmonary epithelium. In the saccular stage, capillaries surround the future air spaces like sleeves. The intersaccular walls or primary septa contain a capillary bilayer. At the deposition site of elastic fibers within the primary septa crests of up-folded tissue of the intersaccular walls are developed. These crests increase in height and become the so-called secondary septa, which subdivide the saccules into smaller units, the alveoli. However, all these walls still contain a double capillary layer, which is suboptimal for gas exchange. It has been shown in rats that during the end of the third postnatal week (after the peak of alveolarization), the number of cells in the interstitium, especially of fibroblasts, are reduced predominantly by apoptosis [Schittny JC, et al. 1998]. This thinning of interstitial tissue may trigger the process by which the two capillary layers merge into one [Kimura S, et al. 1996]. Most of the molecular mechanisms are still unknown. Genetic analyses have demonstrated that cell-extracellular matrix and cell-cell interactions as well as growth factors and transcription factors are involved in vascular development. Members of the VEGF [Healy AM, et al. 2000], angiopoietin [Colen KL, et al. 1999] and ephrin [Hall SM, et al. 2002] families appear to be key players in the control of pulmonary vascularization.

Cell and Gene Therapy

Gene therapy can be defined as the use of genetic material to modify a patient's cells, by correcting an existing abnormality or providing cells with a new function, for the treatment of an inherited or acquired disease.

Already in 1966, Tatum hypothesized that viruses could be used as effective tools for introducing genes [Kay MA, et al. 2001]. Also, shortly after, Kornberg suggested that it might be feasible to attach a gene to a harmless viral DNA and to treat monogenic deficiency diseases by putting the virus/gene complex into the patient's cells [Kornberg A 1971]. However, the concept of gene therapy was introduced only in the late 1970s after the development of recombinant DNA technology. In 1987, Cline [Cline MJ 1987] still pointed out two major obstacles for the application of this technology to human marrow cells: extremely inefficient expression of a new gene in the host cell, and the lack of a convenient and clinically acceptable method of returning the genetically altered cells to the patient. Nevertheless, only in 1990 the first gene therapy protocol would be approved, and the era of clinical gene therapy officially launched [Wivel NA 2001]. Since then, gene therapy has been applied in clinical trials to treat a variety of diseases, including diseases that are caused by a small number of inherited genetic changes, such as cystic fibrosis or muscular dystrophy. By administration of DNA rather than a drug, many different acquired diseases are currently being investigated as candidates for gene therapy, including cardiovascular disease, neurodegenerative disorders such as Parkinson's and infectious diseases such as viral hepatitis and HIV infection in addition to cancer (Table 1).

Current applications and clinical trials

The vast majority (83.9%) of gene therapy clinical trials to date have addressed cancer, cardiovascular disease and inherited monogenic diseases; the first two because of their enormous prevalence, impact and potentially fatal outcomes, the latter because the concept of replacing a well-defined defective gene with its correctly functioning counterpart has an obvious appeal and rationale. Interestingly, trials targeting cardiovascular disease have outnumbered trials for monogenic disease since 2004, although the greatest successes of gene therapy to date have been achieved in the latter group. Is interesting to notice that the range of indications for which gene therapy trials have been approved so far has widened [Edelstein ML, et al. 2007].

Table 2 - Conditions for which human gene transfer trials have been approved [Edelstein ML, et al. 2007].

Monogenic disorders	Cancer
Cystic fibrosis	Gynaecological
Severe combined immunodeficiency	breast, ovary, cervix
Alpha-1 antitrypsin deficiency	Nervous system
Haemophilia A and B	glioblastoma, leptomeningeal carcinomatosis, glioma,
Hunter syndrome	astrocytoma, neuroblastoma
Huntington's chorea	Gastrointestinal
Duchenne muscular dystrophy	colon, colorectal, liver metastases, post-hepatitis liver
Becker muscular dystrophy	cancer, pancreas
Canavan disease	Genitourinary
Chronic granulomatous disease	prostate, renal
Familial hypercholesterolaemia	Melanoma
Gaucher disease	Head and neck
Fanconi's anaemia	nasopharyngeal carcinoma
Purine nucleoside phosphorylase deficiency	Lung
Ornithine transcarbamylase deficiency	adenocarcinoma, small cell, non small cell
Leukocyte adherence deficiency	Mesothelioma
Gyrate atrophy	Haematological
Fabry disease	leukaemia, lymphoma, multiple myeloma
Familial amyotrophic lateral sclerosis	Sarcoma
Junctional epidermolysis bullosa	Germ cell
Wiskott-Aldrich syndrome	
Lipoprotein lipase deficiency	Neurological diseases
Late infantile neuronal ceroid lipofuscinosis	Alzheimer's disease
RPE65 mutation (retinal disease)	Carpal tunnel syndrome
Mucopolysaccharidosis	Cubital tunnel syndrome
	Diabetic neuropathy
	Epilepsy
Cardiovascular disease	Multiple sclerosis
Peripheral vascular disease	Myasthenia gravis
Intermittent claudication	Parkinson's disease
Critical limb ischaemia	Peripheral neuropathy
Myocardial ischaemia	
Coronary artery stenosis	Ocular diseases
Stable and unstable angina	Age-related macular degeneration
Venous ulcers	Diabetic macular edema
Vascular complications of diabetes	Glaucoma
Pulmonary hypertension	Retinitis pigmentosa
Heart failure	Superficial corneal opacity
	Other diseases
Infectious disease	Inflammatory bowel disease
HIV/AIDS	Rheumatoid arthritis
Tetanus	Chronic renal disease
Epstein-Barr virus	Fractures
Cytomegalovirus infection	Erectile disfunction
Adenovirus infection	Anaemia of end stage renal disease
Japanese encephalitis	Parotid salivary hypofunction
Hepatitis C	Type I diabetes
Hepatitis B	Detrusor overactivity
Influenza	Graft versus host disease

Cancer

Thus far, most of the clinical trials in gene therapy have been aimed at the treatment of cancer (66.5% of all gene therapy trials). Many different cancers have been targeted throughout the years, including lung, gynaecological, skin, urological, neurological and gastrointestinal tumors, as well as haematological malignancies and paediatric tumors. A range of different strategies has been applied to cancer gene therapy, from inserting tumor-suppressor genes (e.g. expression of the wild-type p53 tumor-suppressor gene and Ref), to immunotherapy (e.g. intra-tumoral injection of vectors encoding cytokines or major histocompatibility molecules), to gene-directed enzyme pro-drug therapy [Edelstein ML, et al. 2007].

Cardiovascular diseases

Gene transfer for the therapeutic modulation of cardiovascular diseases is an expanding area of gene therapy. During the last decade several approaches have been designed for the treatment of hyperlipidemias, post-angioplasty restenosis, hypertension, and heart failure, and for protection of vascular by-pass grafts and promotion of therapeutic angiogenesis. Adenoviruses and adeno-associated viruses (AAV) are currently the most efficient vectors for delivering therapeutic genes into the cardiovascular system. Gene transfer using local gene delivery techniques have been shown to be superior to less-targeted intra-arterial or intra-venous applications. To date, no gene therapy drugs have been approved for clinical use in cardiovascular applications. In preclinical studies of therapeutic angiogenesis, various growth factors such as VEGFs snd FGFs, have shown positive results; but also factors such as PDGF and HIF have been studied [Edelstein ML, et al. 2007]. Gene therapy also appears to have potential clinical applications in improving the patency of vascular grafts and in treating heart failure. The development of technologies that can ensure long-term, targeted, and regulated gene transfer, and a careful selection of target patient populations, will be very important for the progress of cardiovascular gene therapy in clinical applications [Rissanen TT and Ylä-Herttuala S 2007].

Inherited monogenic diseases

The ultimate aim in treating monogenic diseases by gene therapy is the correction of the disorder by the stable transfer of the functioning gene into dividing cells (stem cells) to ensure the permanence of the correction. Cystic fibrosis (CF) is with no doubt the inherited monogenic disease that arouses more interest in gene therapy: one-third of the 109 trials for inherited monogenic disorders targeted this disease [Edelstein et al. 2007]. CF, is the most common inherited genetic disease in Europe and

the USA and the average life expectancy of patients with CF is less than 40 years, hence the interest in this disease as a prime target for gene therapy. The second most common group of inherited diseases targeted has been the severe combined immunodeficiency syndromes, representing about 20% of the trials for monogenic diseases. This is a group of diseases in which gene therapy has shown lasting and clinically meaningful therapeutic benefit [Cavazzana-Calvo M, et al. 2000; Gaspar HB, et al. 2004; Ginn SL, et al. 2005; Gaspar HB, et al. 2006]. Another monogenic immunodeficiency, chronic granulomatous disease, has also been the target of a successful trial [Ott MG, et al. 2006]. Around 20 other monogenic diseases have been treated (see Table 1) and most of the trials have shown transient expression of the gene transferred, with detectable protein in some cases, but as yet no obvious therapeutic benefit.

Neurological disorders

Neurological disorders such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, multiple sclerosis, stroke, and spinal cord injury are caused by a loss of neurons and glial cells in the brain or spinal cord. Cell replacement therapy and gene transfer to the diseased or injured brain have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases [Valori CF, et al. 2008; Kim SU and de Vellis J 2009]. To date, there are 20 registered phase I and II trials aimed at a variety of neurological diseases [Edelstein ML, et al. 2007].

Ocular disorders

Ocular gene therapy can be used for replacement of mutant genes or it can be used to achieve sustained local delivery of therapeutic proteins in the eye. The advantage of gene replacement is that it has the potential of correcting the primary defect and hence eliminates the source of a particular retinal disease [Campochiaro PA. 2002; 2007]. There are many potential applications of gene delivery in the eye including expression of antiangiogenic proteins in eyes of patients with neovascular diseases, expression of survival factors or anti-apoptotic proteins in eyes of patients with retinal degenerations, and expression of components of the antioxidant defense system in eyes of patients with diseases caused or exacerbated by oxidative stress [Nguyen QD, et al. CLEAR-AMD 1 Study Group. 2006; Campochiaro PA, et al. 2006]. Ocular pathologies have been tackled with 12 trials to date focused on conditions including retinitis pigmentosa, glaucoma and age-related macular degeneration [Edelstein ML, et al. 2007].

Pulmonary Gene Therapy

Gene therapy is currently being evaluated for a wide range of acute and chronic lung diseases. CF, SP-B deficiency and α_1-antitrypsin deficiency are diseases that are associated with single gene defects and represent the obvious rationale for gene therapy of replacing the defective or absent gene. Chronic acquired respiratory disorders such as chronic obstructive pulmonary disease (COPD), asthma, or interstitial lung diseases are considered to be the product of a variety of endogenous (polygenic) and exogenous influences, and less obviously are associated with gene replacement therapy. These chronic inflammatory conditions likely arise from an imbalance between destructive and protective mechanisms, such that transient gene therapy, or gene therapeutics, can be useful to reconstitute a homeostatic balance by the short-term overexpression of protective genes or the suppression of damaging genes. Gene therapy is also considered a potential therapeutic approach for correction of pathological lung conditions, such as pulmonary hypertension. Kamezaki and co-workers demonstrated that extracellular super-oxide dismutase ameliorated pulmonary hypertension induced in the monocrotaline rat model [Kamezaki F, et al. 2008]. Recent advances in cancer biology have identified a number of tumor-associated antigens, and these can be targeted by cell-based and gene-based therapeutic vaccines for immunotherapy interventions in patients with lung cancer [Kolb M, et al. 2006]. To date, at least 90 gene therapy trials for lung conditions have been undertaken [Edelstein ML, et al. 2007]

Cystic fibrosis. Since the discovery of the cystic fibrosis transmembrane receptor (CFTR) gene in 1987, CF has been considered the major lung disease for intervention through gene therapy. However, 16 years after the first clinical trial for CF [Zabner J, et al. 1993] there is still no satisfactory treatment options. Several reasons can be considered for this failure. The ideal vector system has not yet been developed. Strategies to treat CF have employed vectors derived from adenovirus, AAV and oncoretroviruses [Engelhardt JF, et al. 1992; Zsengellér ZK, et al. 1999], but the utility of these vectors is limited by either host immune response, restricted packaging capacity, or low transduction efficiency, respectively. Even if adenoviral vectors, AAV vectors, and liposomes have been shown to transfer sufficient amounts of gene to the lung, but expression is always transient and the levels of expression are never high enough or lasting [Middleton PG and Alton EW 1998; Boucher RC 1999; Griesenbach U, et al. 2004]. The problems associated with repeat administration are yet unsolved. Harvey et al. delivered three doses of Ad-CFTR to the lungs of CF patients 3 months apart and demonstrated that after the third administration vector specific CFTR mRNA was no longer detectable [Harvey BG, et al. 1999]. Also, a phase 2B trial investigating the repeat administration of AAV-CFTR did not show significant improvement in lung function over time [Moss RB, et al. 2007].

The answers to the questions of which pulmonary region and which cells are the best targets for CF gene therapy still must be elucidated. Clinical observations have shown that CF is initially localized in small airways, which are likely not within reach by the aerosols currently in use [Boucher RC 1999]. Mucous plugs and local infections may be real obstacles for gene transfer. The main target tissue for gene transfer is the superficial epithelium, which exhibits all ion transport functions of CFTR and is best accessible via the topical administration of vectors. However, the constitutively highest level of CFTR gene expression is localized in bronchial submucosal gland cells. These glands may be better accessed by the vasculature and systemic vector application [Boucher RC 1999; Griesenbach U, et al. 2004]. Another attempt to deliver gene vectors to these cells is to use adjuncts that can open tight junctions between surface epithelial cells (eg, sodium caprate) [Griesenbach U, et al. 2004]. The replacement of the CFTR gene in the airways alone will probably not compensate for all functional defects in CF patients. Data from the past few years have also suggested an impaired ability to clear bacterial airway infections, partly due to malfunctioning antimicrobial peptides (e.g., human β-defensin 1). Gene transfer could be used to deliver cytokines to the lung as an adjuvant therapy and thereby support the host response against bacteria. In pneumonia models, the survival of animals was improved by transient transgene expression of interleukin (IL)-12 and interferon (IFN)-γ, resulting in enhanced clearance of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [Standiford TJ, et al. 2000]. This fact has important clinical relevance because of the increasing antibiotic resistance of *P aeruginosa*, which persistently colonizes airways in almost all CF patients.

α1-Antitrypsin deficiency is a second pulmonary disease with an underlying single gene defect and a target for gene therapy. It is still unclear whether the replacement of α1-antitrypsin in patients with emphysema affects the course of the disease [Abboud RT, et al. 2001]. Most attempts at gene replacement have seen unsuccessful because of the short-term expression and the high concentrations of protein required for therapeutic efficacy [Song S, et al. 1998]. However, using a nonhuman primate serotype of AAV, high levels of the persistent expression of α1-antitrypsin were seen in mice, even if there was preexisting immunity to human AAV [De BP, et al. 2006].

Surfactant protein B deficiency is a rare disease caused by mutation in the Sp-B gene [Nogee LM, et al. 1994; Hamvas A, et al. 1995]. Because SP-B is normally made by type II epithelial cells in the lung, the onset of pulmonary function at birth causes affected newborns to develop progressive respiratory failure refractory to current therapeutic modalities. The SP-B gene mutation results in absent SP-B protein as well as perturbation of phospholipid, SP-A and C production in type II alveolar epithelial cells [Whitsett JA, et al. 1995]. Distal airspaces accumulate proteinaceous,

eosinophilic material, which is pathognomonic for congenital pulmonary alveolar proteinosis. Currently, the only curative therapeutic option for survival is lung transplantation. Prenatal gene therapy for SP-B deficiency could potentially prevent the fatal consequences of surfactant dysfunction. Prenatal diagnosis is currently possible. Although late gestational approaches have been successfully used [Hamvas A, et al. 1997; Pryhuber GS, et al. 1991], early gestational diagnosis via polymerase chain reaction-based techniques should facilitate prenatal treatment. Prior to birth the SP-B deficient lung is thought to develop normally, and SP-B-deficient mice show normal morphological lung development [Clark JC, et al. 1995]. The vector bearing SP-B should be delivered into the fetal lung with widespread transduction of type II cell precursors. The ultimate goal of *in utero* SP-B gene therapy is to correct the functional deficit even if in a temporary and/or partial way. This would prevent the onset of disease at birth and act as a bridge for lung transplantation.

Primary Ciliary Dyskinesia is a heterogeneous genetic disease that is characterized by cilia dysfunction of the epithelial cells lining the respiratory tracts, resulting in recurrent respiratory tract infections. Despite lifelong physiological therapy and antibiotics, the lungs of affected patients are progressively destroyed, leading to respiratory insufficiency. There is no treatment as of today that could restore normal ciliary beating. Recessive mutations in Dynein Axonemal Intermediate chain type 1 (DNAI1) gene have been described in 10% of cases of Primary Ciliary Dyskinesia. Chinn et al. have recently published a report showing that it is feasible to transfer DNAI1 gene ex vivo to human airway epithelial cells with a lentiviral vector based on Simian Immunodeficiency Virus (SIV) pseudotyped with Vesicular Stomatitis Virus Glycoprotein (VSVG). More important, defective cells that had immotile cilia due to compound heterozygous mutations in the DNAI1 gene recovered ciliary beating after treatment with a lentivirus containing a normal DNAI1 gene. This was the first report on gene therapy focusing in Primary Ciliary Dyskinesia [Chhin B, et al. 2009].

COPD and Asthma Although there is no directed research in gene therapy for COPD, several possibilities are imaginable. Currently, the most accepted theory for the development of COPD is protease/ antiprotease imbalance similar to emphysema due to hereditary α_1-antitrypsin deficiency. Some have shown that the pathogenesis of COPD involves not only elastases but also collagenases and gelatinases studies [Segura-Valdez L, et al. 2000]. Thus, the reestablishment of the balance by overexpressing antiprotease genes is theoretically beneficial, and the levels of antiproteases required should be lower, as in patients with $\alpha\alpha_1$-antitrypsin deficiency. Experimental models have suggested a role for $_1$-antitrypsin and secretory leukoprotease inhibitor in the treatment of this disorder [Tomee JF, et al. 1998; Rogers DF and Laurent GJ 1998]. Neutrophils are a major source of

proteases and reactive oxygen, and, because of their overabundance in COPD, gene therapy could also target adhesion molecules for neutrophils to reduce the influx of inflammatory cells into the lung parenchyma. The benchmark for all future treatments of asthma are inhaled corticosteroids and bronchodilators, which are an established therapy for the majority of asthmatic patients [Alton EW, et al. 1999]. Transient gene therapy could bring some benefit for asthmatic patients with severe disease who require high doses of systemic corticosteroids and for patients with corticosteroid-resistant asthma. Another potential area for gene therapy might be in patients with steroid-resistant asthma. A study [Mathieu M, et al. 1999] has shown that the transfer of the glucocorticoid receptor gene *in vitro* mediated the inhibition of NF-κB activities even in absence of exgenous corticosteroids, and the authors suggested hat this approach could restore corticosteroid sensitivity in patients.

Lung Cancer This is an area of development with gene based vaccines and immunotherapy of lung cancers. Here, access to the tumor is limited, and the intent is to stimulate the host immune response against unknown antigenic epitopes expressed by cancer cells in an effort to control cell growth and metastases. A nonviral vector expressing IL-12 was administered through intranasal instillation in mice to inhibit lung metastatic growth of osteosarcoma [Duan X, et al. 2006], while a granulocyte-macrophage colony-stimulating factor, gene-modified, autologous tumor cell vaccine elicited vaccine-induced immune activation in patients with advanced-stage non-small cell lung cancer [Nemunaitis J, et al. 2006]. Adenoviral vectors have been used to deliver intrapulmonary IFN-β to mice bearing an orthotopic graft of bronchogenic adenocarcinoma of the lung [Wilderman MJ, et al. 2005] and to deliver suicide genes (herpes simplex virus thymidine kinase) to the pleural space in patients with malignant pleural mesothelioma, eliciting some surprising clinical responses [Sterman DH, et al. 2005]. These are examples of developments that will increase as the efficacy and specificity of gene transfer to the lung increases [Rüttinger D, et al. 2006].

Gene Therapy Methods

In gene therapy studies have to consider many different issues, ranging from the medical aspects of a specific disease (type of genetic modification, cellular target), to the basic laboratory work concerning the appropriate gene construct design (type of vector, size of transgene, type of promoter, reporter and, eventually, type of envelope) and delivery systems (administration route, timing, optimal titer) [Lazo PA and Yunta M 2000]. Each of these aspects will be discussed further on with special focus on pulmonary gene transfer.

Types of vectors

Several viral and non-viral gene transfer agents have been evaluated for a range of conditions in animal models and in the clinic (Figure 4). Both physical and immunological barriers exist, and these must be tackled. Although encouraging progress has been made, particularly for lung cancer and CF, all these studies are plagued by low levels of gene transfer. Given the diversity of disease targets that are potentially amenable to gene transfer, it has become clear that there can be no single vector that is suitable for all applications. It is unlikely that a single vector would be optimal for all lung gene therapy applications. Perhaps the only characteristics that are required by all vectors are (a) the abilities to be reproducibly and stably propagated and purified to high titers, (b) to mediate targeted delivery to the tissue or organ of interest without widespread dissemination, and (c) to mediate gene delivery and transgene expression without inducing harmful side effects [Thomas CE, et al. 2003]. Ultimately, all disease targets will benefit from an improved understanding of the biology of vector delivery, uptake and expression. Improvements in vector design will gather rewards in the clinic [Gill DR, et al. 2004].

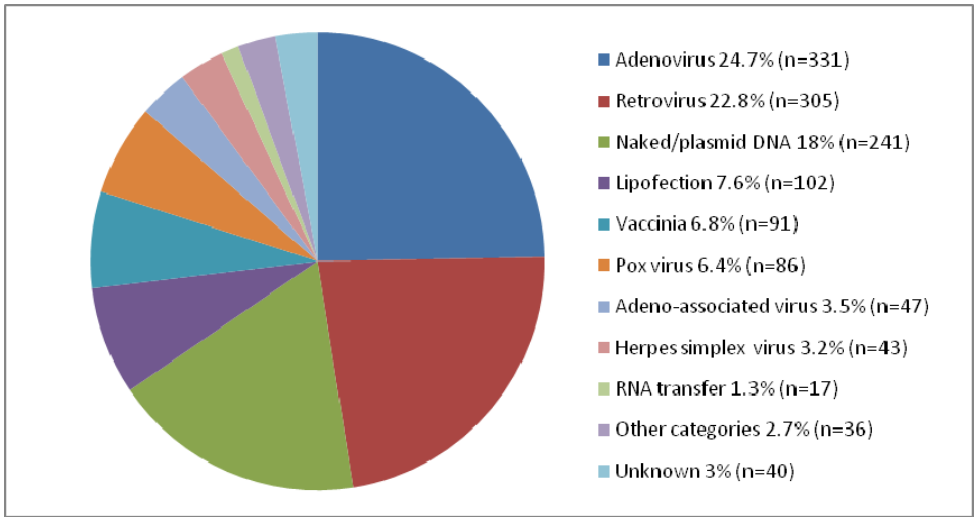

Figure 4 – Vectors used in gene therapy clinical targets [Edelstein ML, et al. 2007].

Viral vectors

Viruses are highly evolved biological machines that efficiently gain access to host cells and exploit the cellular machinery to facilitate their replication. Ideal virus-based vectors for most gene-therapy applications harness the viral infection pathway but avoid the subsequent expression of viral genes that leads to replication and toxicity. This is achieved by deleting all, or some, of the coding regions from the viral genome, but leaving intact those sequences (usually the terminal repeat sequences) that are required *in cis* for functions such as packaging the vector genome into the virus capsid or the integration of vector DNA into the host chromatin. The expression cassette of choice is then cloned into the viral backbone in place of those sequences that were deleted. The deleted genes encoding proteins that are involved in replication or capsid/envelope proteins are included in a separate packaging construct to provide helper functions *in trans*. The packaging cells into which the vector genome and packaging construct are co-transfected then produce the recombinant vector particles [Thomas CE, et al. 2003]. After production in a packaging cell line, the recombinant vector particles are purified and quantified. Purification strategies have traditionally relied on the separation of vector particles from cellular components by density gradient centrifugation (usually a caesium chloride gradient); however, this process is laborious, difficult to scale up for industrial purposes and can sometimes damage the vector particles and reduce the infectious titre of the vector stock. Advances in column-chromatographic methods for the purification of several classes of vector have alleviated these concerns [Clark KR, et al. 1999; Green AP, et al. 2002] and most of the main classes of vector that are described here are now able to be grown and purified to the high titers

required for administration to humans. The number of different viruses that are under development as gene-therapy vectors is steadily increasing, but there are, at present, five main classes of clinically applicable viral vector that are derived from adenoviruses, AAVs, oncoretroviruses, lentiviruses, parainfluenza viruses.

Adenoviral vectors

Adenovirus has been one of the most extensively studied recombinant viral systems for gene transfer to the lung. Adenovirus has a large, complex linear 36-kb DNA genome and is attractive for gene therapy to the lung because it efficiently transduces dividing and non-dividing cells of the airway [Kovesdi I, et al. 1997; Cao H, et al. 2004]. Their viral coat proteins are organized in an icosohedral, non-enveloped capsid. The adenoviral genes are divided into early and late classes, based on the time of their expression during the viral lifecycle. Among the DNA regions for early RNA transcripts, E1a and E1b encode proteins for trans-activating other viral genes or regulating the host cell cycle, E2 for viral DNA replication, E3 for modulating host immune responses, and E4 for inhibiting host cell apoptosis [Parks RJ 2000]. Because the vector genomes persist as episome and do not integrate, repetitive dosing would be required in any gene therapy protocol for inherited genetic deficiencies. Numerous regulator and structural viral genes have made this virus one of the more complex vector systems to engineer in a fashion that does not invoke a cellular immune response.

The first generation adenoviral vectors (FGAd) have been the most extensively used vector for pulmonary gene transfer, namely with the goal of treating CF. FGAd vectors are characterized by E1 viral gene deletion that inhibit, but do not completely prevent, viral gene expression and replication. Transgene sequences are normally inserted into the E1-deleted region but can also be inserted into the E3 region. In most FGAd, the E3 region has also been deleted to make room for the insertion of a transgene cassette. Although once thought to be an ideal vector for CF gene therapy, more than a decade of research has revealed a number of serious shortcomings and the enthusiasm for FGAd as diminished for several reasons. First, pulmonary delivery of FGAd in small animals, large animals, and humans is inefficient [Grubb BR, et al. 1994; Harvey BG, et al. 1999; Zuckerman JB, et al. 1999; Joseph PM, et al. 2001; Perricone MA, et al. 2001]. It was discovered that the cellular receptor for adenovirus, the Coxsackie Adenovirus Receptor (CAR), resided on the basolateral surface of the airway epithelial cells and that the tight junctions prevented vector–receptor interactions required for transduction [Pickles RJ, et al. 2000]. Even if previous studies in mice and cotton rats have

demonstrated efficient gene transfer to the airway for both CFTR [Rosenfeld MA, et al. 1992; Yang Y, et al. 1994] and α-1 antitrypsin gene [Rosenfeld MA, et al. 1991], comparison with human models has demonstrated that transduction from the apical surface of human airway cells is much less efficient than that found in rodents [Engelhardt JF, et al. 1993]. In part, this difference is thought to be the result of species-specific differences in the abundance of CAR [Bergelson JM, et al. 1997], on the apical surface of airway cells [Walters RW, et al. 1999]. V5 integrin, an identified co-receptor for adenovirus type-2, is also localized to the basolateral membrane of human airway epithelium and may be responsible for the low efficiency of recombinant adenovirus infection in polarized airway epithelia [Goldman MJ and Wilson JM. 1995; Pickles RJ, et al. 1998]. A significant finding was that transient disruption of the tight junctions could significantly increase the efficiency of transduction thus dramatically decreasing the vector dose required to achieve therapeutic levels of transduction. Various strategies have been proposed to improve adenoviral entry into airway epithelia including calcium phosphate coprecipitates [Lee JH, et al. 1999], EGTA [Chu Q, et al. 2001], EDTA [Wang G, et al. 2000], polycations [Kaplan JM, et al. 1998], polidocanol [Parsons DW, et al. 1998], sodium caprate [Johnson LG, et al. 2003], L-a-lysophosphatidylcholine [Koehler DR, et al. 2005], and other agents. A controversial issue is whether such tight junction openers can be used clinically in diseases like CF, given the heavy bacterial colonization in the lung and the attendant risk of systemic invasion. The second main reason that restrained the enthusiasm regarding adenoviral vectors is that gene expression from first-generation vectors is also accompanied by an intense cellular immune response to virally expressed genes [Kovesdi I, et al. 1997]. This has been attributed to expression of the viral genes present in the vector backbone of FGAds, which is directly cytotoxic and also provokes an adaptive cellular immune response against the transduced cells consequently resulting in transient transgene expression and longterm, chronic toxicity (through major histocompatibility complex class I-restricted antigen presentation and subsequent activation of CD4C and CD8C cytotoxic T-lymphocytes) [Yang Y, et al. 1995a; Dai Y, et al. 1995; Morral N, et al. 1997]. Second-generation vectors have been generated in an attempt to reduce this cellular immune response [Yeh P and Perricaudet M 1997; O'Neal WK, et al. 1998]. In such vectors, deletions or temperature-sensitive mutants of E2a, E2b, and/or E4 have been tested with some success [Engelhardt JF, et al. 1994; Lusky M, et al. 1998]. Complex complementing cell lines that express E2 and/or E4 are needed to propagate second-generation viruses [Gorziglia MI, et al. 1999]. Some of these gene products require regulated expression because they are toxic to cells, thus making large-scale clinical production of virus challenging. These second-generation vector systems have improved the longevity of transgene expression by reducing cellular immune responses to the vector, but they have not completely solved associated immunologic problems [Engelhardt JF, et al. 1994].

Third-generation adenoviral vector systems, also called gutted or helper-dependent adenoviral vectors (HDAd), are devoid of all viral genes and therefore able to mediate long-term, high-level transgene expression in the absence of chronic toxicity [Parks RJ 2000; Zhou H, et al. 2002; Palmer DJ and Ng P 2005]. In addition, because the HDAd genome exists episomally in transduced cells, the risks of germline transmission and insertional mutagenesis leading to oncogenic transformation are negligible [Hillgenberg M, et al. 2001]. Moreover, the deletion of the viral sequences permits a large cloning capacity of ~37 kb allowing for the delivery of whole-genomic loci, multiple transgenes, and large cis-acting elements to enhance, prolong, and regulate transgene expression [Flotte TR, et al. 2007]. The major hurdles for this vector system are production and generation of replication-competent adenovirus during passaging and propagation. Several strategies using CRE/Lox [Parks RJ, et al. 1996] or FLP/frt [Ng P, et al. 2001] helper adenoviral vector systems have aided in increasing the quantity and purity of vector preparation [Palmer D and Ng P 2003]. Although helper-dependent adenoviral vectors are devoid of all viral genes and, hence, lack cellular immunity to foreign viral antigens, cellular immunity for foreign transgenes remains a concern. Additionally, adenoviral vectors invoke a substantial humoral immune response that inhibits repeat administration to the lung [Yang Y, et al. 1995a]. Several strategies to reduce both humoral and cellular responses have been tested in animal models and include the use of immunomodulatory agents or antibodies [Yang Y, et al. 1995a; 1995b; 1996]. However, the death of a patient undergoing liver-directed gene therapy for ornithine transcarbamylase deficiency with second-generation adenoviral vectors has suggested that the immunology of adenovirus in humans may be poorly understood [Raper SE, et al. 2002]. For example, a recent report has described complement activation with adenoviral particles in the presence of pre-existing antibodies [Cichon G, et al. 2001]. Such mechanisms may play a role in the acute toxicity seen in clinical trials, and further investigation into acute innate immune response to viral capsid proteins is needed.

Several *in vitro* and *in vivo* studies have assessed the safety and efficacy of HDAd in pulmonary gene transfer, with particular interest in CF models [Fisher KJ, et al. 1996; Koehler DR, et al. 2003; Toietta G, et al. 2003]. Koehler et al. used HDAd-K18-CFTR bearing the human CFTR cDNA expressed from the K18 control elements [Koehler DR, et al. 2003]. This vector was found to express properly localized CFTR in cultured cells and in the apical airway epithelia of mice after intranasal administration (preceded by EGTA pretreatment). These results indicate that HDAd can express properly localized CFTR in the appropriate target cell types for CF gene therapy *in vivo* as well as suggest that this vector could benefit CF patients by reducing susceptibility to opportunistic pathogens. Despite some encouraging results, several outstanding challenges remained in the transition to larger animals models: production of the large quantities of HDAd, the need for a

pretreatment with a tight-juntion disruptor (e.g. EGTA), increasing procedure time and compromising transduction efficiency, optimization of vector delivery (intranasal administration not efficient in large animal models).

Adeno-associated viral vectors

AAVs are members of the Dependovirus genus of the Parvovirus Family. AAV consists of a very compact 20-nm non-enveloped icosahedral capsid surrounding a 4.7-kb single-stranded DNA molecule with inverted terminal repeats (ITR) of 145 nucleotides at either end. Between these ITR are the two viral genes, rep, which encodes functions required for replication and gene regulation, and cap, which encodes the capsid proteins, known as VP1, VP2, and VP3 [Berns KI 1996; Flotte TR and Berns KI 2005]. Members of this genus require helper virus coinfection for active replication in cell culture or in non-human primate experimental infections, and in the absence of a helper virus (such as an adenovirus or herpes virus) AAV will establish stable latency within cells without a significant risk of pathological effects [Berns KI, et al. 1975; Cheung AK, et al. 1980]. Primate AAV exists in well over 100 distinct variants, some defined as serotypes and others as genomovars, being AAV serotype 2 the most studied serotype. In addition to primate AAVs, a variety of other AAVs have been identified, including avian, canine, and bovine strains. Interestingly, many of these strains possess divergent tissue tropism, while seemingly retaining the basic properties of stability and non-pathogenicity [Flotte TR, et al. 2007]. AAV derived vectors aroused as promising tool in gene therapy studies due to their genome simplicity, good safety profile, broad tissue tropism, long duration of expression, and suggestion of their superior escape from immune system surveillance compared with other viruses [Griesenbah U, et al. 2004]. Most recombinant AAV vectors have rep and cap deleted. Cells that express rep and cap and helper virus genes will package recombinant AAV genomes if they are flanked by ITRs and are 5 kb or less in length. Packaging is generally efficient with the capsid of any AAV serotype, so long as the ITRs and the rep gene are from the same serotype (usually both are type 2) [Hermonat PL and Muzyczka N 1984; Tratschin JD, et al. 1985]. Progress in the development of recombinant AAV as a gene therapy vector has been developed to solve several long-standing hurdles in the use of this vector system: viral production and purification, limited packaging capacity (< 5kb). Knowledge of the molecular mechanisms by which AAV vector converts its single-stranded DNA genome into large concatamers has also led to the development of novel techniques, including cis-activation, trans-splicing and homologous recombination, to expand the packaging capacity of this vector system [Yan Z, et al. 2000; Sun L, et al. 2000; Nakai H, et al. 2000; Duan D et al. 2001; Halbert CL, et al. 2002]. The basic principle of

these techniques is to split the therapeutic cDNA and required promoter elements, and package them into two viruses, which when transfecting the same cell may recombine and generate a full-length therapeutic gene.

The lung was one of the first sites for *in vivo* transduction with first-generation AAV2 vectors. As with many other sites of delivery, initial transduction efficiency was relatively low, but gene transfer was noted to be stable and safe [Flotte TR, et al. 1992; Wagner JA, et al. 1998]. Early studies with lung cells in culture and *in vivo* also provided the initial evidence that rep-deleted recombinant AAV persists as a stable episome, rather than as an integrated provirus [Kearns WG, et al. 1996]. Most of the AAV serotypes have been tested for lung gene transfer. Although subtle differences in the efficiencies are found between various laboratories, the general consensus is that transduction efficiencies to airway epithelia follow this general order AAV-5 > AAV-6/AAV1 > AAV-2 > AAV-3 > AAV-4 vectors [Walters RW, et al. 2000; Duan D, et al. 2000; Auricchio A, et al. 2002; Halbert CL, et al. 2000]. AAV2 with capsids from serotypes 1, 5, 6 and 9 (AAV2 genome plus AAV1, 5, 6 or 9 capsids) have been evaluated for lung gene therapy and appear to be more efficient in transducing airway epithelial cells than AAV2 [Sirninger J, et al. 2004; Virella-Lowell I, et al. 2005].

Several lung-specific limitations for the use of AAV derived vectors in the pulmonary system are easily pointed out: (1) small packaging capacity, (2) paucity of AAV receptors in the apical surface of airway epithelial cells, (3) rapid turnover of airway epithelium implies limited persistence of the vector. The small packaging capacity of AAV precludes the use of this vector for transfer of larger genes as CFTR. Although there is enough space for the CFTR cDNA (over 4.4kb), it is not possible to include potent promoter/enhancer elements. Several strategies were and are being developed, including design of compact promoter elements [Haberman RP, et al. 2000], trans-splicing and homologous recombination [Duan D, et al. 2001; Halbert CL, et al. 2002], truncation of the therapeutic transgene [Ostedgaard LS, et al. 2005; Mueller C, et al. 2007]. Several primary attachment receptors and co-receptors have been identified for AAV: heparan sulfate proteoglycan, the primary attachment receptor for AAV-2, Vß5-integrin and human FGFR1, co-receptors critical for AAV-2 entry [Summerford C and Samulski RJ 1998; Summerford C, et al. 1999; Qing K, et al. 1999]. Despite the identification of these receptors and co-receptors, current studies in the field reveal that airway epithelial cells present few of these in their apical surface. Detailed studies in polarized epithelial cells have clearly indicated that apical entry of the vector is possible through an alternate import pathway [Duan D, et al. 2000; Sanlioglu S, et al. 2001] and treatment of airway epithelium with proteasome inhibitors facilitates nuclear transport of the vector. This combined vector/small molecule approach might be a feasible alternative to enhance AAV mediated gene transfer to the lung [Yan Z, et al. 2004]. Since the airway epithelium is turning over once over several months in

normal individuals and perhaps four times faster in CF patients, it is unclear how any episomal version of recombinant AAV would be able to persist for more than a few months. Studies attempting to improve vector persistence focus on the development of vectors capable of a greater degree of integration, whether it is Rep-mediated site-specific integration or some less-specific form of integration that might occur by blocking host pathways involved in the formation of AAV episomes [Flotte TR 2007]. Besides, AAV-mediated lung transduction efficiency may be improved by astute selection of the promoter/enhancer elements. Halbert et al. demonstrated that the use of an AAV vector with a hybrid promoter consisting of a CMV enhancer, a β-actin promoter and splice donor, and a β-globin splice acceptor, was most effective and lead to more than 90% transduction efficiency in mouse airways [Halbert SL, et al. 2007]. Liu et al. showed that rAAV was capable of transducing airway epithelial progenitors that had the capacity to clonally expand, both in culture and in vivo following lung injury. These studies suggest that recombinant AAV may be a useful vector for gene targeting of airway stem/progenitor cells [Liu X, et al. 2009].

Oncoretroviral vectors

Retroviruses are lipid-enveloped particles comprising a homodimer of linear, positive-sense, single-stranded RNA genomes of 7 to 11 kb. Following entry into target cells, the RNA genome is retro-transcribed into linear doublestranded DNA and integrated into the cell chromatin. This family of viruses includes several varieties being exploited for gene therapy: the mammalian and avian C-type retroviruses (hereafter also referred to as oncoretroviruses), lentiviruses (immunodeficiency viruses) and spumaviruses. They tend to establish chronic infection that is usually well tolerated by the host but may also cause latent diseases ranging from malignancy to immunodeficiency [Coffin J, et al. 2000]. All retroviral genomes have two long terminal repeat (LTR) sequences at their ends. LTR and neighboring sequences act *in cis* during viral gene expression, and packaging, retro-transcription and integration of the genome. The LTR sequences frame the tandem *gag*, *pol* and *env* genes encoding the structural proteins, nucleic-acid polymerases/integrases and surface glycoprotein, respectively. The viral envelope glycoprotein dictates the host range of retroviral particles through its interaction with receptors on target cells. Maloney murine leukemia virus (MLV)-based vectors were the first type of recombinant retrovirus used for gene delivery. Other examples of vectors derived from oncoretroviruses are Spleen Necrosis Virus, Rous Sarcoma Virus and Avian Leukosis Virus - based vectors [Hu WS and Pathak VK 2000]. Recombinant oncoretrovirus-based genomes are composed of two LTR at either end of the genome, and a packing sequence (ψ). The transgene

cassettes (up to 8 kb) are inserted in place of the three viral genes *gag*, *pol* and *env*. A useful property of retroviral vectors is the ability to integrate efficiently into the chromatin of target cells. Although integration does not guarantee stable expression of the transduced gene, it is an effective way to maintain the genetic information in a self-renewing tissue and in the clonal outgrowth of a stem cell. This is advantageous for gene transfer applications requiring sustained expression in proliferating targets, such as hematopoietic stem and progenitor cells. However, integration can have important effects on the engraftment, proliferation and survival of transduced cells because the integrated proviral DNA can either activate or disrupt host cell genes, resulting in immortalization [Calmels B, et al. 2005], clonal dominance [Fehse B and Roeder I 2007], and in worst case scenario, malignant transformation [Hacein-Bey-Abina S, et al. 2003; Seggewiss R, et al. 2006]. The initial assumption was that activation of proto-oncogenes via replication-incompetent retrovirus vectors was extremely unlikely when vectors integrated only once or a small number of times per target cell. This assumption was refuted after four of the ten patients enrolled in the severe combined immunodeficiency (SCID)-X1 gene therapy trial [Cavazzana-Calvo M, et al. 2000], developed clonal vector–containing T-cell lymphoproliferations resembling *de novo* acute T-cell leukemias [Hacein-Bey-Abina S, et al. 2003]. Subsequently, several large scale surveys of murine retrovirus and integration profiles were carried out in cell lines, and uncovered preferences for integration within or near expressed genes, indicating that the risk of adjacent proto-oncogene activation might be higher than previously estimated based on a random integration model [Baum C, et al. 2003; Wu X, et al. 2003; Métais J-Y and Dunbar CE 2008]. Disruption of the nuclear membrane is required for the pre-integration complex to gain access to the chromatin, and productive transduction by retroviral vectors is strictly dependent on target cell mitosis shortly after entry. Since retroviral vectors can only transduce diving cells and because only a fraction of cells pass through mitosis at any given time, this severely limits the applications of retroviral vectors in gene therapy [Roe T, et al. 1993; Miller DG, et al. 1990; Halene S and Kohn DB 2000]. Pseudotyping consist in the substitution of one viral Env by another from a different virus. Such an approach can expand the host-range of retroviral vectors by incorporating sequences from unrelated viruses. For example, vectors pseudotyped with the VSVG can infect most cells, are particularly stable, and can be concentrated to high titers (excedding 1×10^{10}t.u./ml).

The first clinical trial of human gene therapy used a MLV-based vector, to correct a genetic disorder known as adenosine deaminase deficiency [Blaese RM, et al. 1995.]. Also, one of the first successful trials of gene therapy used γ-retroviral vectors and demonstrated full correction of SCID-X1 phenotype in 10 of 11 patients [Cavazzana-Calvo M, et al. 2000]. Unfortunatly, beginning 3 years after reinfusion of transduced cells, four of the ten patients developed acute T-cell leukemias, related

with proto-oncogene activation, has mentioned above [Hacein-Bey-Abina S, et al. 2003]. Nowadays, insertional mutagenesis continues to be a major concern in hematopoietic stem cell gene therapy. Non-conventional gene transfer vectors, as Avian Sarcoma Leukosis Viruses (ASLV), with more favorable integration features, are being developed and optimized [Hu J, et al. 2008].

The application of this vector system for *in vivo* gene delivery in the lung has been hindered by the inability of this virus to infect non-dividing cells of the airway epithelium. Nonetheless, MLV-based vectors have been proposed for application of *in utero* gene transfer, where epithelial proliferation is high [Duan D, et al. 1998a].

Lentiviral vectors

Lentiviruses have a more complex genome than other retroviridae: in addition to the *gag*, *pol* and *env* genes, they encode two regulatory genes, *tat* and *rev*, essential for expression of the genome, and a variable set of accessory genes. Unlike retroviruses, they rely on active transport of the pre-integration complex through the nucleopore by the nuclear import machinery of the target cell [Bukrinsky MI and Haffar OK 1999]. The lentiviral strategy for nuclear targeting enables infection of non-dividing cells, an attractive attribute for a gene therapy vector. Also, the lentiviral integration profile shows a reduced propensity to hit the promoter-proximal window or regulatory motifs such as DNAse 1 hypersensitive sites when compared to vectors based on the gammaretrovirus MLV [Schröder AR, et al. 2002; Mitchell RS, et al. 2004; Derse D, et al. 2007]. Nevertheless, lentiviral insertion pattern with its profound preference for active genes should not represent a reliable safeguard against the potential risk of insertional mutagenesis [Schambach A and Baum C 2008]. Replication-defective vectors were originally derived from human immunodeficiency virus (HIV)-1 to transduce lymphocytes, but it was a VSVG pseudotyped lentiviral vector with expanded tropism [Naldini L, et al. 1996] that spurred applications for gene therapy. The genetic information required to package a functional lentiviral core in the vector was then found to be only a fraction of the parental genome [Vigna E and Naldini L 2000]. An important approach to alleviate biosafety concerns is the use of self-inactivating transfer vectors (SIN) [Zufferey R, et al. 1998; Miyoshi H, et al. 1998]. These vectors contain a deletion in the downstream LTR that when transduced into target cells, results in the transcriptional inactivation of the upstream LTR and diminishes substantially the risk of vector mobilization and recombination [Bukovsky AA, et al. 1999]. As the non-required genes are critical for viral pathogenesis, new generations of “minimal” packaging constructs have been adopted to increase vector biosafety: the so called third-generation lentiviral vectors. These are lentiviral SIN vectors containing both Rev-responsive element (RRE) and Tat cognate motif and being dependent

upon the co-expression of Rev in packaging cells. When using strong promoters (e.g. the promoter from RSV) to drive the expression of the genomic vector message in packaging cells, Tat is not essential to achieve efficient expression of genomic RNA [Dull T, et al. 1998]. Hybrid lentiviral vectors have also been derived from nonhuman lentiviruses: Simian Immunodeficiency Virus (SIV), Equine Infectious Anemia Virus (EIAV), Feline Immunodeficiency Virus (FIV), Caprine Arthritis/Encephalitis Virus and Bovine Jembrana Disease Virus, following similar approaches to those used for HIV-derived vectors, on the rationale that they would be more acceptable for clinical application because the parental viruses are not infectious to humans [Olsen JC 1998; Poeschla EM, et al. 1998; Mitrophanous K, et al. 1999]. However, because of the lower number and yet unclear role of accessory genes in non-primate lentiviruses, the actual gain in biosafety of these advanced design remain to be established [Vigna E and Naldini L 2000]. The obligatory RNA step in the retroviral lifecycle poses great constraints on the viral genome and on its exploitation for gene transfer purposes. The transgene expression cassette must be of limited size (8 kb), without introns and internal polyadenylation signals. Together with the exposure to loco-regional differences in the structure and activity of chromatin consequent to random integration, these factors combine to limit expression of the transduced genes. VSV-pseudotyped lentiviral vectors can be delivered directly *in vivo*. Lentiviral vectors efficiently transduce several non-dividing, differentiated epithelial tissues of rodents, humans and other species, isolated or dissociated ex vivo [Vigna E and Naldini L 2000]. Direct transduction *in vivo* appears to be more sensitive to tissue barriers limiting vector access, as in the case of respiratory mucosa [Johnson LG, et al. 2000] and to intracellular conditions (for example, cell cycle status) as in the case of hepatocytes [Park F, et al. 2000].

The ability of lentiviral vectors to transduce a great variety of non-dividing cells, and the substantial flexibility in the design of the expression cassettes, remain the most important rationales for their clinical use. The scope of potential clinical targets includes retinal or other sensory epithelium, neurons, cardiac or skeletal muscle, hepatocytes, endothelial cells, dendritic cells, hematopoietic stem cells, and dormant tumor stem cells. The ability to transduce non-dividing cells has also triggered the potential clinical development of integration-defective lentiviral vectors, sometimes addressed as non-integrating lentiviral vectors [Yáñez-Muñoz RJ, et al. 2006; Philpott NJ and Thrasher AJ 2007]. The first clinical trial using lentiviral vectors was conducted to treat individuals suffering from the acquired immunodeficiency syndrome caused by infection with HIV-1 [Dropulic B and June CH 2006; Levine BL, et al. 2006]. The first clinical trial using a non-HIV based lentiviral vector has started December 2007. For the treatment of Parkinson's disease, the company Oxford Biomedica has developed a lentiviral vector, ProSavin, based on EIAV, expressing the three key dopamine biosynthetic enzymes (tyrosine hydroxylase, aromatic L-amino acid decarboxylase and

GTP cyclohydrolase 1). In this phase I/II clinical trial the lentiviral vector was directly administered bilaterally into the sensorimotor putamen of late stage Parkinson's patients.

The development of lentiviral and pseudotyped lentiviral vectors, which can infect nondividing cells, has overcome some of the limitations of MLV-based vectors and has renewed interest for this class of retroviridae for *in vivo* gene delivery to the lung [Naldini L, et al. 1996]. As with other viral vectors, factors at the airway surface pose barriers to retroviral and lentiviral gene transfer. Airway epithelial gene transfer efficacy with lentiviral vector system has previously been restricted by a paucity of pseudotypes, which can be produced at high titres as the commonly used VSVG. However, when pseudotyped with VSVG, lentiviruses can only enter airway epithelial cells via the basolateral membrane. Therefore, apical transduction by lentivirus has been developed by using a number of viral envelopes from diverse origins, both *in vitro* and *in vivo*, including filovirus [Medina MF, et al. 2003; Sinn PL, et al. 2003], baculovirus [Sinn PL, et al. 2005], influenza [McKay T, et al. 2006] and parainfluenza [Kobayashi M, et al. 2003] viruses. Jaagsiekte Sheep Retrovirus envelope proteins have been shown to stabilize pseudotyped retrovirus in the presence of lung surfactant [Coil DA, et al. 2001] and evaluated for pulmonary gene transfer [Liu S-L, et al. 2004]. For example, the Ebola virus envelope glycoprotein has been used successfully to achieve efficient transduction of the murine lung epithelium and human explants [Kobinger GP, et al. 2001; Lim FY, et al. 2003] although generation of consistently high viral titres has been problematic. Sinn et al. [Sinn PL, et al. 2003] also showed that transgene expression after lung application of feline immunodeficiency virus pseudotyped with the baculoviral gp64 envelope applied in a viscoelastic gel formulation was significantly higher than observed with VSVG pseudotyped construct. Buckley et al. [Buckley SM, et al. 2008] performed a comparative study using HIV-based lentiviral vectors pseudotyped with the baculovirus gp64 envelope versus VSVG to transduce fetal, neonatal or adult airways. They concluded that gp64 pseudotyped lentivirus efficiently transduces airway epithelial cells after both fetal and neonatal administration, whereas adult administration resulted in low level transduction in this tissue but efficient transduction of alveoli. Intra-amniotic administration of gp64 pseudotyped lentivirus appeared to be the most efficient mode of airway epithelial transduction in the murine model. Nevertheless, Kremer et al. [Kremer KL, et al. 2007] did not see increased apical uptake of gp64/HIV when compared to VSVG.

Parainfluenza viral vectors

Parainfluenza virus has a negative-strand RNA genome and replicate in the cytoplasm. They do not go through a DNA intermediate and do not enter the nucleus. This family of viruses includes several varieties being exploited for gene therapy: the murine parainfluenza virus type 1 or Sendai virus, the human Respiratory Syncytial Virus and the human Parainfluenza Virus type 3 (PIV3). All of these have been shown to efficiently transfect airway epithelial cells via the apical membrane using sialic acid and cholesterol, which are abundantly expressed on the apical surface of airway epithelial cells [Ferrari S, et al. 2003; Zhang L, et al. 2005]. Only Sendai virus has been assessed in animal models *in vivo*. SeV-mediated gene expression is transient (lasting for about 7 days) and currently repeated administration is inefficient. Several groups are assessing a variety of immuno-modulatory strategies to improve the use of SeV for chronic lung diseases, as CF.

Non-viral vectors

Gene delivery systems based on non-viral vectors mainly comprise cationic liposomes [Bennett CF, et al. 1992; Ropert C, et al. 1993; Thierry AR, et al. 1993], DNA-protein complexes [Ryser HJ and Shen WC 1978] and mechanic administration of naked DNA [Tascon RE, et al. 1996; Ulmer JB, et al. 1993]. These systems are relatively easy to manipulate, are not infectious and are not very toxic. Furthermore, nonviral vectors allow for the delivery of large DNA fragments and are also particularly suitable to deliver oligonucleotides to mammalian cells, which is an excellent feature for the application of antisense strategies to downregulate the expression of certain genes. A number of obstacles have severely limited the application of nonviral-based vectors in therapy and preclinical studies [Romano G, et al. 1999]. The lack of specific targeting, the low transfection efficiency and the fact that transgene expression is only transient make difficult the *in vivo* applications of nonviral gene delivery systems.

Cationic liposomes

Cationic lipossomes as a vehicle for delivery of DNA, have also been extensively studied in animal models of lung gene therapy. Major attractions of liposome mediated gene delivery include (a) an easily scalable gene transfer formulation for clinical trials, (b) no apparent size limitation to the DNA transgene being delivered, and (c) the absence of exogenous protein in the delivered complexes that should reduce immune responses to the vector. Despite these theoretical advantages, progress in developing clinically efficacious protocols has been poor owing to the inherently low efficiency of

gene transfer, the transient nature of transgene expression, and immunogenicity to unmethylated CpG dinucleotides in plasmid derived bacterial DNA [Tan Y, et al. 1999]. The mechanism of gene transfer with cationic liposome/DNA complexes is poorly understood, but several general features appear to be important in the efficacy of gene expression with these reagents including (a) the lipid composition, (b) the complex charge density determined by the liposome/DNA ratio, and (c) the size of the complex. All these factors appear to be important for efficient endocytosis and/or intracellular escape from endosomes, which ultimately affect the efficiency of gene transfer [Lee ER, et al. 1996; Felgner PL 1996]. A major obstacle in the use of liposome/DNA complex-mediated gene transfer has been the apparent low level of endocytosis from the apical membrane of differentiated polarized airway epithelia [Matsui H, et al. 1997]. Despite these limitations, studies in CFTR knockout mice have demonstrated the ability of CFTR-expressing liposome/DNA complexes to correct ion transport abnormalities [Alton EW, et al. 1993; Hyde SC, et al. 1993]. However, studies in CF human bronchial xenografts have demonstrated that cationic liposome can mediate complementation of mucus sulfation defects in CF epithelia but not ion transport abnormalities [Zhang Y, et al. 1998]. Improvements in cationic liposome-mediated gene delivery were made possible by a massive functional screen of cationic lipids for gene transfer to the lungs of mice. In this study, the Genzyme Corporation identified a cationic lipid called GL-67 that was 100 times more effective than previously evaluated common cationic liposomes [Lee ER, et al. 1996]. However, even with the most effective GL-67 lipid formulation, gene transfer was maximal at 2 days post-transfection and quickly diminished thereafter. GL-67 lipid formulation was the basis of the first lung clinical trail using aerosolized cationic lipids in normal volunteers [Chadwick SL, et al. 1997]. Luton and coworkers administrered cationic cholesterol derivatives to sheep fetuses via surgical replacement of the fetal airway fluid by the transfection mixture followed by tracheal occlusion. Reporter gene expression was detected in both trachea and lung epithelial cells and some mesenchymal cells. However, the level of transgene expression was relatively low and there was evidence of acute toxic effects under lipofection conditions [Luton D, et al. 2004].

Other non-viral vectors

Other nonviral DNA delivery systems have been described, including polymers and small synthetic beads. Even naked DNA diluted in hypotonic fluids can produce some gene delivery to lung epithelium. However, the utility of these delivery systems into the lung, particularly *in vivo*, has not yet been proven. For all of these systems, it appears that gene expression efficiency is quite low. For diseases such as CF, this may be a major limitation, whereas for other diseases, low-level expression may be adequate [West J and Rodman DM 2001]. Several groups are assessing a

variety of physical delivery methods, including electroporation, magnetism, ultrasound and vibration, in an attempt to increase the transfection efficiency of nonviral formulations. Electroporation has been successfully used to enhance transfection in a variety of organs including muscle [Griesenbah U, et al. 2004]. Some results for lung gene transfer are encouraging and demonstrate that the transfection efficiency of naked DNA can be enhanced in the presence of electrical fields [Dean DA, et al. 2003]. Clearly, important technical questions and safety considerations have to be resolved.

Vector promoter

Many gene delivery vectors use viral promoters such as the cytomegalovirus-immediate early (CMV-IE) promoter because this promoter can drive transgene expression in many cell types. Studies focusing on pulmonary gene transfer have used CMV-IE for this reason because it has yet to be resolved which of the 40 cell types in the respiratory tract needs to be targeted for each of the pulmonary disorders that may benefit from genetic therapy [Davies JC, et al. 1998]. However, many studies of fetal gene transfer [Bestor TH 2000; Rivella S and Sadelain M 1998; Tarantal AF, et al. 2001] have provided evidence of transgene silencing with this promoter. In addition, the development of pulmonary gene transfer strategies may require selective targeting of transgene expression to specific cells of the developing lung to ultimately be curative. Lung-specific promoters such as surfactant protein-C (SP-C; targets type II cells) or CC10 (targets Clara cells) are of interest for fetal gene transfer because SP-C mRNAs appear at early human fetal gestational ages in association with the epithelium where airways branch, and SP-C mRNA and proSP-C are present in distal lung epithelial cells before differentiation into type II cells occurs [Khoor A, et al. 1994; Wert SE, et al. 1993]. Whitsett et al. used the gene CFTR under the direction of the SP-C and CC10 promoters in transgenic mice and evaluated the effects of expression *in utero* [Whitsett JA, et al., 1992]. The use of SP-C and CC10 promoters in these studies ensured CFTR expression in more differentiated cell types because these proteins become specific markers of their respective cell types (type II and Clara cell) as they differentiate.

Table 3 — Advantages and disadvantages of currently used vector systems for lung gene transfer [Kolb M, et al. 2006].

Vector	Advantage	Disadvantage
Viral		
Retrovirus / Lentivirus	Viral genes removed, no viral proteins made, integrates into host DNA (retrovirus)	Possible insertional mutagenesis, cell division necessary (retrovirus), low titers
Adenovirus	Efficient, transduces nondividing cells, produced in high titers	Prior exposure, immune response, inefficient with repeated application
AAV	Virus genes removed, no viral proteins made, safe, transduces nondividing cells	Production labor-intensive, small packaging capacity for foreign DNA
Parainfluenza virus 1 Sendai virus	RNA genome, targets apical surface of epithelium, replicates in cytoplasm	Particles are inflammatory, induces immunity
Non-viral		
Naked DNA	Simple, nonimmunogenic, inexpensive, safe	Inefficient transduction
Cationic liposomes	Nonimmunogenic, repeated application possible, safe	Gene expression transient and low

Delivery systems

Vectors are the vehicles that carry the genetic information into the patient's cells for treatment of diseases. Each vector system has unique properties. Therefore, different vectors will be required for treating the variety of diseases amenable to gene transfer. Vectors can be introduced into cells from the patient's body (e.g. blood cells) and then reintroduced into the patient, or the vectors can be administered directly into the body through injection or even possibly by mouth. Further discussion of gene delivery systems: routes and time of administration, experimental models, will be restricted to lung directed *in utero* gene therapy.

In utero gene therapy

The current approaches of gene therapy into mature organisms are confronted with several problems including the following: (1) the underlying genetic defect may have already caused irreversible pathological changes; (2) the level of sufficient protein expression to ameliorate or prevent the disease requires prohibitively large amounts of gene delivery vector; (3) adult tissues may be poorly infected by conventional vector systems dependent upon cellular proliferation for optimal infection, for example, oncoretrovirus vectors; (4) immune responses, either preexisting or developing following vector delivery, may rapidly eliminate transgenic protein expression and prevent future effective intervention. Early gene transfer, in the neonatal or even fetal period, may overcome some or all of these obstacles. [Waddington SN, et al. 2004a; 2005]. The major advantages of fetal and neonatal gene therapy are (1) prevention of irreversible pathological processes; (2) easier access to certain organs (in the case of the fetal lung, a fluid-filled structure is easier to disperse the gene transfer reagent) (3) reduced amount of vector required due to high ratio of vector particles to cells (4) ideal environment for infection of abundant stem cells and other progenitors (5) immaturity of immune system.

Therapeutic gene transfer during development could be of benefit for those disorders that compromise prenatal structure and function and have significant morbidity soon after birth [Kawada T, et al. 2002; Melo LG, et al. 2004; Ryan K, et al. 2004]. Similarly, CF or 1-antitrypsin deficiency can result in significant morbidity and mortality [Ratjen F and Döring G 2003]. For these diseases, if treatment can be accomplished before severe damage occurs, then, theoretically, healthy newborns can be delivered free of disease at term. Lysosomal storage diseases such as the mucopolysaccaridoses, including Sly, Hunter and Hurler syndromes, Tay-Sachs disease and globoid cell leukodystrophy, demonstrate fetal pathology; nevertheless, substantial therapeutic benefits have been observed in mouse and dog models following neonatal gene therapy [Waddington SN, et al. 2004b].

From birth to adulthood, body mass increases approximately 20-fold in humans, therefore, a relatively much lower amount of virus will infect a higher percentage of cells when introduced early rather than late in life. Also, in adult gene therapy studies of factor IX adenovirus vectors, larger animals (e.g. haemophiliac dogs) require far higher doses per kg body mass of viral vector to achieve the same levels of transgenic factor IX expression than the smaller mouse models, making it difficult to scale vector doses from small to large species based on body mass alone .

Many genetic disorders, the organ can be difficult to target after birth, for example the lung in cystic fibrosis, the brain in urea-cycle disorders, or the skin in epidermolysis bullosa. Fetal treatment can take advantage of developmental changes to access organs that are inaccessible after birth [David AL and Peebles D 2008].

Gene transfer to the developing fetus targets rapidly expanding stem-cell populations, providing a large population of transduced cells to provide a therapeutic effect. Many cell types, such as myoblasts, central nervous system stem cells, mesenchymal stem cells, and hematopoietic stem cells are more prevalent in early gestation and decrease in frequency with age. The genetic integration of a transgene into a pluripotential stem cell would potentially result in tremendous expansion of the transduced cell population with fetal and postnatal growth [Yang EY, et al. 1999a; 1999b]. For example, after intravascular administration of lentivirus vectors to fetal mice, expression of a marker gene appeared to be distributed in the liver in focal clusters, suggesting they may have arisen from individual progenitors [Waddington SN, et al. 2003; MacKenzie TC, et al. 2002].

The fetus has a functionally immature immune system compared to an adult, which might be to its advantage. Worldwide, up to 50% of adults have pre-existing humoral immunity to adenovirus and adeno-associated virus serotypes from which commonly used gene therapy vectors are derived [Bessis N, et al. 2004] Even in the absence of a pre-existing immune sensitivity, vector administration to adults often results in the development of an immune response that reduces the duration and level of transgene expression [Gilchrist SC, et al. 2002]. Weaker fetal immune response towards a transgenic protein may be due to the following: (i) the reduced number of immune cells in early life; (ii) the developmental immaturity of cells participating in the immune response; (iii) the deviance of the early immune response from that of the adult, with particular bias towards a TH2 rather than a TH1 response; and (iv) the absence of memory cells due to the naivety of the immune system. Immune tolerance to exogenous protein can be induced in the fetus if the protein is introduced before the immune system is competent. Tolerance also requires that the exogenous protein expression is maintained, even if at low level, and so the ability of the vector to give long-term expression is vital. As an example, in a mouse animal model of haemophilia B, one study showed that the functionally immature fetal immune system does not respond to the product of the introduced gene, and therefore immune tolerance can be induced [Waddington SN, et al. 2004b]. This means that treatment could be repeated after birth, if a single fetal treatment was not sufficient to cure the individual of the disease.

Safety and ethical issues

Various safety issues in relation to in-utero gene therapy need to be addressed before such therapy can be applied clinically [Fletcher JC and Richter G 1996; U.S. National Institutes of Health. Recombinant DNA Advisory Committee 2000]. There is a theoretical risk that the therapeutic gene product or vector that is required later in life to correct a genetic disease could interfere with normal fetal development. This has been suggested in the case of cystic fibrosis, where in-utero infection of rats at 16–17 days gestation with a recombinant adenovirus carrying the human cystic fibrosis transmembrane receptor gene resulted in altered lung development and morphology [Morrow SL, et al. 1998]. The effects of a transgenic protein on developmental processes will be difficult to predict, depending on the time of gestation and the type of protein introduced, which will require careful long-term monitoring. An established risk factor of integrating viral vectors is insertional mutagenesis (see "Oncoretroviral vectors"). The fetal system might be particularly sensitive to such events because integrating vectors prefer to insert their genomes into chromatin in open configuration. Whereas one of the aims of prenatal gene therapy is to achieve immune tolerance to the transgene and delivery system, vectors must be designed to be sufficiently different to the wild-type so that the immune system remains able to mount an effective immune response against wild-type virus infection. Hypothetical germ-line transmission is another potential concern, even if it was never reported, nor for the fetus neither for the mother. Any fetal therapy or procedure poses risks of infection, immune reactions and the induction of preterm labour for the fetus and the potential to harm the mother. A conflict of interest might potentially arise because treating the fetus might not be in the mother's best interest [David AL and Peebles D 2008].

Future perspectives

Positive results of pre-clinical studies in rodent models have emerged to show proof-of-concept for a postnatal therapeutic effect of in utero gene delivery. Rucker et al. used an AAV serotype 1 vector (AAV-2/1) to deliver human α-glucosidase to the diaphragm of mice deficient in this enzyme, a condition which often results in death from respiratory failure in humans [Rucker M, et al. 2004]. Normal contractile function was restored for up to 6 months postpartum. In 2004, Dejneka et al. used the same AAV serotype to deliver human retinal pigment epithelium 65 to the retinal pigmented epithelium of Rpe65-/- mice and demonstrated restoration of visual function [Dejneka NS, et al. 2004]. Karolewski and Wolfe also used AAV-2/1 to treat a mouse model of mucopolysaccharidosis type VII. This vector carrying the human β-glucuronidase cDNA was injected into the ventricle of the fetal brain and resulted in widespread gene expression in the brain and spinal cord and a significant improvement in survival after one year [Karolewski BA and Wolfe JH 2006]. Also in 2004,

Waddington and coworkers injected VSVG-pseudotyped HIV vector into the fetal vitelline vessels, which resulted in predominant expression of human Factor IX in the mouse liver (the site of endogenous synthesis) and achieved lifelong correction of the bleeding diathesis [Waddington SN, et al. 2004b]. Seppen et al achieved intraperitoneal and hepatic delivery of the transgene from direct intrahepatic HIV-based lentiviral vector injection. This significantly ameliorated the metabolic defect for one year [Seppen J, et al. 2003].

In the short term, two intermediate strategies for possible *in utero* gene therapy are emerging. The first involves the use of "advanced" non-integrating vectors, including helper-dependent adenovirus, integration-deficient lentivirus, and new adeno-associated virus serotypes [Waddington SN, et al. 2007]. These may be applied to tissues from which the vector genome is slowly lost, such as muscle [Rucker M, et al. 2004], neuronal tissue in the central nervous system [Karolewski BA and Wolfe JH 2006], and the retina [Dejneka NS, et al. 2004] for longer-term correction of genetic disease. They may also be applied to rapidly dividing tissues, such as the liver, to permit transient correction of genetic diseases which manifest perinatally until a postnatal treatment can be applied; and may also be applied to the fetus, placenta or extraembryonic membranes for treatment of pregnancy-associated diseases such as pre-eclampsia [Koyama S, et al. 2006] or bronchopulmonary hypoplasia [Larson JE and Cohen JC 2006]. The second fetal gene therapy strategy is ex vivo transduction and subsequent re-implantation. This may now exploit the technological advances in maximizing safety and efficacy of ex vivo transduction, novel and safer integrating retroviral vectors, and advances in stem cell technology [Rio P, et al. 2005; Chan J, et al. 2005]. Recent advances in fetal medicine—particularly in imaging and minimally invasive intervention—have opened the way, in large animal models, for clinically relevant delivery of gene therapy vectors to virtually any fetal organ. However, although postnatal gene therapy has seen its first successful clinical applications, no trials are planned for human gene therapy *in utero*, now or in the near future. Indeed, many view the concept as having only academic value, with no potential for translation into the clinic [Coutelle C 2008].

In utero lung gene transfer

The fetal lung is an attractive target organ for fetal gene transfer. There are a number of genetic and acquired disorders with peri- or postnatal pulmonary manifestations. These include monogenetic diseases like CF [Larson JE, et al. 2000] or SP-B deficiency that would presumably require long-term expression of the deficient or defective gene. However, there are also abnormalities of lung growth, such as congenital diaphragmatic hernia (CDH), or lung maturation, such as respiratory distress syndrome of prematurity, that could potentially benefit from strategies that achieve transient gene expression in specific pulmonary distributions. Additionally, an important advantage of the use of non-integrating viral vectors with transient gene expression is the avoidance of many of the current concerns regarding integrating vectors in the fetus, i.e., insertional mutagenesis, developmental abnormalities, and germline alteration. Success of gene therapy will depend on the choice of vector, defining the optimal site, route and timing of gene delivery during lung development [Yu ZY, et al. 2007]. One additional advantage of targeting the fetal lung is that is more efficient to disperse the gene transfer reagent in a fluid-filled structure rather than across air-liquid interface.

Adenoviral vectors have been used in most *in utero* investigations, except those using retroviral vectors [Douar AM, et al. 1997; Pitt BR, et al. 1995]. Although having the advantage of being able to be concentrated in high titers and providing efficient gene transfer, adenoviral vectors may lead to substantial inflammation and even fetal loss [Iwamoto HS, et al. 1999; Yang EY, et al. 1999a]. Retroviral vector use in *in utero* applications has been limited because retroviral infectivity is reduced by amniotic fluid [Douar AM, et al. 1996]. Previous work, however, suggests that AAV gene transfer to respiratory epithelia is limited by vector entry and postentry interactions of cellular components with vector DNA [Duan D, et al. 1998b]. In particular, well-differentiated pulmonary epithelial cells may lack the factors necessary for efficient AAV-mediated gene expression. AAV-mediated gene expression is much more efficient in undifferentiated pulmonary cells [Bals R, et al. 1999]. These observations suggest that one strategy for generating higher degrees of vector entry and gene expression in AAV may be targeting rapidly dividing undifferentiated epithelial cells with an *in utero* or neonatal approach. The neonatal approach has been studied [Rubenstein RC, et al 1997; Zeitlin PL, et al. 1995] but studies of *in utero* administration of AAV are limited to recent investigations of the effect of intraperitoneal and intramuscular delivery in a mouse model [Lipshutz GS, et al. 2001; Mitchell M, et al. 2000]. Pulmonary applications of *in utero* lentiviral gene transfer *in vivo* have broadened. Following successful demonstration of *in vivo* gene transfer to human xenografts

[Goldman MJ, et al. 1997] and rabbit airways [Wang G, et al. 1999] groups have demonstrated efficacy of *in utero* vector delivery. Following intrapulmonary delivery of an HIV vector encoding green fluorescent protein (GFP) into rhesus monkey fetuses, Tarantal et al. [Tarantal AF, et al. 2001; 2005] reported gene expression in pulmonary epithelia confirmed by direct fluorescence and Q-PCR. Lim et al. [Lim FY, et al. 2003] had similar success in targeting epithelia in human fetal tracheal xenografts using HIV-LacZ. Transgene expression remained detectable 9 months posttransduction.

Route of administration

If fetal gene therapy is to be clinically applicable, developments in vector technology must be accompanied by improvements in minimally invasive methods of delivering vectors to the fetus. Traditionally, invasive surgical techniques such as maternal laparotomy or hysterotomy have been performed to access the fetus in small- and even large-animal models. However, in clinical practice, minimally invasive techniques such as ultrasound-guided injection, or even fetoscopy, could be used to deliver gene therapy to the fetus with less morbidity and mortality.

Experiments that use intra-amniotic injection of oligodeoxynucleotides have demonstrated excellent expression in fetal skin, but no expression in the fetal lung [Hayashi SI, et al. 1996]. Intra-amniotic administration of adenovirus showed low level pulmonary expression of transferred marker genes in 3 of 4 studies [Sekhon HS and Larson JE 1995; McCray PB Jr, et al. 1995; Douar AM, et al. 1997; Holzinger A, et al. 1995]. In contrast, much higher levels of gene transfer were observed in the proximal gastrointestinal tract (oropharynx and esophagus) because of fetal swallowing of amniotic fluid. Thus, the net efflux of fetal hmg fluid probably predominates over any influx of fluid with fetal breathing movements, preventing vector access to the lung. An alternate approach is direct administration of vector into the fetal trachea. This has been performed by open fetal surgical [McCray PB Jr, et al 1995; Vincent MC, et al 1995] or fetoscopic techniques [Sylvester KG, et al. 1997].

Intra-tracheal injection of retroviruses, which are much less efficient at transduction than adenovirus, resulted in only spotty single-cell areas of gene transfer [Pitt BR, et al. 1995]. Results with recombinant adenovirus have been much more successful. Several studies have documented high efficiency marker gene transfer in the lung parenchyma of late gestation sheep within 7 days after intratracheal injection of first generation recombinant adenovirus [McCray PB Jr, et al 1995; Vincent MC, et al 1995; Sylvester KG, et al. 1997]. Although only 1 study quantified transduction of distal alveolar cells as high as 12% [McCray PB Jr, et al 1995], areas of higher level transduction were observed in other studies. A notable finding was that the distal lung parenchyma appeared to be the major site of gene transfer. In 2 of 3 studies, no expression could be detected in any large airways

[Vincent MC, et al 1995; Sylvester KG, et al. 1997]. Tracheal gene transfer was only observed in I study and may have been due to concomitant epithelial injury due to instrumentation or vehicle toxicity [McCray PB Jr, et al 1995]. One interpretation is that the late gestation fetal airways are well differentiated and similar to adult airways in terms of tight junctions and basolateral distribution of viral receptors. This conclusion is substantiated by electron microscopy studies [Schneeberger EE, et al. 1978]. Although discouraging for the purposes of treating CF, an encouraging finding was that parenchymal gene transfer was specifically localized to type II alveolar epithelial cells by colocalization with sheep SP-B protein [Sylvester KG, et al. 1997]. Thus, recombinant adenovirus can target the appropriate cell type for treating SPB deficiency. Despite the initially high gene transfer efficiency with recombinant adenovirus, long-term results have been disappointing. The appearance of cellular inflammation at about 7 to 10 days heralds loss of transferred gene expression [McCray PB Jr, et al. 1995; Yang EY, et al. 1999c]. This suggests that the late gestation fetus reacts in a manner very similar to adult animals in response to first generation adenoviruses. With the use of completely deleted recombinant adenoviruses, which are devoid of adenoviral structural genes, and with application of fetal gene therapy earlier in gestation, it is conceivable that transferred gene expression can be prolonged. In summary, initial results with first generation adenoviruses in the fetal lung have been promising. Clearly, administration of vector can be achieved via minimally invasive, fetoscopic methods. Distribution of vector is broad throughout the lung, and gene transfer occurs in a high percentage of cells. The limited pattern of gene expression in parenchymal tissues would be favorable for the purposes of treating SP-B deficiency, yet because conducting airways generally did not show transferred gene expression, recombinant adenovirus may not be the optimal vector for treating CF. With further improvements in reducing the immunogenicity of recombinant adenovirus and further analysis of the optimal timing of vector delivery during gestation, prolonged gene expression may be obtainable [Yang EY, et al. 1999b].

Time of administration

Accessing the respiratory epithelium prior to acquisition of a fully differentiated phenotype, that has proven resistant to virus-mediated transduction, and the possibility of successfully targeting epithelial progenitor cells with integrating vector systems such that durable therapeutic benefit results [Waddington SN, et al. 2004a]. Gene delivery via the airway lumen becomes theoretically possible from the beginning of the pseudoglandular stage of fetal lung development, occurring between 5 and 17 weeks of gestation in humans (term 40 weeks), but is more realistically achievable during the subsequent canalicular phase, which extends through to week 26 of gestation [Jeffrey PK 1998].

During the pseudoglandular stage airway branching is completed and epithelial differentiation and maturation begins in the proximal airway and progresses distally, with the upper lung lobes developing slightly ahead of the lower lobes. In the monkey the pseudoglandular stage occurs between 55 and 110 days (term 165 days) and the canalicular stage between 110 and 138 days gestation; in the fetal lamb, where lung development has been well characterised and shown to parallel that of the human fetus, the pseudoglandular stage of lung development occurs between 40 and 80 days of gestation (term 147 days) [Alcorn DG, et al. 1981].

Experimental models

Fetal gene transfer studies on rodents take advantage of the wide range of disease models, short gestation time and the need for relatively small amounts of vector. However, it is important to translate these studies to larger animal models if the goal of human fetal gene therapy is to be achieved. Larger animal models are valuable since they are more relevant to humans in terms of gestation time, maturation of the fetal immune system, and fetal mass as well as in relation to the vector application technology. The importance of a nonhuman primate model for these studies is related to the similarity of rhesus monkeys to humans developmentally and anatomically. For example, the fetal monkey lung passes through the same stages of development at similar gestational time points compared to the human fetus, and during prenatal and postnatal life, the human and nonhuman primate lung are still developing [Fannuchi MV and Plopper CG 1997]. In addition, human and nonhuman primates share a variety of pulmonary cell phenotypes not found in other species, and the overall structure of the monkey lung is more similar to that in the human than any other mammalian species [Plopper CG and Hyde DM 1992]. It is likely that non-human primates will be the ultimate animal model that will be used for safety studies in the immediate preparation for a clinical trial of fetal gene therapy. However, the high maintenance costs and breeding conditions prohibit their use in the routine development of novel injection techniques. Sheep are much easier to breed and maintain and are a well established animal model of human fetal physiology.

Table 4 - *In utero* lung gene transfer studies' synopsis.

Route	Type vector	Development stage	Targeted compartment	References and experimental model
Intrapulmonary (US)	Lentiv	Embryonic	Airway epitehlium	Tarantal 2001 (Rhesus monkey)
		Pseudogland and early Canalic	Airway epithelium (type I and II cells), endothelium, macrophages	Tarantal 2001 and 2005 (Rhesus monkey)
Intratracheal	Adv	Canalic to Saccular	Trachea and bronchial epithelium (including type II cells)	David 2003, 2006, Peebles 2004 (Transduction enhancers), Sylvester 1997 (TO); sheep.
	Lentiv	Saccular	Bronchiolar airway	Yu 2007 (HIV; sheep)
	Oncoretro	Saccular	Airway and respiratory epithelium; submucosal space	Pitt 1995 (MLV; sheep)
	Cationic lipossome	Canalic	Trachea and bronchial epithelium; few mesenchymal cells	Luton 2004 (sheep; TO)
Intraamniotic	Adv	Embryonic	No significant expression	Douar 1997 (mouse), Buckley 2005 (mouse)
		Pseudogland	Low levels of expression	McCray 1995 (mouse)
			Trachea and bronchioles epithelium	Buckley 2005 (mouse)
			Airway epithelium (except trachea and alveoli)	Douar 1997 (mouse), Sekhon and Larson 1995 (rat), Cohen 1998 (mouse) Morrow 1998 (rat; CFTR); Cohen and Larson 2005, 2006 (rat; CFTR/ASCFTR; NI-CDH, TIUKO)
			Some cells in the parenchyma surrounding small airways	Cohen 2008 (mouse; CFTR)
		Canalic	Trachea, epithelial cells (including type I and II cells), airway	Cohen 2008 (mouse; CFTR) Larson 2000 (Rhesus monkey; CFTR)
		Saccular	Trachea, airway epithelium (small bronchus and bronchiolus) and alveoli	Holzinger 1995 (sheep), McCray 1995 (sheep; CFTR; TO)
	AAV	Canalic	Pulmonary epithelium including type I and II cells	Garrett 2003 (mouse, rat and Rhesus monkey)
		Saccular	Trachea, pulmonary epithelium including type II cells	Boyle 2001 (rabbit)
	Lentiv	Pseudogland	Airway epithelium	Buckley 2008 (HIV; mouse)
Intravenous (yolk sac vessels)	Adv	Embryonic Pseudogland Saccular	Epithelium; Endothelium	Schachtner 1999 (mouse)
	AAV	Pseudogland	(Lung)	Bilbao 2005 (mouse)
	Lentiv	Pseudogland	Interstitial cells	Waddington 2003 (EIAV; mouse)
Intraperitoneal	AAV	Pseudogland	Airway	Lipshutz 2001, Bilbao 2005 (mouse)
	Oncoretro	Canalic to Saccular	Epithelium, fibroblasts, macrophages of alveoli, SMC	Porada 2005 (MSLV; sheep)

AAV- adeno-associated viral vector; Adv- Adenoviral vector; ASCFTR- Antisense CFTR; Canalic- canalicular stage of lung development; CFTR- cystic fibrosis transmembrane receptor; EIAV- equine infectious anemia virus; HIV- human immunodeficiency virus; Lentiv- Lentiviral vector; MLV- murine leukemia virus; NI-CDH- Nitrofen-induced Congenital Diaphragmatic Hernia; Oncoretro- oncoretroviral vector; Pseudogland- pseudoglandular stage of lung development; SMC- smooth muscle cells; TIUKO- transient *in utero* knockout; TO- tracheal occlusion; US- ultrasound guided.

Aims

Unique therapeutic opportunities occur during fetal life, related with normal developmental events, which favor prenatal over postnatal treatment. These opportunities create a compelling rationale for the prenatal treatment of pulmonary fetal, pediatric and even adult disorders, with potentially less morbidity and mortality than that associated with currently available portnatal treatments. The combination of advances in maternal screening for fetal diseases, fetal imaging, molecular diagnosis and gene chip technology shift the diagnosis window, of most anatomic and genetic diseases, for early gestation. Fetal treatment offers many advantages: developmental plasticity allow restoration of function after genetic correction; immature immune system allow the development of tolerance to the vector and transgenic protein; higher frequency and more accessible populations of stem cells and progenitor cells; high vector/cell ratio reduces the amount of vector to be administred.

There are a number of genetic and acquired disorders with peri or postnatal pulmonary manifestations. These include monogenetic diseases like cystic fibrosis or surfactant protein B deficiency, congenital diaphragmatic hernia, congenital cystic adenomatoid malformation, respiratory distress syndrome of prematurity that could potentially benefit from prenatal strategies. Rather than consider gene replacement therapy to monogenetic diseases, we intented to modulate lung growth and maturation, envisaging therapeutic applications in diseases like CDH or respiratory distress syndrome of prematurity. Congenital diaphragmatic hernia (CDH) is a malformation that remains with high mortality mainly due to fetal lung hypoplasia and pulmonary hypertension, despite sophisticated postnatal clinical approach. Now, it's possible to stratify the fetuses into low- and high-risk groups and offer antenatal therapy for the later ones, in order to promote prenatal lung growth and/or modulate pulmonary hypertension.

The developing lung has been considered an attractive target organ for fetal gene transfer, however to achieve our purposes we needed to develop a new method that selectively target the fetal lung early in gestation. Several issues should be addressed: route of administration, type of vector, safety and vector biodistribution, gene transfer efficiency and kinetics (transient or sustained), cellular target. Following the establishment of a new model of intrapulmonary in utero gene transfer, it was our intent to modulate lung development acting upon key players envisaging a potential induction of growth in hypoplastic lungs and/or maturation in abnormally immature lungs.

Thus, the specific aims of this study are:

1. To develop a new approach to fetal lung gene therapy

   1.1. To selective target rat fetal lung using ultrasound guided microinjections

   1.2. To compare different viral vectors for fetal lung gene therapy

2. To study the effects of FGF10 gene transfer in modulation of fetal lung growth

   2.1. To induce over-expression of FGF10 by adenoviral gene transfer

   2.2. To characterize the effects of FGF-10 on fetal lung growth

   2.3 To clarify the regulatory mechanisms by which FGF-10 acts on fetal lung grow**th**

CHAPTER 2

TARGETED FETAL LUNG GENE TRANSFER

original article

Targeted Gene Transfer to Fetal Rat Lung Interstitium by Ultrasound-guided Intrapulmonary Injection

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In utero gene transfer to the developing lung may have clinical or research applications. In this study, we developed a new method for specifically targeting the fetal rat lung with adeno and lentiviral vectors encoding the enhanced green fluorescence protein (EGFP) marker gene at E15.5 using ultrasound biomicroscopy (UBM). Survival rate, morphometric parameters, viral biodistribution, and lung transduction efficiency were analyzed and compared to the intra-amniotic route of administration. Expression of EGFP started as early as 24 and 72 h after the injection of adenoviral and lentiviral vectors, respectively. Both vectors transduced lung parenchyma with gene expression limited to interstitial cells of the injected region, in contrast to intra-amniotic injection, which targeted the pulmonary epithelium. Expression of EGFP was most intense at E18.5 and E21.5 for adenoviral and lentiviral vectors, respectively. In contrast to lentivirus, adenoviral expression significantly declined until final analysis at 1 week of age. This study demonstrates the feasibility of targeting the fetal rat lung interstitium with viral vectors under UBM guidance during the pseudoglandular stage. This model system may facilitate *in vivo* studies of dynamic lung morphogenesis and could provide insight into the efficacy of prenatal gene transfer strategies for treatment of specific lung disorders.

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INTRODUCTION

Prenatal gene transfer may offer a number of unique biological advantages relative to postnatal gene transfer.[1–4] The relatively high frequency and accessibility of stem cells and progenitors and their rapid proliferation in the fetus may offer efficiency advantages for stem-cell-targeted gene transfer. The low total cell number in the fetus allows relatively high vector to target cell ratios, allowing the use of small amounts of vector. The immunologic immaturity of the early gestational fetus may induce tolerance to vector-associated or transgene-encoded proteins. Finally, *in utero* gene transfer has the potential to treat a disease before its clinical manifestations. Advances in prenatal diagnosis of genetic and congenital disorders with progressively more sensitive techniques may increase opportunities for consideration of prenatal gene therapy.[5,6]

The fetal lung is an attractive target organ for fetal gene transfer. There are a number of genetic and acquired disorders with peri- or postnatal pulmonary manifestations. These include monogenetic diseases like cystic fibrosis[7] or surfactant protein B (SP-B) deficiency that would presumably require long-term expression of the deficient or defective gene. However, there are also abnormalities of lung growth, such as congenital diaphragmatic hernia, or lung maturation, such as respiratory distress syndrome of prematurity, that could potentially benefit from strategies that achieve transient gene expression in specific pulmonary distributions. Additionally, an important advantage of the use of non-integrating viral vectors with transient gene expression is the avoidance of many of the current concerns regarding integrating vectors in the fetus, *i.e.*, insertional mutagenesis, developmental abnormalities, and germline alteration. We therefore anticipate the future need for a variety of lung-targeting strategies using different gene transfer technologies to achieve optimal therapeutic results.

In this study, we describe a new method for the direct injection of viral vectors into the rat fetal lung as early as the pseudoglandular phase of lung development. Using this technique, we assessed the efficiency and distribution of transduction in the lung, the biodistribution of transduction, and the toxicity of equine infectious anemia virus (EIAV) and adenovirus vectors expressing the enhanced green fluorescence protein (EGFP) marker gene after *in utero* injection. In addition, we compare the direct injection technique to intra-amniotic

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injection with respect to the efficiency of lung transduction, and organ, and cellular distribution.

RESULTS

The technique of ultrasound-guided injections

Ultrasound biomicroscopy (UBM) was used to scan the fetus to identify the lung (**Figure 1**). E15.5 was the earliest stage that we could perform *in utero* injections with a high survival rate and with accurate targeting of the developing lung. At E13.5 and E14.5, the lung bud was very difficult to visualize and mortality was unacceptably high. Important technical components of this procedure were the performance of a maternal laparotomy to expose the gravid uterus, and the application of pre-warmed sterile ultrasound gel over the uterus. This allowed manipulation of the fetal position into the axial orientation, which we found was the optimal plane for injection. The lung was best visualized between the level of the cardiac apex and upper part of the liver. Applying this imaging technique, we could inject small volumes (25–350 nL) of a viral vector suspension into the fetal lung parenchyma without any visually apparent injury to the lung or fetus. Injection of larger volumes was associated with the disruption of fetal lung architecture. The fetus and micropipette were manipulated so that the tip of the micropipette traversed only the uterine wall, amniotic membrane, amniotic cavity, fetal skin, and thoracic cavity (**Figure 1** and **Supplementary Video S1**), avoiding the placenta, limbs, heart, or liver.

Safety of *in utero* gene transfer

Viral vectors used in this study were first-generation adenoviral vector and second-generation EIAV. To determine whether there was inherent toxicity from the viral preparation or from the early expression of the transgene, we compared survival after the intrapulmonary injection of viral vectors with injection of an equivalent volume of phosphate-buffered saline (PBS). The overall survival rate was 81±6, 82±5, and 87±4% for the PBS, EIAV, and adeno groups, respectively. Survival rates were calculated per dam by the formula: (number of injected fetuses alive/total number of injected fetuses per dam) × 100. There was no statistical difference among the groups at the studied time points (**Table 1**).

In total, 53 dams were used and 527 fetuses were injected. There were no maternal deaths. There was no evidence of transplacental transfer of vector or transfected cells when maternal blood was analyzed for EGFP by flow cytometry (data not shown), although maternal tissues were not analyzed in detail. Fetuses delivered by cesarean section or at term were viable and without gross anomalies. Morphometric parameters, namely body weight, lung-to-body and heart-to-body weight ratios were similar and there were no statistical differences among the groups (**Table 1**). Macroscopic structure of the lung lobes, diaphragm, heart, and thoracic cavity was normal. Pups

Table 1 Survival and somatic growth

	PBS	EIAV	Adeno
E16.5			
Survival (%)	85±9	82±7	78±12
Body weight (g)	0.49±0.02	0.53±0.01	0.51±0.02
Lung weight (mg)	1.82±0.08	1.98±0.04	1.78±0.14
Heart weight (mg)	0.94±0.07	0.86±0.05	0.98±0.8
E18.5			
Survival (%)	94±5	87±6	89±1
Body weight (g)	1.60±0.04	1.74±0.10	1.52±0.09
Lung weight (mg)	3.19±0.10	3.27±0.12	3.23±0.12
Heart weight (mg)	0.75±0.04	0.62±0.02	0.72±0.05
E21.5			
Survival (%)	72±16	85±10	96±4
Body weight (g)	6.10±0.24	5.85±0.13	5.35±0.10
Lung weight (mg)	2.50±0.10	2.56±0.10	2.66±0.09
Heart weight (mg)	0.76±0.06	0.69±0.06	0.77±0.02
P7			
Survival (%)	72±11	82±12	82±7
Body weight (g)	15.19±0.13	14.00±0.68	16.43±0.64
Lung weight (mg)	2.26±0.05	2.15±0.08	2.29±0.06
Heart weight (mg)	0.67±0.01	0.76±0.02	0.70±0.02

EIAV, equine infectious anemia virus; PBS, phosphate-buffered saline. Survival is presented as mean±SE of survival rate per dam calculated by the formula: number of injected fetuses alive/total number of injected fetuses per dam × 100.

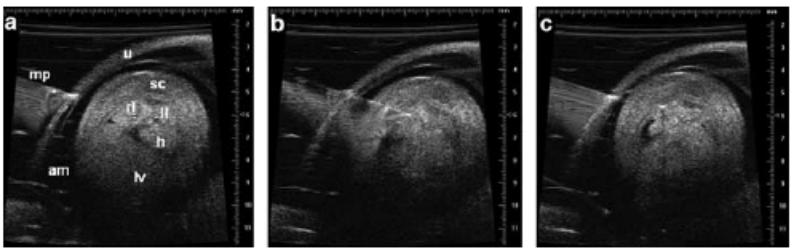

Figure 1 Fetal lung injections guided by UBM. (**a**) The micropipette (mp) is aligned to target the fetal right lung (rl). (**b**) The micropipette is advanced through uterine wall (u), and amniotic cavity (am) into the fetal lung; a small volume of viral vector suspension is injected into the pulmonary parenchyma. Other organs can be clearly visualized: heart (h), liver (lv), left lung (ll), and spinal cord (sc). (**c**) After injection, the micropipette is removed.

that survived until the seventh day after birth displayed overtly normal development and activity levels.

Efficiency of gene transfer to the lung and kinetics of gene expression

To determine the overall pattern of gene expression, we used stereoscopic fluorescence microscopy. After intrapulmonary injections, we observed that expression of EGFP, with the volumes of injectate utilized, was almost entirely limited to the injected lung and the ipsilateral pleural space after intrapulmonary injection (**Table 2** and **Figure 2**). The time course of EGFP expression differed for the two vectors. EGFP expression was detected as early as 24 h after injection (E16.5) for adenoviral vector whereas the first expression did not appear until 72 h after injection for the EIAV-derived lentiviral vector (**Figure 2**). Maximum expression was observed at E16.5–E18.5 for adenovirus and at E21.5–P7 for lentivirus. For adenovirus, the intensity of lung EGFP fluorescence gradually decreased until P7 when it was barely discernable. For EIAV at P7, the intensity of the fluorescence was maintained, but the area of fluorescence was smaller presumably owing to the growth of the non-transduced lung and the relatively small area of initial transduction. In the adenoviral group, expression of EGFP was also seen in some fetuses in the skin and eyes, whereas in both groups the contralateral non-injected lung was positive in a few fetuses (**Table 2**).

Table 2 Viral vector biodistribution by fluorescence stereomicroscopy

	E16.5		E18.5		E21.5		P7	
	EIAV	Adeno	EIAV	Adeno	EIAV	Adeno	EIAV	Adeno
n injected	14	20	31	24	22	26	19	23
Lung injected[a]								
–	7	1	2	1	1	0	1	0
+	5	1	18	3	11	11	5	12
++	1	8	8	10	8	13	10	10
+++	1	10	3	11	2	2	3	1
Lung non-injected	0	2	1	4	4	4	1	0
Brain	0	0	0	0	0	0	0	0
Diaphragm	0	0	0	0	0	0	0	0
Eye	0	6	0	5	0	9	0	2
Gonads	0	0	0	0	0	0	0	0
Heart	0	0	0	1	0	0	0	0
Intestine	0	0	0	0	0	0	0	0
Kidney	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0
Muscle	0	0	0	0	0	0	0	0
Skin	0	2	0	3	0	4	1	0
Spleen	0	0	0	0	0	0	0	0
Stomach	0	0	0	0	0	0	0	0
Trachea	0	0	0	0	0	0	0	0

EGFP, enhanced green fluorescence protein; EIAV, equine infectious anemia virus. [a]Numbers represent the number of injected lungs that were negative or positive for EGFP fluorescence at the time points stated. Positive animals were subjectively ranked on a scale of + to +++ for intensity of fluorescence.

To confirm the fluorescence observations and to provide a better quantitative assessment of changes in gene expression over time in the injected lungs, we performed real-time polymerase chain reaction (PCR) (**Figure 3a**). Evaluation of the number of vector copies per lung revealed the presence of the vector at E16.5 in both EIAV and adenoviral groups. However, the number of vector copies was statistically higher for adenoviral vector at E16.5 and E18.5. Whereas adenoviral-associated copy number progressively decreased after E16.5, copy number from EIAV gene transfer increased after E18.5 and was statistically greater than adenovirus at P7. It is important to note that this is not a direct comparison of transduction efficiency between the two viral vectors owing to the differing volumes and titers injected. Approximately 25 nL of adenoviral vector at a titer of $2\text{–}8 \times 10^{11}$ i.p./mL was injected versus 350 nL of EIAV vector at a titer of 10^8–10^9 i.p./mL. Thus, even at the extremes of volume allowed by the model, there was at least a 100-fold greater dose of adenoviral vector injected. However, this quantitative data confirm the fluorescence observation of the rapid diminution in EGFP expression for adenoviral-transduced lungs over the interval between E16.5 and P7. In contrast, copy numbers, although relatively low, were maintained in the EIAV group throughout the duration of the study.

Biodistribution of viral vectors

To confirm our impression that EGFP gene expression was relatively limited to the lung after intraparenchymal injection and to more globally assessed gene expression in the injected fetuses, we performed quantitative PCR on several tissues at E21.5 (**Figure 3b**). A statistically higher number of vector copies relative to PBS controls were observed in the heart and in the tract of the micropipette through the thoracic wall in both the EIAV-EGFP and Adeno-EGFP groups. For all other tissues analyzed, there were no statistical differences in EGFP expression above the PBS-injected controls.

Cellular distribution of gene transduction within the lung

EGFP-positive cells were predominantly found in the pulmonary interstitium for both the EIAV and adenoviral groups, after intrapulmonary injections (**Figure 2i–p**). There was no expression of reporter gene in the surface epithelium of airways or in the vascular endothelium. EGFP-positive cells neither stained for vimentin, a mesenchymal-derived cell marker (**Figure 4a** and **b**), nor for SP-B, a surfactant protein expressed in Type II pneumocytes (**Figure 4c** and **d** and **Supplementary Figure S2**). Therefore, we demonstrated that transduced cells were located within the interstitial compartment of the lung and that they were not epithelial. This is in distinct contrast to the epithelial-restricted expression seen with intra-amniotic vector injections (**Figure 4e** and **f**).

DISCUSSION

We report for the first time, the feasibility of using UBM to perform *in utero* intrapulmonary injections in rats at the pseudoglandular stage of lung development. Both adenoviral and lentiviral vectors efficiently transduced lung parenchyma, although different expression patterns were observed following

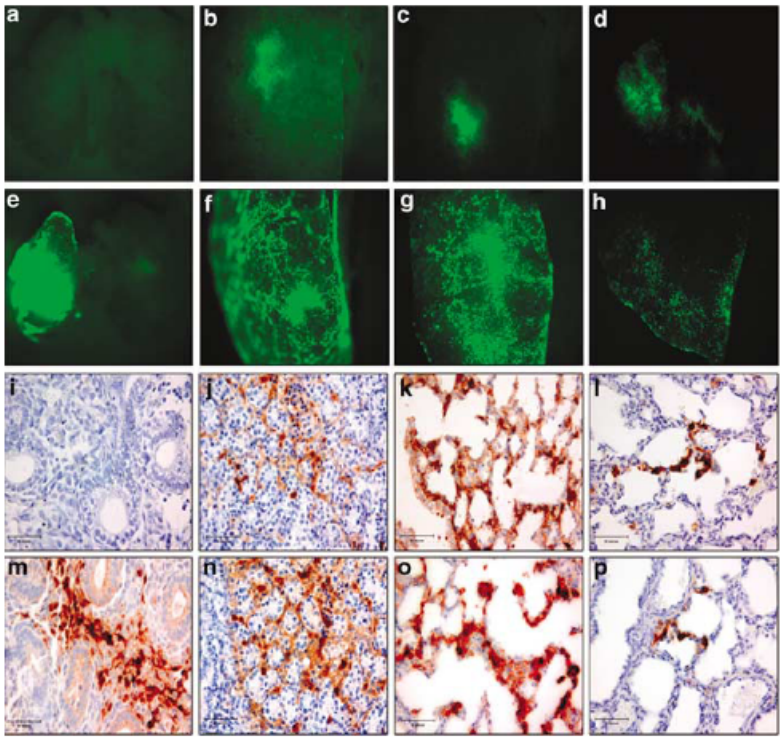

Figure 2 Fluorescence stereomicroscopy and lung immunostaining for EGFP. Injected fetuses at E15.5 were analyzed at E16.5, E18.5, E21.5, and P7. Representative examples of fluorescence stereomicroscopy of the lungs at each analysis time after injection with (**a–d**) lentiviral vector and (**e–h**) adenoviral vector are shown. Viral vector transduction can be detected by green fluorescence in an area around the location of the injection. Representative images of immunostaining for EGFP from the lungs injected with (**i–l**) lentiviral vector and (**m–p**) adenoviral vector at the same analysis times are presented. (**i–l**) Lentiviral vector was only detected at (**j**) E18.5, with more EGFP expression being detected at (**k**) E21.5 and maintained until (**l**) P7. (**m–p**) Adenoviral vector induced an earlier expression of EGFP with maximal expression at (**m, n**) E16.5–E18.5. EGFP expression was analyzed by immunoperoxidase (brown staining) and both vectors were expressed in interstitial cells. No epithelial expression was detected. Bar = 50 μm.

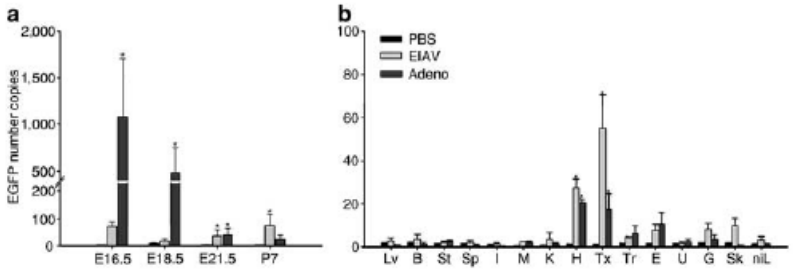

Figure 3 Quantitative PCR for EGFP. (**a**) Number of vector copies detected by quantitative PCR in lung samples after PBS, adenoviral, and lentiviral injections, at different time points of gestation. (**b**) Number of vector copies detected by quantitative PCR in non-pulmonary tissues and non-injected lung samples after PBS, adenoviral, and lentiviral injections at E21.5. Lv, liver; B, brain; St, stomach; Sp, spleen; I, intestine; M, muscle; K, kidney; H, heart; Tx, thorax; Tr, trachea; D, diaphragm; E, eye; G, gonads; Sk, skin; niL, non-injected lung of injected fetuses. *Significant differences = $P \leqslant 0.05$ compared to PBS control.

injection. As expected, adenoviral vector expression appeared quickly and was transient, whereas lentiviral vector expression was relatively delayed and persisted through the time period of this study. Unexpectedly, in contrast to intra-amniotic injections, both vectors selectively transduced interstitial cells and not alveolar or airway epithelial cells, or vascular endothelial cells.

Several routes of vector administration have been utilized to achieve prenatal gene transfer to the lung including intra-amniotic,[8–11] systemic,[12–14] intratracheal,[15–17] and intrapulmonary.[18,19] Intra-amniotic and systemic approaches share the same limitations of being nonspecific for fetal lung. Intratracheal and intrapulmonary delivery, although more specific to the lung,

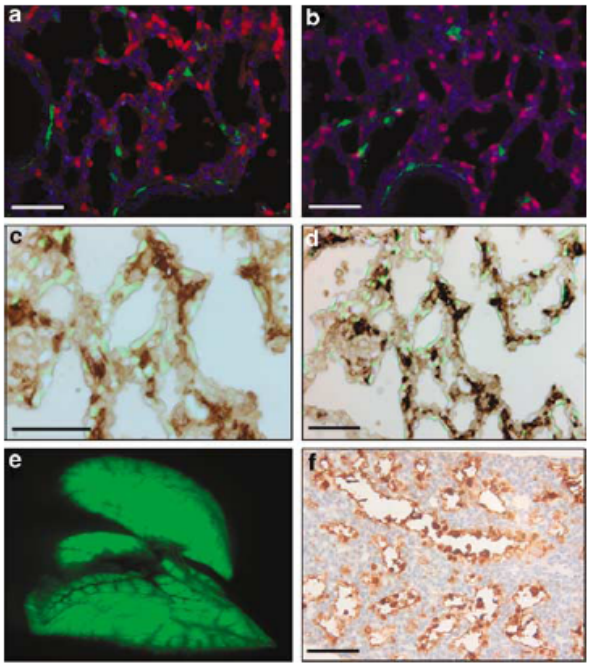

Figure 4 Interstitial localization of tranduced cells after intraparenchymal injection. (**a**, **b**) Lung double immunofluorescence for EGFP (green) and vimentin (red) in fetuses injected with (**a**) lentiviral and (**b**) adenoviral vectors. Lungs were harvested at P7. (**c**, **d**) Lung immunoperoxidase staining for EGFP (brown) and immunofluorescence for SP-B (green). Images represent an overlay of bright light and fluorescent images at original magnification × 60 and × 40 for **c** and **d**, respectively. Merged images were created with Adobe Photoshop software and adjusted for brightness, contrast, and opacification to allow optimal visualization of both markers (original images before manipulation can be seen in **Supplementary Figure S2**). EGFP-positive cells did not colocalize with vimentin, or SP-B, and were localized within the interstitial region of the parenchyma. (**e**, **f**) Epithelial localization of transduced cells after intra-amniotic injection. Intra-amniotic injections were performed at E16.5 and the fetuses were harvested at E21.5. (**e**) Fluorescence stereomicroscopy and (**f**) lung immunostaining for EGFP are presented demonstrating the transduction of only epithelial cells. Bar = 50 μm.

have previously only been performed in large animal models. Tarantal *et al.*[18] demonstrated, in a non-human primate model, that intrapulmonary injections during the pseudoglandular stage allowed specific targeting of the lung relative to injections performed during the embryonic stage. In this study, we used UBM to perform *in utero* intrapulmonary injections. The use of this relatively new imaging technology made injection of the fetal rat lung at E15.5. UBM utilizes high-frequency (20–100 MHz), pulse-echo ultrasound for imaging live tissues and organs, allowing near microscopic resolution and has been previously utilized for fetal brain injections.[20,21] Using UBM, we could successfully target the rat lung during the mid-point of the pseudoglandular stage of lung development with a high fetal survival rate.

First-generation adenovirus and EIAV lentivirus-based vectors were used in this study. We chose to study first-generation adenoviral vectors rather than adeno-associated viral vectors because we wished to see rapid, high-level expression of the marker gene. We used EIAV owing to the fact that human immunodeficiency virus-1-based lentiviral vectors have not shown high efficiency transduction[22,23] in many tissues and owing to the successful application of EAIV vectors in fetal rodent models by Waddington *et al.*[14] Adenovirus has been one of the most extensively studied recombinant viral systems because of its high transduction efficiency, rapid expression, accommodation of large transgene inserts, and high titers.[23–26] The primary problem with adenovirus is its high immunogenicity and propensity to invoke strong immune responses. We previously documented that this is a problem with intratracheal administration in late gestational fetal lambs; however, in the same model, no significant inflammation is observed with adenoviral administration during the preimmune phase of lamb immunologic development.[27] Similarly, as we would predict from the stage of rat immune development at E15.5, we saw no overt inflammatory response in the lung by histology in these experiments. However, as the purpose of this study was not induction of immune tolerance, we did not perform a detailed analysis of immune response to adenoviral products or transgene. In contrast to adenovirus, lentiviral vectors are relatively non-toxic and minimally immunogenic and can stably integrate transgene into dividing and non-dividing cells with subsequent long-term gene expression.[28–30] The main disadvantages are the low titers that are usually obtained *in vitro*.[23] This proved somewhat limiting in the current study. With EIAV vector titers of only 10^7–10^8 infectious particles per mL, we needed to inject the maximal tolerated volume of 350 nL of EIAV to achieve significant transduction.

The rapid loss of EGFP expression in the adenoviral-transduced lungs is in keeping with the known episomal location of adenoviral gene expression, the small volume of vector administered (25 nL), and the rapid proliferation of fetal lung tissue. Our data are also consistent with the known capacity of EIAV to stably integrate transgene into the host genome. In the EIAV-transduced lungs, there was, if anything, a slight increase in copy number at P7. We would conclude from this analysis that adenovirus would be the most appropriate vector to use when the goal is to induce rapid and transient overexpression of a gene in this model, whereas, lentivirus would be a more suitable vector to induce sustained and long-term gene expression.

Gene transfer was localized to the injected lung with the only exceptions being the needle track through the thorax and a very low copy number in the heart. This could be due to inadvertent injection of the heart but is more likely due to the small amount of intravascular injection associated with this technique. A few animals had EGFP expression noted by stereoscopic fluorescent analysis in the skin or eye. However, in the animals selected for PCR analysis, these tissues did not have statistically higher gene copy numbers than PBS controls. This discrepancy likely represents inconsistent amounts of leakage of the vector into the amniotic space during removal of the micropipette. The lack of pulmonary epithelial transduction in these animals despite intra-amniotic leakage is most likely explained by the very minimal volume of leakage into the amniotic space relative to the volume of injectate in the intra-amniotic injection

experiments as well as the timing of the injection. In our experience, and that of others,[8] efficient transduction efficiency of pulmonary epithelium after the intra-amniotic delivery of vector is limited to a narrow gestational window related to the onset of fetal respiratory movements. In this study, intrapulmonary injections were performed 1 day before the time point when efficient transduction to pulmonary epithelium is seen after intra-amniotic injection.

One of the most interesting findings in our study was the specificity for the interstitial compartment seen with both the vectors utilized. There was no expression of reporter gene in the surface epithelium of airways or in the vascular endothelium. The obvious question is, what population of cells was transduced? Unfortunately, we were not able to definitively identify the cells in this study owing to a lack of specific markers for cell types in the interstitium. Although we suspected that the cells were mesenchymal in origin, they do not stain with vimentin, a common mesenchymal and fibroblast marker. Attempts to colocalize EGFP staining with SP-B, a surfactant protein expressed in Type II pneumocytes, confirmed that the transduced cells were not epithelial, and that they were located within the interstitial compartment of the lung. This is in distinct contrast to the epithelial restricted expression seen with intra-amniotic vector injections. Although the obvious explanation is the route of injection, the needle tip must traverse all layers of lung parenchyma on insertion and withdrawal and one would expect to see epithelial transduction as well. We do not have an explanation for this observation but it was seen consistent in all lungs examined and with both vectors utilized. In contrast, in the two previous studies of direct lung transduction in primates using human immunodeficiency virus-1 lentiviral vectors,[18–19] only epithelial expression was described, so this appears to be a model-dependent observation. Further characterization of the type of cells that were transduced will require additional studies. Nevertheless, to our knowledge, this is the first observation of this pattern of parenchymal expression with any reports of prenatal lung gene transfer. The observation of transduction of distinct cell populations within the lung with different routes of transduction raises the possibility of manipulating gene expression in specific and separate cell populations within the developing lung. This may have interesting applications toward understanding mesenchymal epithelial inductive interactions during the pseudoglandular phase of lung development.

The ability to achieve gene transfer by direct intrapulmonary injection in a rat model provides a novel tool for the exploration of potential therapeutic strategies for lung disorders and for biological studies examining the effects of specific genes on lung development. Obvious advantages of this technique include ease of manipulation, minimal expense, and, if translatable to the mouse, the availability of well-characterized murine models of human lung diseases.[31–33] Although this study was performed in rats, we now also have preliminary experience with this approach in murine fetuses with similar success (data not shown). Finally, the possibility of targeting the lung during the pseudoglandular stage may present unique experimental and, ultimately perhaps, clinical opportunities. This stage, characterized by intense branching morphogenesis, is the period of greatest overall growth of the airways and vasculature of the fetal lung, and corresponds to a stage of immunologic immaturity and thymic processing of self-antigen. Therefore, gene transfer during this period has the potential to have major effects on the key elements of lung growth with minimal potential for detrimental immune responses.

MATERIALS AND METHODS

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and followed guidelines set forth in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Viral vector preparation. EIAV-derived lentivirus vector expresses the EGFP reporter gene from an internal cytomegalovirus immediate-early promoter. The packaging plasmid, pEV53, and the SIN EIAV transfer vector[34,35] were kindly provided by Dr Bruce A Bunnell (Tulane University, New Orleans, LA). The plasmid pFL85, containing the EIAV provirus,[36] was kindly provided by Dr Robert Stephens (National Cancer Institute, Frederick, MD). The transfer plasmid, pZEK-CMV-*eGFP*, was reassembled retaining the left and right long terminal repeats and the extended packaging was inserted after the left long terminal repeat.[37] All Woodchuck hepatitis virus sequences were removed and replaced with the modified Woodchuck hepatitis post-transcriptional regulatory element.[38] Viral vector pseudotyped with the vesicular stomatitus virus G-protein (VSV-G) envelope was generated by three plasmid co-transfections in 293T cells as described previously.[29] The initial transfection conditions were: 18 μg pE2KEGFP + 18 μg CMVΔR8.91 + 12 μg VSV-G + 96.9 μL 2 M $CaCl_2$ + 2.5 mM HEPES, pH 7.3 up to a total volume of 780 μL added to 780 μL of 2 × HEPES buffer solution (HeBS) (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.0). The lentivirus vector supernatants were concentrated by ultracentrifugation at 28,000 r.p.m. for 90 min at 4°C (SW-28 rotor, Beckman, Palo Alto, CA). Titers were determined by plaque-forming assay. Vector particle titers obtained range from approximately 1×10^8 to 1×10^9 infectious particles per mL.

The *E1–E3* replication-deficient recombinant adenovirus, based on human serotype 5, contains *eGFP* under the human cytomegalovirus immediate-early promoter (rAd-CMV-*eGFP*) and was obtained from the vector core facility of the Gene Therapy Program (Division of Medical Genetics, University of Pennsylvania, Philadelphia, PA).[13] Recombinant vectors were prepared as previously reported.[39–41] Adenoviral vector was stored at −80°C in PBS with 5% glycerol. Before administration, vector aliquots were resuspended in fresh PBS at a concentration of 2-8 × 10^{11} infectious particles per mL.

Viral vectors administration. *Ultrasound-guided injections.* Time-dated pregnant Sprague-Dawley rats (Charles-River, Wilmington, MA) at 15.5 days *post coitum* (E15.5) were anesthetized with isoflurane (3.5% for induction, 2% for maintenance) and laid supine on a platform. The body temperature was monitored via a rectal probe (Indus Instruments, Houston, TX) and maintained at 36–38°C. The surgical site was chemically depilated and disinfected. A 2-cm ventral midline incision was made and the uterus containing one or two fetuses was partially exposed and covered with a pre-warmed sterile ultrasound gel (Aquasonic, Parker Laboratories, Fairfield, NJ). Fetuses were positioned to obtain axial views of the lungs in B-mode using a 40 MHz probe (VisualSonics Vevo 660, Toronto, Canada). Glass microcapillary pipettes (outer diameter 1.14 mm, inner diameter 0.53 mm, Humagen, Marlton, NJ) were backfilled with mineral oil (Sigma, St Louis, MO), connected to the micropipette holder that was attached to a three-axis micro-injector unit (VisualSonics Vevo 660), filled with the PBS or viral vector

suspension (5 μL), and aligned with the scanhead. Under two-dimensional imaging, the micropipette tip was physically advanced through the uterine wall and amniotic cavity into the lung and a specific volume was injected. The micropipette was then physically retracted and the next fetus was positioned and the procedure was repeated. Between six and 10 fetuses were injected per dam. The maximum time of the surgical procedure (between maternal abdominal incision and its closure) was 60 min. The abdomen was closed and dams recovered in a warming chamber. Fetuses were harvested at E16.5, E18.5, E21.5 (by cesarean), and P7. Fetuses or pups were inspected for the presence of macroscopic abnormalities and body, wet lung, and heart weights were recorded. Tissue samples were collected for histological and PCR analysis.

Intra-amniotic microinjections. Time-dated pregnant Sprague–Dawley rats (Charles-River, Wilmington, MA) at 16.5 days *post coitum* (E16.5) were anesthetized as described above. A midline laparotomy was made and one horn of the uterus was exposed at a time. The injections were performed under direct vision with glass microcapillary pipettes connected to a microinjector. Pipettes were backfilled with adenovirus suspension and 5 μL were injected into the intra-amniotic cavity of each fetus. Maximum time of the surgical procedure was 20 min. After injections, the abdomen was closed in two layers and dams recovered in a warming chamber. Fetuses were harvested at E21.5 (by cesarean) and lungs were collected for analysis.

Fluorescence stereomicroscopy. Injected fetuses were visualized under fluorescence stereomicroscopy (MZ16FA, Leica, Heerburgg, Switzerland) immediately after harvesting, to evaluate EGFP expression, transduction efficiency, and biodistribution of the vector. After removal of the fetus from the amniotic sac, the eyes and skin were inspected for fluorescence. Thoracotomy was performed and the heart and lungs were visualized. The liver, spleen, stomach, intestines, kidneys gonads, brain, and muscles of the lower limbs were also inspected.

Histology and immunohistochemistry. Tissue specimens collected for histology and immunohistochemistry were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. To evaluate and localize EGFP protein in the harvested lungs, 4 μm serial sections were rehydrated and placed in 1% sodium borohydride in PBS. Slides were blocked for specific protein with goat serum (1:10 dilution) and incubated with monoclonal rabbit anti-GFP IgG fraction (1:200 dilution; Molecular Probes, Eugene, OR). Slides were then blocked for peroxidase with Dakocytomation (S-2001; Dako, Carpenteria, CA) followed by incubation with biotinylated goat anti-rabbit IgG (1:200 dilution; Vector Lab PK-4001, Burlingame, CA). Slides were incubated with avidin–biotin complex (1:200 dilution; Vector Lab, Burlingame, CA) developed with the peroxidase substrate kit (SK-4100; Vector Lab, Burlingame, CA) and lightly stained with Harris hematoxylin. For double immunofluorescence of GFP and vimentin, slides were blocked with horse serum (1:10 dilution) followed by incubation with monoclonal mouse anti-vimentin, Clone V9, M0725 (1:50 dilution; Dako, CA) at 4°C and incubation with secondary antibody Alexa Fluor A555 goat anti-mouse (1:100 dilution; Molecular Probes, Eugene, OR). Slides were blocked with goat serum (1:10 dilution) followed by incubation with monoclonal rabbit anti-GFP IgG fraction (1:200 dilution; Molecular Probes, Eugene, OR) at 4°C and incubation with secondary antibody Alexa Fluor A488 (1:200 dilution; Molecular probes, Eugene, OR). Sections were coversliped with 4′,6′-diamidino-2-phenylindole (Molecular Probes, Eugene, OR), visualized under the microscope (Leica, DMRBE) to analyze the distribution of GFP and the colocalization of GFP and vimentin (Iplab, Scientific Imaging Software, BD Biosciences Bioimaging, Rockville, MD). Double staining for GFP (immunoperoxidase) and SP-B (immunofluorescence) was performed using the techniques described above. For SP-B, slides were incubated with primary antibody (1:200 dilution, Chemicon, Temecula, CA) followed by incubation with secondary antibody Alexa Fluor A488 goat anti-rabbit (1:100 dilution, Molecular Probes, Eugene, OR). Bright light microscopic and fluorescent images were then merged to produce the images seen in **Figure 4c** and **d**.

Real-time PCR analysis. Samples from the injected lungs were collected at E16.5, E18.5, E21.5, and P7. Extrapulmonary tissues were collected at E21.5 and included the brain, diaphragm, eye, gonads, heart, intestine, kidney, liver, muscle, skin, spleen, stomach, and trachea. All tissues collected for PCR were immediately frozen in liquid nitrogen and stored at −80°C. Genomic DNA was isolated from tissues using the DNeasy Tissue kit (Qiagen, Valencia, CA). Real-time PCR assays were performed on the ABI PRISM 7900s Sequence Detection System. Sequence data were obtained from the GenBank accession nos. U55763 for the cloning vector pEGFP-C1 and NC005111 for *Rattus norvegicus* β-actin region of chromosome 12. The internal probes of both TaqMan systems were labelled at the 5′ end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxytetramethylrhodamine) (IDT, Coralville, IA). The primers and probe for the pEGFP-C1 were as follows: forward 5′-GGGCACAAGCTGGAGTACAACT-3′; reverse 5′-TCTGCTTGTCGGCCATGA-3′; probe: 5′-FAM-ACAGCCACAACGTCT-TAMsp-3′. The amplicon size was 61 bp. The β-actin TaqMan system originated an amplicon with 92 bp and consisted of primers and probe as follows: forward 5′-GTATTCCTTTCTCTACAGATCATG-3′; reverse 5′-CCAGAGGCATACAGGGACAAC-3′; probe: 5′-FAM-AGCCATGTACGTAGCCATCCAGGCTG-TAMsp-3′. Amplification conditions were identical for all reactions: 2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The amplification reactions were set in a final volume of 20 μL, containing 1 × TaqMan Universal PCR Mastermix (Applied Biosystems), 50 ng of the gDNA sample, 900 nM of each primer, and 250 nM of the respective probe. Negative control was included in all the runs. Standard amplification curve for *eGFP* was made with pEGFP-C1, serially diluted from 2×10^4 to 0.2 copies/μL. In all the samples, the calculated pEGFP concentration was normalized for β-actin as internal control. For each experimental group studied, three samples were analyzed in triplicate. Amplification data were analyzed by the sequence detection system software (SDS 1.2 version, Applied Biosystems).

Statistical analysis of data. The results were presented as mean ± SEM. To ascertain the statistical significance of the differences in the levels of transduction in each tissue at different time points, one-way analysis of variance was performed. Differences between groups were considered to be statistically significant when $P < 0.05$.

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SUPPLEMENTARY MATERIAL

Video S1. Fetal lung injections guided by UBM.
Figure S2. Fluorescent and bright light images of the merged images in Figure 4c and d.

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CHAPTER 3

MODULATION OF LUNG DEVELOPMENT BY FGF10 GENE TRANSFER

Cystic Adenomatoid Malformations Are Induced by Localized FGF10 Overexpression in Fetal Rat Lung

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Fibroblast growth factor-10 (FGF10) is a mesenchymal growth factor, involved in epithelial and mesenchymal interactions during lung branching morphogenesis. In the present work, FGF10 overexpression was transiently induced in a temporally and spatially restricted manner, during the pseudoglandular or canalicular stages of rat lung development, by trans-uterine ultrasound-guided intraparenchymal microinjections of adenoviral vector encoding the *rfgf10* transgene. The morphologic and histologic classification of the resulting malformations were dependent upon developmental stage and location. Overexpression of FGF10 restricted to the proximal tracheobronchial tree during the pseudoglandular phase resulted in large cysts lined by tall columnar epithelium composed primarily of Clara cells with a paucity of Type II pneumocytes, resembling bronchiolar type epithelium. In contrast, FGF10 overexpression in the distal lung parenchyma during the canalicular phase resulted in small cysts lined by cuboidal epithelial cells composed of primarily Type II pneumocytes resembling acinar epithelial differentiation. The cystic malformations induced by FGF10 overexpression appear to closely recapitulate the morphology and histology of the spectrum of human congenital cystic adenomatoid malformation (CCAM). These findings support a role for FGF10 in the induction of human CCAM and provide further mechanistic insight into the role of FGF10 in normal and abnormal lung development.

Keywords: fibroblast growth factor-10; congenital cystic adenomatoid malformation; adenoviral vector; lung development; gene transfer

CLINICAL RELEVANCE

Our results demonstrate that overexpression of a single gene induces cystic lung lesions resembling the entire spectrum of congenital cystic adenomatoid malformation (CCAM). These results provide unique insight into developmental mechanisms that may contribute to CCAM formation.

Fibroblast growth factors (FGFs) comprise a family of potent mitogens that regulate cellular proliferation, migration, and differentiation (1, 2). Two members of the FGF family, FGF7 and FGF10, are expressed in lung mesenchymal cells in distinct spatial and temporal patterns. FGF7 expression begins at Embryonic Day (E)14.5 and is expressed throughout the mesenchyme surrounding the developing lung tubules (3, 4). FGF10 expression is initiated earlier at the onset of lung organogenesis and is restricted to the mesenchyme surrounding the distal tips of the branching tubules (5). Both FGF7 and FGF10 bind with high affinity to the same FGF receptor isoform, FGFR2b (6, 7). FGFR2 expression is restricted to the epithelial cells of the developing lung at the onset of lung organogenesis at E9.5, and moves more peripherally in epithelial distribution with ongoing lung development (7). These distinct patterns of expression of FGF7 and FGF10 in lung mesenchyme and the simultaneous expression of FGFR2 in respiratory epithelial cells implicate a primary role for FGF mesenchymal/epithelial signaling in lung morphogenesis. The control of the bud size and shape during branching is achieved by an intricate exchange of signals between the growing bud and the surrounding mesenchyme. As the bud elongates, FGF10/FGFR2 signaling induces expression of Spry2 and Bmp4 in the distal epithelium. Spry2 is one of the earliest factors to be induced and limits the proliferation of the lung epithelium (8, 9). Bmp4 disrupts distal budding when expression is activated via an autocrine mechanism (10, 11).

Much of what is currently known about the role of the FGFs and other individual mediators of lung morphogenesis has been derived from analysis of phenotypes induced by either targeted disruption, or ectopic transgenic overexpression of the candidate genes. Targeted disruption of *fgf10* results in absence of lung structures distal to the mainstem bronchi, and this phenotype is replicated in *fgfr2b* knockout mice, confirming a critical role for this signaling pathway in lung development (12, 13). In contrast, mice with targeted disruption of *fgf7* have normal lungs (14), indicating that this polypeptide is not required for normal lung morphogenesis or that the FGF peptides have redundant functions. Ectopic overexpression of either factor in respiratory epithelium using epithelium specific promoter genes results in marked perturbations of lung morphogenesis. FGF7 overexpression is uniformly lethal by E16, inducing massive cysts of the pulmonary parenchyma reminiscent of the macrocystic form of cystic adenomatoid malformation (15). Conditional ectopic overexpression of FGF10 in the respiratory epithelium at later stages of gestation markedly perturbs lung morphogenesis and causes dense, adenomatous malformations (16). These effects appear to be mediated by the proliferative and chemoattractant effects of FGFs on lung epithelium. FGF7 has potent mitogenic effects on pulmonary epithelial cells inducing proliferation of bronchial epithelial and mature type II alveolar cells (4, 17). FGF10 acts as a mitogen and chemoattractant, inducing directional growth of the lung buds in close proximity (5, 18, 19) during the earliest stages of lung development. While the above studies are elucidating in many

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ways, they also emphasize that the effects of specific lung growth factors on normal or abnormal lung development are highly dependent upon timing, duration, and distribution of expression. Global, ectopic expression of these highly spatially and temporally restricted growth factors may induce artifactual effects not seen with more compartment-appropriate overexpression, including autocrine effects and absence of normal directional induction. We reasoned that a more physiologic and discrete approach to dissection of FGF10 effects on lung development would be to induce transient, mesenchymal, overexpression in a localized area of the developing lung during specific stages of lung development.

We recently described an efficient method for gene transfer to fetal lung interstitial cells during the pseudoglandular stage of lung development by direct ultrasound-guided intrapulmonary injection of viral vectors (20). Furthermore, we demonstrated that adenoviral vector induced a rapid onset but relatively transient expression of the reporter gene relative to lentiviral vector. It occurred to us that this model might be valuable for investigation of the effects of transient and localized mesenchymal overexpression of specific growth factors on lung development.

In the present work, we hypothesized that focal FGF10 mesenchymal overexpression during lung development would cause focal abnormalities of epithelial proliferation. To test this hypothesis, we injected a bi-cistronic adenoviral vector encoding the *rfgf10* and *egfp* transgenes (AdGFP-FGF10) into targeted areas of fetal rat lung parenchyma, at specific stages of lung development. We observed that FGF10 overexpression resulted in the induction of consistent patterns of malformation, the appearance of which were developmental stage and location dependent. These malformations, in total, appear to closely recapitulate the morphology and histology of the entire spectrum of human congenital cystic adenomatoid malformation (CCAM).

MATERIALS AND METHODS

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Viral Vector Preparation

Cell culture. 293 cells were kindly provided by the Gene Therapy Program (Division of Medical Genetics, University of Pennsylvania, Philadelphia, PA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies/Invitrogen) at 37°C in a 5% CO_2 humidified atmosphere.

Rat FGF10 cds and recombinant adenovirus constructions. A cDNA library was prepared from newborn Sprague-Dawley lung using Trizol Reagent and Superscript (Invitrogen, Carlsbad, CA) as per manufacturers' recommendations. The rat *FGF10* cds was amplified using the forward primer 5′-gcggccgcc atgtggaaatggatactgacacattg-3′ and reverse primer 5′-ctcgaggctagcctatgagtggaccaccatg-3′ using standard methods and confirmed by sequence analysis (GenBank Sequence Database accession number NM_012951). The first-generation recombinant human adenovirus serotype 5 vector expressing the rat *FGF10* cds was synthesized as previously reported (21) with the following modifications. The rat *FGF10* cds was subcloned into a previously modified pShuttle-CMV vector containing a multiple cloning site and IRES-GFP cassette downstream to the promoter. Approximately 1 μg of the *Pme*I linearized shuttle vector DNA was electroporated into *Escherichia coli* BJ5183 harboring the pAdEasy-1 vector. Selected colonies were screened by restriction digests for the presence of size correct homologous recombined plasmids. The pAd-CMV-Rat FGF10-IRES-GFP plasmid DNA transformed the *recA–E. Coli*, DH10B, by electroporation for large-scale amplification. Five micrograms of *Pac*I-linearized pAd-CMV-Rat FGF10-IRES-GFP DNA was combined with Maxfect (Mediatech, Herndon, VA) as per manufacturer's instructions and used to transfect 293 cells. Transfected cells were monitored for GFP expression and cytopathic effect. Twelve days later, cells and cell debris were removed, pelleted, and after three cycles of freeze/thawing a viral lysate was obtained, identified as Ad-ratFGF10. To assure the production of rat FGF10 protein, 293 cells were infected with the viral lysate, and 48 hours later growth media was assayed for the presence of rat FGF10 by Western blot using a rabbit anti-Rat FGF10 antibody (H-121:sc-7919; Santa Cruz Biotechnology, Santa Cruz, CA) at 1/200 dilution. Propagation, concentration, and purification of the Ad-ratFGF10 was performed using Vivapure AdenoPACK 500 kit (Sartorius, Edgewood, NY) following manufacturer's recommendations. The viral pellet was resuspended in storage buffer (20 mM Tris-HCl, 25 mM NaCl, 2.5% glycerol, pH = 8) and stored at −80°C. Titer was determined in triplicate using serial dilutions of viral stock on 293 cells. Two days later, titers were determined by counting the number of GFP-positive colonies/field.

Ultrasound-Guided Injections

Time-dated pregnant Sprague-Dawley (Charles-River, Boston, MA) rats at 15.5 (E15.5; $n = 42$) and 18.5 (E18.5; $n = 18$) days *post-coitum* were anesthetized and a laparotomy was performed. Ultrasound-guided injections were performed as previously described (20). Briefly, fetuses were positioned to obtain axial views of the lungs in B-mode (VisualSonics, Toronto, Canada). The tip of the micropipette was brought into the image plane and was physically advanced through the uterine wall and amniotic cavity into the lung. A volume of 25 nl was injected using a remote control injector. After injections, dams were allowed to recover in a warming chamber. Two types of injections were performed with respect to the level of the bronchopulmonary tree. Injections performed near the main bronchus were defined as proximal. Injections performed at the peripheral basal area of the lung were defined as distal.

Ultrasound and Magnetic Resonance Imaging

Fetuses to be analyzed at E16.5, E18.5, and E21.5 were first inspected for lung morphology by ultrasound biomicroscopy. After performing laparotomy, the uterus was partially removed from the abdomen exposing one or two fetuses. Fetuses were covered with warmed sterile ultrasound gel and positioned to obtain axial views of the lungs in B-mode using a 40-MHz probe (VisualSonics).

Lung morphology of pups 1 week old (P7) was examined by magnetic resonance imaging using a Bruker Advance 400 wide-bore NMR spectrometer (Bruker AXS Inc., Madison, WI) equipped for microimaging. The Bruker self-shielded gradients were used with a 25-mm-diammeter RF coil to image the whole pups. After isoflurane overdose and neck dislocation, lungs were instilled intratracheally with perfluorocarbon to increase contrast and reduce susceptibility artifacts. Pups were inserted vertically into an 18-mm glass tube filled with Fomblin (perfluoropolyether), a liquid that approximately matches the susceptibility of tissue and does not give any background signal. Axial, coronal, and sagittal slices through the thoracic cavity were acquired with a spin echo T2 weighted pulse sequence, in which each image represents the sum of four echoes. For each orientation, 16 contiguous slices of 0.5 mm thickness were acquired with a square field of view (FOV) of 18 mm, a square matrix of 256 × 256 giving an in-plane resolution of 70 μm^2. The images were acquired using the following parameters: bandwith 50 kHz, pulse length 2 ms, repetition time 2 s, echo time 12 ms, and 2 excitations. Fetuses were screened inside the uterus at E16.5, E18.5, E19.5, and E21.5 dpc, and after being anesthetized, at 1 week (P7). The fetuses were positioned to get axial views of the lungs in B-mode and were screened in 3D-mode. After screening, fetuses and pups were harvested and lungs collected for stereomicroscopy, histology, and biochemical analysis.

Stereomicroscopy

Injected fetuses were visualized under a fluorescence stereomicroscope (MZ16FA; Leica, Heerburgg, Switzerland) immediately after harvesting, to evaluate enhanced green fluorescent protein expression, transduction efficiency, and biodistribution of the vector within lungs, trachea, heart, and diaphragm.

Histology and Immunohistochemistry

Specimens for histologic analysis were fixed in formalin (10%) and processed for paraffin sections. Immunostainings were performed on 4-μm-thick paraffin-embedded sections. After dewaxing in xylene and rehydration in ethanol, slides were blocked for specific serum (1:10 dilution) for 30 minutes at room temperature (RT) followed by incubation at 4°C with each primary antibody. The primary antibodies were monoclonal mouse EPOS anti–α-SMA (U.7033; Dako, Carpinteria, CA), polyclonal rabbit anti–Clara cell secretory protein (CCSP) (1:800 dilution; Seven Hills Bioreagents, Cincinnatti, OH), polyclonal rabbit anti-pro–surfactant protein (SP)-C (1:100 dilution; Chemicon, Temecula, CA), and polyclonal rabbit anti-GFP (1:100 dilution; Invitrogen). Samples were incubated with 3% hydrogen peroxide in methanol for 30 minutes at room temperature to quench endogenous peroxidase. Incubation with secondary antibody biotinylated goat anti-polyvalent (UltraVision HRP Detection System; Lab Vision, Fremont, CA) was performed at room temperature for 30 minutes. Slides were incubated with avidin–biotin complex (1:200 dilution; Vector Laboratories, Burlingame, CA) and developed with 3, 3′-diaminobenzidine (DAB) substrate kit (SK-4100; Vector Laboratories). Sections were counterstained with Harris hematoxylin. The sections were visualized under the Olympus BX61 microscope and photographed using an Olympus DP70 camera (Olympus America, Center Valley, PA). Adjacent sections were also stained with hematoxylin and eosin for morphologic studies.

In Situ Hybridization

Digoxigenin-labeled rat FGF10 antisense and sense probes were produced from the 648-bp sequence cloned in pIBI31, linearized using *NheI* or *NotI*, and transcribed with T3 or T7 polymerases, respectively. The following mouse cDNAs were used to generate digoxigenin-labeled riboprobes: 1.0 kb *Bmp4* and 948 bp *Spry2* (kindly provided by Dr. Saverio Bellusci). Lungs were fixed overnight at 4°C in 4% formaldehyde-2 mM EGTA, rinsed in PBS, dehydrated through a methanol series, and stored in 100% methanol at −20°C. Whole-mount (FGF10) and tissue section (BMP4 and Spry2) *in situ* hybridization were performed according to procedures described previously (22, 23). Lungs were visualized as whole mounts in PBT (PBS, 0.1% Tween20) under a dissecting microscope stereomicroscope and photographed with an Olympus 2100 camera. Whole mount lungs were embedded in 2-hydroxyethyl methacrylate (Technovit 7100; Heraeus Kulzer GmbH, Hanau, Germany) and processed for sectioning at 15 μm thickness using a rotary microtome (Leica RM 2155). Hybridized lungs sections were photographed using an Olympus DP70 camera coupled to an Olympus BX61 microscope.

Quantitative PCR

Samples from injected lungs collected at E16.5, E18.5, and E21.5 were placed in 1.5 ml microcentrifuge tubes, immediately frozen in liquid nitrogen, and stored at −80°C.

To examine rFGF-10 gene expression, total RNA from each lung specimen was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA), reverse transcribed, and subjected to quantitative polymerase chain reaction (qPCR) analysis as previously described (24–26). First-strand cDNA was prepared from 1 μg total cellular RNA isolated above. Total cDNA was used for the relative quantification by real time-PCR of rFGF10 and of the reference gene, rβ-actin, using the ABI PRISM 7700 s Sequence Detection System (Applied Biosystems, Foster City, CA). Sequence data were obtained from the GenBank Sequence Database (accession numbers: NM_031144, *Rattus norvegicus* rβ-actin; NM_012951, *R. norvegicus* FGF10 mRNA). Pre-designed TaqMan Gene Expression Assays were used for rFGF10 (Rn00564115_m1; Applied Biosystems), β-actin (Rn00667869_m1; Applied Biosystems). Amplification conditions were identical for all reactions. The thermal profile was as follows: 2 minutes at 50°C, 10 minutes of denaturation at 95°C, 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. The amplification reactions were set in a final volume of 20 μl, containing 1× TaqMan Universal PCR Mastermix (Applied Biosystems), 100 ng of the cDNA sample, 900 nM of each primer, and 250 nM of the respective probe. Negative control was included in all the runs, which consisted of omitting the cDNA and performing a melting curve analysis. β-actin, rFGF10, standard amplification curves (ST curve) were made with randomly selected cDNA samples (setting at $r = 0.99$). For each experimental group studied, three samples were analyzed, in triplicate, and mean result was used for further analysis. Amplification data were analyzed by the sequence detection system software (SDS 1.2 version; Applied Biosystems).

Statistical Analysis

The results were presented as mean ± SE. To ascertain the statistical significance of the differences in the levels of mRNA, one-way ANOVA was performed. Upon statistically significant differences, a Student-Newman-Keuls test was selected to perform pairwise multiple comparisons. Survival rates were compared by the log-rank test. Differences between groups were considered to be statistically significant when $P < 0.05$.

RESULTS

Intraparenchymal Injection of AdGFP-FGF10 during the Pseudoglandular Stage Results in Pulmonary Morbidity

We investigated the effects of localized FGF10 overexpression on fetal rat lungs during the pseudoglandular and canalicular stages of lung development by performing transuterine ultrasound-guided microinjections of AdGFP-FGF10 at E15.5 and E18.5 by our previously described technique. Pre- and postnatal imaging analysis revealed the presence of cystic lesions in the injected lungs without obvious other abnormalities. External morphology of injected fetuses among all the groups was normal, as was the macroscopic structure of diaphragm, heart, and thoracic cavity. At cesarean, we detected a prenatal mortality rate of 26% and 10% associated with lung injections at E15.5 and E18.5, respectively, with control PBS injections. This was comparable to our previous study (20) using the same methodology and comparing PBS with the identical AdGFP vector minus the FGF10 transgene. In that study, survival to cesarean section was 81 ± 6% and 87 ± 4% for PBS and AdGFP, respectively. It is also important to note that in that study, no pulmonary cystic changes were seen in any animals injected with PBS or AdGFP vector alone. When AdGFP-FGF10 was injected at E15.5, there was an additional mortality during the first hours of postnatal life that reduced the survival rate to 33% by the seventh day after birth. Some pups that were injected at E15.5 were cyanotic and displayed gasping respiratory efforts within a few hours after birth, with subsequent demise. Autopsies or magnetic resonance imaging (MRI) examination revealed very large cysts that occupied almost all of the ipsilateral hemithorax. In contrast, when AdGFP-FGF10 was injected at E18.5, the survival rate was not statistically different from that of PBS-injected controls and no macrocystic lesions were seen. Pups that survived until the seventh day after birth displayed overtly normal activity levels and appeared developmentally normal. There were no maternal deaths.

Phenotype of the Lung Cystic Malformation Is Developmental Stage Dependent

To assess the effect of focal FGF10 overexpression on lung development during critical periods of lung morphogenesis, we performed *in utero* gene transfer during the rat pseudoglandular (E15.5) and canalicular (E18.5) stages of lung development. Lungs were analyzed by ultrasound, MRI, gross appearance, and fluorescence stereomicroscopy at different pre and postnatal time points (Figure 1).

When FGF10 gene transfer was performed at E15.5, malformations containing large cysts were detected as early as 24 hours after injection (Figures 1a–1c). The malformations persisted during the remainder of gestation (Figures 1d–1i) and at

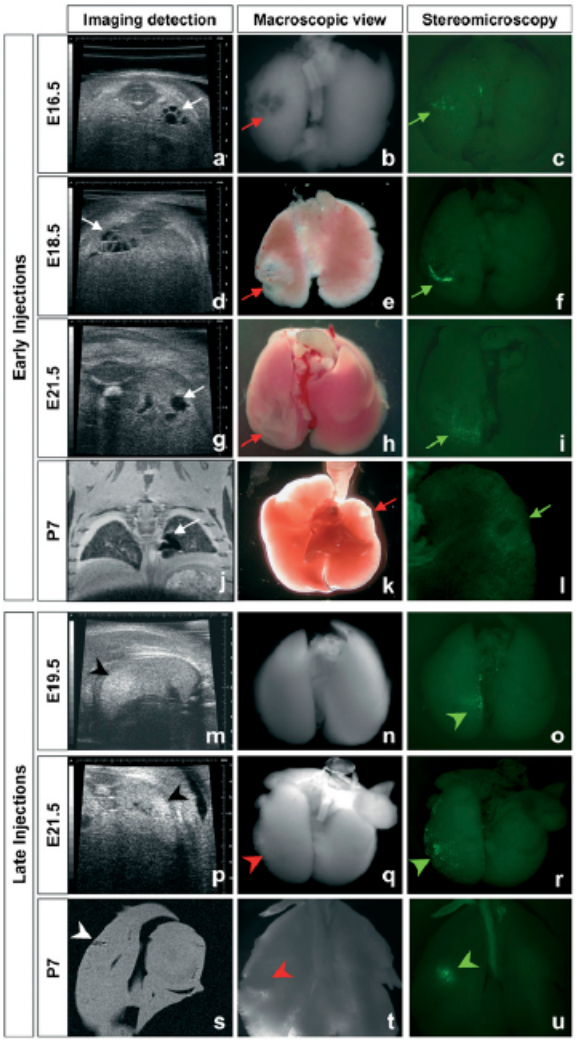

Figure 1. Developmental stage dependence of cystic malformations. Fetuses injected at Embryonic Day (E)15.5 were analyzed at E16.5, E18.5, E21.5, and Postnatal Day (P)7 (*a–l*). Representative images of injected lungs demonstrate macrocystic malformations (*white arrows*) by ultrasound at prenatal time points and magnetic resonance imaging (MRI) at P7. The macrocysts appeared within 24 hours after injection (*a–c*) and persisted until P7 (*j–l*) and were large enough to be easily appreciated by gross examination (*red arrows*). The expression of enhanced green fluorescent protein (EGFP) coincided with the affected area (*c, f, i, l, green arrows*). Fetuses injected at E18.5 were analyzed at E19.5, E21.5, and P7 (*m–u*). Microcystic malformations were detected by ultrasound at E19.5 (*m*) and E21.5 (*p*) as an area of increased echogenicity (*black arrowheads*) and were visualized as small cysts on MRI at P7 (*s, white arrowheads*). The malformations were present by E19.5 and persisted until P7, with the area of the malformation coinciding with the area of EGFP expression (*o, r, u, green arrowheads*).

1 week after birth (Figures 1j–1l). Imaging of these lesions revealed fluid-filled areas of low echogenicity with thin intervening septa surrounded by normal lung parenchyma. Gross inspection revealed lung malformations characterized by a single or usually multiple large cysts that were lined by a thin membrane. The cysts were always restricted to a single lobe of the lung. Analysis by fluorescence stereomicroscopy demonstrated that the affected area coincided with the area of gene transfer, as confirmed by GFP expression. At birth after ventilation of the neonatal lung, the cysts were immediately filled with air. The communication between the main airways and the cysts was confirmed by bronchofluoroscopy (data not shown).

In contrast, when FGF10 gene transfer was performed at E18.5, prenatal ultrasound imaging revealed hyperechogenicity in the area injected within 24 hours after injection, with only rare instances of visible cysts by ultrasound or gross inspection (Figures 1m–1r). These malformations were not easily detected by gross inspection; however, postnatal MRI revealed restricted areas with several small cysts (Figure 1s).

Phenotype of the Malformation Is Spatially Dependent

To explore the effect of FGF10 gene transfer at different levels of the bronchopulmonary tree, injections localized to either the proximal or the distal areas of the developing lung were analyzed at term (E21.5) (Figure 2). When the injections were performed at E15.5, proximal injections (Figures 2a–2c) induced larger cysts than distal injections (Figures 2d–2f), but the number of cysts were greater in the distal injection group. In contrast, injections into either the proximal or distal lung parenchyma at E18.5 induced only small cysts on microscopic analysis (Figures 2g–2l). Relative to the early injections, the affected area was more restricted and no macroscopic cysts were clearly seen. In all groups, lesions were well demarcated histologically from the adjacent normal lung parenchyma.

Histologic Characterization of Induced Cystic Lung Malformations

Specimens from lungs injected either at E15.5 or E18.5 were harvested at E21.5 and processed for histologic analysis and immunohistochemistry for α-smooth muscle actin (α-SMA), CCSP, SP-C, and green fluorescence protein (GFP) (Figure 3 and Table 1). Lungs injected at E15.5 targeting the proximal bronchopulmonary tree demonstrated one to four large predominant cysts surrounded by normal lung parenchyma (Figures 3f–3j). These cysts were lined by tall columnar pseudostratified epithelium encircled by a well-defined muscular wall composed of two to three cell layers. Most of the cells lining the cysts were positive for CCSP, whereas only a few cells stained for pro–SP-C, indicating a predominance of Clara cells and a paucity of Type II pneumocytes, respectively. Cartilage and skeletal muscle were absent. In lungs injected at E15.5 with gene transfer to the distal lung parenchyma, several large cysts appeared in the injected area which were lined with cuboidal to tall columnar epithelium (Figures 3k–3o). Smooth muscle fibers could be detected surrounding the cysts forming an incomplete layer. Within the cysts there was a sharp boundary between positive and negative areas for CCSP, corresponding to the transition between terminal and respiratory bronchioles. There was an intense staining for pro–SP-C in the epithelial lining of the cysts. Cystic malformations in fetuses injected at E15.5 were separated by thin septa with only a small amount of interstitial tissue.

Injections targeting the proximal lung parenchyma at E18.5 resulted in multiple relatively small-size cysts compared with the early injection time point (Figures 3p–3t). These cysts were lined by cuboidal epithelium with few Clara cells and rare type II pneumocytes. Smooth muscle fibers could be detected surrounding the cysts forming an incomplete layer. Injections targeting the distal lung parenchyma at E18.5 resulted in several types of small cysts (Figures 3u–3y). Some resembled bronchioles, while others contained intricate folds. The lining

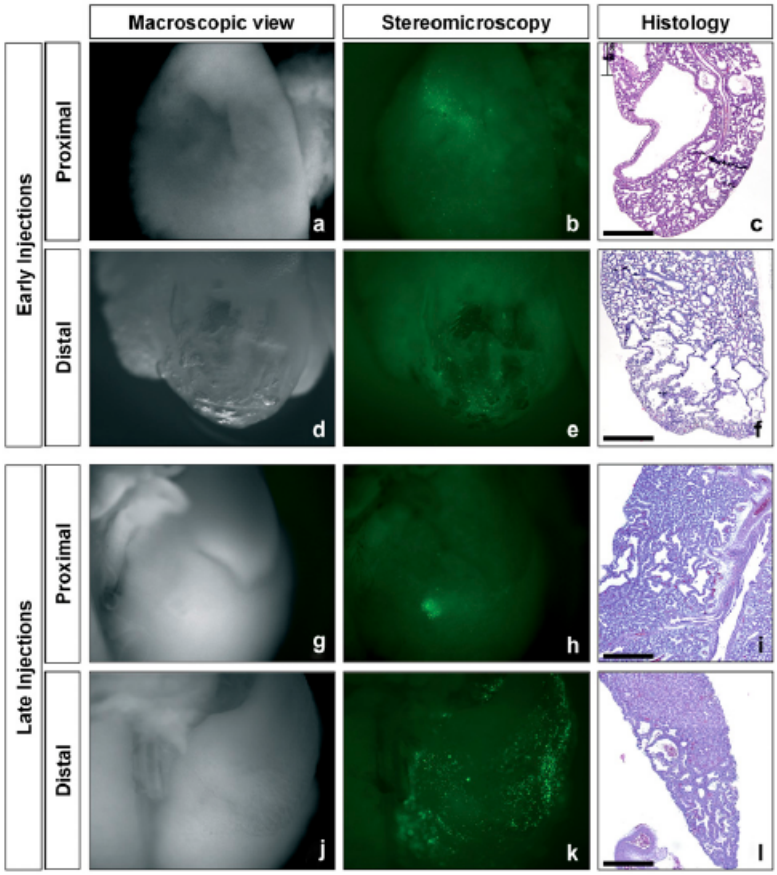

Figure 2. Spatial dependence of cystic malformations. Fetal injections of viral vector targeted proximal versus distal areas of the pulmonary tree and lungs were analyzed at E21.5. Early proximal injections of AdGFP-FGF10 (*a–c*) induced the formation of a few large cysts within the central lung parenchyma easily detectable by macroscopic inspection. Early distal injections of AdGFP-FGF10 (*d–f*) induced the formation of several large cysts visible in the periphery of the lung, but the cysts were smaller than with proximal injections. Late FGF10 gene transfer (*g–l*) induced the formation of small cysts that were barely macroscopically detectable. Once again, the cysts were larger with proximal injections (*g-i*) than distal injections (*j–l*). The overall affected area in late injections was more restricted than that inearly injections. In all groups, GFP expression (*b, e, h, k*) coincided with the location of the cysts and the malformations were well demarcated from the adjacent normal lung parenchyma. *Scale bars* correspond to 500 μm.

epithelium was composed primarily of type II pneumocytes. Clara cells were absent. Expression of α-SMA revealed the presence of scattered smooth muscle cells. Relative to the E15.5 injected group, fetuses injected at E18.5 displayed septa between the cysts that contained a moderate to abundant amount of loose mesenchymal-type interstitium. As we noted in our previous study (20) the distribution of gene transfer as confirmed by GFP immunohistochemistry (Figures 3j, 3o, 3t, 3y) appeared to be restricted to the mesenchyme in all lungs injected with AdGFP-FGF10.

Location and Quantification of Lung FGF10 Overexpression

FGF10 mRNA expression was detected by whole mount *in situ* hybridization (wISH) 24 hours after injections in both the early and late injected groups (Figures 4a–4o). The normal pattern of FGF10 expression observed in the PBS-injected control lungs was profoundly altered by FGF10 gene transfer. In all injected lungs examined, an increase in FGF10 mRNA expression was observed in an area that corresponded to the area of expression of GFP. On tissue sections of hybridized lungs, FGF10 overexpression in both early and late injected lungs appeared to be restricted to the mesenchymal compartment of lung parenchyma (Figures 4j and 4o), in agreement with the GFP immunohistochemistry.

Quantification of *FGF10* mRNA levels was performed at E16.5, E18.5, and E21.5 in ipsilateral and contralateral lungs injected at E15.5 and compared with PBS-injected control lungs (Figure 4p). *FGF10* mRNA expression was significantly increased in only the injected lung at 24 hours after injection. Levels of *fgf10* mRNA in injected lungs returned to levels measured in PBS control groups at E18.5 and E21.5.

Expression Pattern of BMP4 and Spry2 in Cystic Malformations

The expression pattern of *BMP4* and *Spry2* was characterized by *in situ* hybridization (ISH) 24 hours after injection in both early and late injected lungs (Figure 5). For early injected lungs, aside from the cystic morphology, the pattern of expression of BMP4 and Spry2 did not seem significantly altered in comparison with the PBS controls (Figures 5a–5d). In contrast, for late injected lungs expression of BMP4 and Spry2 appears significantly increased relative to controls in the epithelium of the cystic malformations (Figures 5e–5h).

DISCUSSION

FGF10 overexpression from the mesenchyme was focally and transiently induced at different locations and developmental stages in the fetal rat lung using our method of intraparenchymal gene transfer. In the area of forced FGF10 expression, pulmonary morphogenesis was markedly perturbed with the very rapid appearance of localized cystic lung malformations. The type of malformation observed was developmental stage and location dependent, with the spectrum ranging from macrocystic

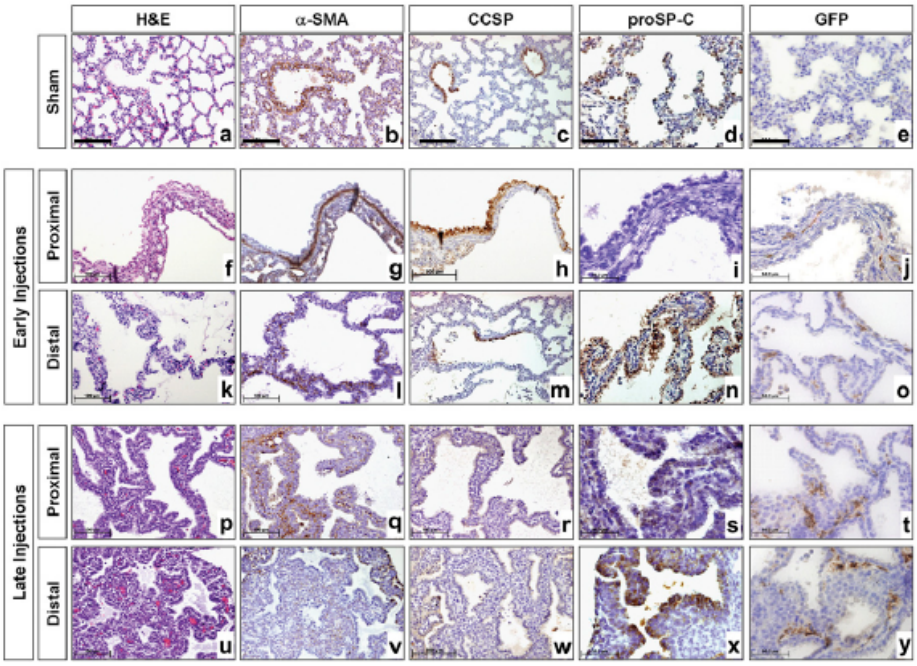

Figure 3. Histologic characterization of cystic malformations. Specimens from fetuses injected in the proximal and distal lung parenchyma at E15.5 (*f–o*) and E18.5 (*p–y*) were analyzed at E21.5. Lungs injected at E15.5 targeting the proximal area of bronchopulmonary tree presented large cysts encircled by a well-defined muscular wall composed of two to three cell layers (*g*). Cysts were lined by tall columnar pseudostratified epithelium (*f*) and the majority of the cells lining the cysts were positive for CCSP (*h*), with very few cells expressing proSP-C (*i*). In contrast, lungs injected into the distal parenchyma at E15.5 demonstrated several large cysts that were lined with cuboidal to tall columnar epithelium (*k*). Subepithelial smooth muscle fibers could be detected forming an incomplete layer (*l*). Cystic epithelium was predominantly positive for pro–surfactant protein (SP)-C (*n*), whereas CCSP immunostaining revealed a sharp boundary between positive and negative areas (*m*). Cystic malformations in fetuses injected at E15.5 were separated by thin septae containing a small amount of interstitial tissue. Injections at E18.5 resulted in relatively small cysts lined by cuboidal epithelium and separated by a moderate to abundant amount of loose mesenchymal type of interstitium (*p, u*). Cystic malformations caused by proximal injections contained few CCSP-positive cells (*r*), rare proSP-C-positive cells (*s*) and an incomplete subepithelial smooth muscle layer (*q*). Cystic malformations secondary to distal injections were lined by proSP-C positive cells (*x*) and scattered smooth muscle cells were present (*v*). In all groups, GFP positive cells were restricted to the interstitial compartment of lung parenchyma (*j, o, t, y*). In all groups cartilage and skeletal muscle were absent. *Scale bars* correspond to 100 μm for hematoxylin and eosin, α-smooth muscle actin, and CCSP and 50 μm for pro–SP-C and GFP immunostaining.

malformations lined by predominantly bronchial epithelium, to microcystic malformations lined by predominantly alveolar epithelium. The malformations were detectable by prenatal ultrasound examination within 24 hours after vector injection, and persisted until at least 1 week after birth. Whether examined grossly, by pre or postnatal imaging, or microscopically, the malformations appeared remarkably similar to the spectrum of human malformations characterized as congenital cystic adenomatoid malformation (CCAM).

The present study differs from previous studies examining the effect of FGF10 overexpression during lung development in several important ways. Expression of FGF10 in this study was focal rather than diffuse, and was expressed in interstitial cells rather than ectopically from respiratory epithelium. In our previous study (20) assessing the distribution of marker gene expression after intraparenchymal injection, we demonstrated that gene expression could be limited to a small area of lung parenchyma by limiting the volume of injection, and that expression was confined to the interstitial compartment of the lung with no demonstrable co-localization with SP-B–expressing epithelial cells. This distribution of expression was confirmed in this study by both GFP immunohistochemistry and wISH for FGF10. Another important difference is that the expression in this study was relatively transient. This was due to the use of adenoviral vector allowing rapid onset of expression with peak expression occurring approximately 24 hours after injection. The expression profile of FGF10 documented in this study is in good agreement with our previous study (20) in which quantitative PCR was used to assess GFP mRNA expression using the AdGFP control vector. The rapid diminution of transgene expression in both studies is consistent with the known episomal location of adenoviral gene expression, the small volume of vector administered (25 nl), and the rapid proliferation of fetal lung tissue. The fact that we observed rapid formation of the cystic lesions and that they persisted, supports the hypothesis that FGF10 is involved in the early inductive events in CCAM formation and that continued presence of FGF10 is not necessarily required in the natural evolution of CCAM malformations.

Although speculative, our results can be interpreted in the context of current models of FGF10 involvement in branching

TABLE 1. HISTOLOGIC CHARACTERIZATION OF CYSTIC MALFORMATIONS

	E15.5 Proximal	E15.5 Distal	E18.5 Proximal	E18.5 Distal
Cyst size	Large	Large	Small	Small
Number of cysts	Few	Several	Few	Several
Epithelial lining	Tall columnar pseudo-stratified	Cuboidal to tall columnar	Cuboidal	Cuboidal
α-SMA	Well-defined muscular layer	Discontinuous bands of smooth mucle	Discontinuous bands of smooth mucle	Scattered expression
CCSP	+++	+	+/−	—
SP-C	+/−	+++	—	+++

Definition of abbreviations: α-SMA, α-smooth muscle actin; CCSP, Clara cell secretory protein; E, Embryonic Day; SP-C, surfactant protein C.

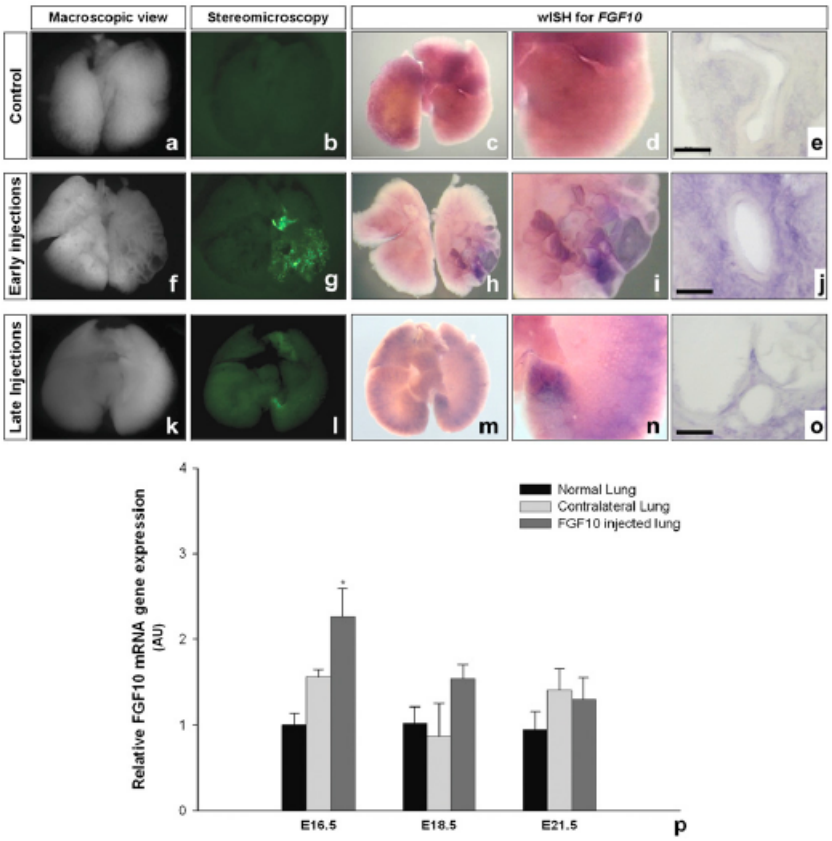

Figure 4. FGF10 mesenchymal overexpression coincides with location of cystic malformations. Lungs from injected fetuses at E15.5 and E18.5 were analyzed by whole mount *in situ* hybridization (wISH) for *fgf10*, 24 hours after injection (E16.5, *f–j*, and E19.5, *k–o*, respectively). The macroscopic view (*a, f, k*) and fluorescence stereomicroscopy images for GFP (*b, g, l*) are depicted alongside the wISH images for comparison. *FGF10* is overexpressed in the mesenchyme of the cystic area (*h–j; m–o*). (*p*) Quantitative PCR for *FGF10* in lung. *FGF10* expression of injected lungs at E15.5 was analyzed at E16.5, E18.5, and E21.5. Quantitative PCR was performed in PBS control, AdGFP-FGF10 injected, and contralateral lungs. *Fgf10* mRNA levels were significantly higher at E16.5 in AdGFP-FGF10 ipsilateral lungs versus PBS and contralateral lungs. However, at E18.5 and E21.5 *FGF10* expression was not significantly different among groups. *Scale bars* correspond to 50 μm for methacrylate sections of hybridized lungs.

morphogenesis (27) in which FGF10 and reciprocal influences such as Bmp4 and Spry2 regulate lung development (Figure 6). Under normal circumstances, cross-talk between the mesenchyme and the endodermal epithelium regulates the spatial and temporal gene expression for *fgf10*, *Bmp4*, and many other morphoregulatory molecules. Highly localized expression of *fgf10* in the mesenchyme adjacent to the tip of the nascent airway stimulates proliferation and growth outward. Simultaneously, FGF10 signaling induces a steady increase in *Bmp4* or other reciprocal factors transcription in epithelial cells. Bmp4 and Spry2 are inhibitory to epithelial cell proliferation/movement arresting the outward movement of the developing airway (8, 10). Branching is then induced by lateral up-regulation of mesenchymal FGF10 inducing dichotomous branching of the more proximal epithelium that remains responsive to FGF10. Thus during early stages of branching morphogenesis, high levels of mesenchymal FGF10 expression would be expected to induce a burst of proliferation and outward migration of the epithelium in segments of the developing airways between branch points that would be expected to be responsive to FGF10, with less proliferation at branch points where Bmp4 and other antagonists were expressed. While reciprocal signals such as Bmp4 would be expected to be diffusely induced in the epithelium, the ultimate size of the cyst would depend upon the balance of proliferative versus inhibitory signaling. *In situ* hybridization for BMP4 and for Spry2 indicated that their regulatory action over FGF10 might not be effective in lungs that were injected at earlier stages, because they are not concomitantly overexpressed. Thus, at the early time point, within the first 24 to 48 hours when FGF10 levels are high, one would

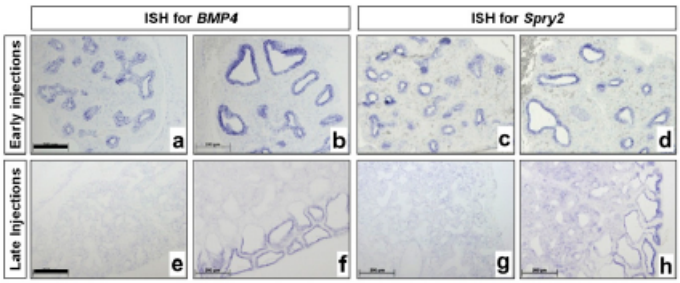

Figure 5. BMP4 and Spry2 expression pattern in cystic malformations. Lungs from injected fetuses at E15.5 and E18.5 were analyzed by *in situ* hybridization (ISH) for *bmp4 and spry2*, 24 hours after injection (E16.5, *a–d*; and E19.5, *e–h*, respectively). For AdGFP-FGF10 early injected lungs (*b, d*) the expression pattern of *BMP4* and *Spry2* does not seem altered in relation to respective controls (*a, c*). For AdGFP-FGF10 late injected lungs (*f, h*) expression of *BMP4* and *Spry2* is increased in the epithelium of the cystic malformations when compared with controls (*e, g*). *Scale bars* correspond to 200 μm.

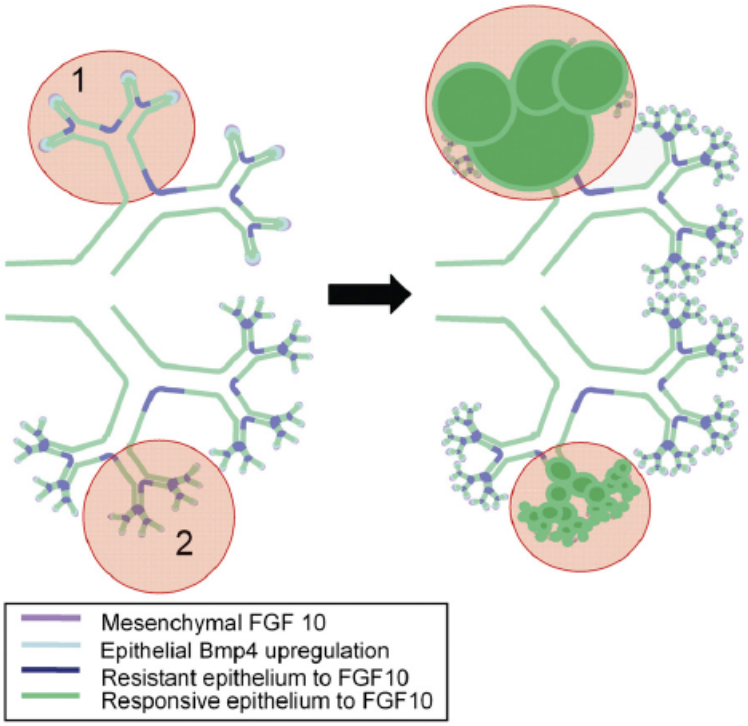

Figure 6. Model of FGF10 induction of cystic adenomatoid malformations. During the pseudoglandular and early canalicular phases the lung consists of repeating branch points with terminal growth determined by mesenchymal epithelial interaction orchestrated by mesenchymal FGF10 expressed at the tips of the elongating airways. By the model of Weaver and coworkers (10), FGF10 induces Bmp4 (or other FGF10 antagonists) in the adjacent epithelium, which renders the epithelium resistant to FGF10, stopping airway extension and creating a branch point with dichotomous branching induced by lateral FGF10 stimulation of the more proximal responsive airway epithelium. By this model, either proximal or early FGF10 overexpression as depicted by *pink circle 1* would stimulate a limited number of more proximal or less differentiated responsive airway segments, resulting in large cystic malformations. Whereas distal FGF10 overexpression as depicted by *circle 2* would stimulate a larger number of distal or more differentiated FGF10-responsive airway segments, respectively, resulting in a large number of smaller cysts. The cysts would tend to enlarge until induction of Bmp4 and other reciprocal factors resulted in epithelial inhibition or the FGF10 concentration diminished. The lining epithelium would be composed of the differentiated progeny of the lining epithelium of the proximal versus distal stimulated segment, that is, either bronchial or alveolar type epithelium, respectively. Although not shown in this cartoon, late FGF10 overexpression would be predicted to act on differentiated epithelium and terminally differentiated airways resulting in more adenomatous hyperplasia rather than cystic malformation.

expect unopposed action of FGF10 with rapid formation of large cysts. In contrast, in late injected lungs it was clear that expression of these two factors increase in the epithelium surrounding cystic areas. In this case, the significant induction of these regulatory molecules might be responsible for the inhibitory effect on FGF10-induced proliferation, leading to the formation of smaller cysts in late injected lungs. Also, in the proximal airways, where fewer branch points exist, and high columnar epithelium predominates, unopposed FGF10 signaling would be predicted to result in large cysts lined by predominantly bronchial epithelium. In distal airways, where multiple branch points are already present and cuboidal epithelium predominates, smaller and more numerous cysts would form lined by predominantly alveolar epithelium. In contrast, during the canalicular stage of lung development only terminal branching occurs and the distal airways are lined predominantly by cuboidal epithelium which is beginning to flatten and differentiate into type I and type II pneumocytes. Thus FGF10 overexpression might stimulate short segments of responsive epithelium into small cyst formation but the predominant effect would be expected to be similar to the effect of conditional FGF10 overexpression in postnatal lungs in which differentiated adenomas lined by abundant Type II epithelial cells form.

Ch'in and Tang identified CCAM as a pathologic entity in 1949 (27). While the pre- and postnatal natural history of CCAM is now well defined (28, 29), the pathogenesis of CCAM remains unknown. Diagnosis of CCAM in fetal life is usually made by ultrasound evaluation, and typically lesions are confined to a single lobe and can be classified as macrocystic or microcystic. In humans, macrocystic CCAMs have large cysts (2–10 cm) that vary in size and number with thin intervening echogenic areas. These multiple cysts are interconnected, and typically communicate with the tracheobronchial tree. The microcystic classification is applied to lesions that appear as a well delineated echogenic area in the fetal lung with no macrocystic spaces being identified. A CCAM categorization between the macrocystic and microcystic "definition" is sometimes used where the cyst spaces are present but small to moderate sized (< 2 cm) with adjacent echogenic tissue areas (30, 31). The ultrasound and MRI appearance, lobar distribution, and ultrasound classification of the malformations observed in this study were strikingly similar to human CCAM. FGF10 gene transfer during the pseudoglandular stage resulted in macrocystic lesions by ultrasound classification that were confirmed by MRI evaluation at Postnatal Day 7. A completely different ultrasound pattern was observed when gene transfer was performed during the canalicular stage—no cysts were visualized, but well-defined areas of increased echogenicity could be identified. MRI performed on microcystic lesions at 7 days after birth demonstrated very small cysts with careful analysis of images. The clinical manifestations of our induced lesions were also suggestive of human CCAM. CCAMs may grow rapidly before birth to massive size, resulting in extreme mediastinal shift and compromise of cardiac output resulting in fetal heart failure (hydrops); they may cause respiratory distress after birth; or they may be asymptomatic. It is not uncommon for CCAMs to undergo a rapid growth phase, only to decrease in size later in gestation. The same spectrum of clinical manifestations was observed in this study ranging from pre- or perinatal death from large, mass displacing tumors to asymptomatic survivors.

Stocker classified CCAMs into five subtypes defined on the basis of clinical features, macroscopic and microscopic criteria, and the site of the defect in the tracheobronchial tree (32). Depending on differences in cytodifferentiation, CCAM can be

divided into two major subtypes: CCAM types 1, 2, and 3, consisting of tissue with a bronchiolar-type epithelial differentiation; and a second subtype consisting of CCAM type 4, which has an acinar-alveolar epithelial differentiation. These two major subtypes have been hypothesized to originate at distinct stages of lung development: the first subtype (CCAM types 1, 2, and 3) may develop at the pseudoglandular stage, and the second subtype (CCAM type 4) may be due to a late event that disrupts branching of the distal acinar structures in the saccular period (33). Our study supports this pathophysiologic hypothesis. In this study we could reproduce the two histologic subtypes of CCAM. When proximal overexpression of FGF10 was induced during the pseudoglandular phase, we induced large cysts lined by tall to columnar epithelium with a high density of Clara cells and only few type II pneumocytes, resembling bronchiolar-type epithelial differentiation. Furthermore, when distal overexpression of FGF10 was induced during the canalicular phase, we observed small cysts composed of cuboidal epithelial that were primarily type II pneumocytes, resembling acinar epithelial differentiation.

There have been a number of efforts to characterize the molecular basis of CCAM in surgical specimens collected in both the pre- and postnatal periods. Cass and collaborators demonstrated that there is increased proliferation and decreased apoptosis in fetal CCAMs (34). *Fgf7* gene expression or protein production was evaluated in CCAMs requiring fetal resection, and no differences were found when compared with normal lungs (34). Platelet-derived growth factor-BB (PDGF-BB) gene expression and protein production were found to be increased in CCAMs that grew rapidly and required *in utero* resection (35), but there is no evidence that this factor is the causative factor for CCAM. Glial cell–derived neurotrophic factor (GDNF) was another factor with abnormal expression in epithelial cells lining CCAM cysts, suggesting a focal arrest in lung maturation during the fetal period (36). Although abnormal Hoxb-5 expression at a level typical of earlier lung developmental stages was observed in CCAM (37), it is presently unclear what initially triggers the cascade of events that ends with these malformations. All these studies have a common major limitation, which is that the analysis was performed in CCAM specimens that were surgically resected, analysis of which is unlikely to reveal the initial inciting events. In the present study, we have been able to evaluate in a prospective fashion the formation of CCAM-like malformations in response to overexpression of FGF10 in the mesenchymal compartment of the developing lung. We have demonstrated that transient overexpression of a single gene in a focal area of the lung is sufficient to reproduce the spectrum of gross and histologic features of human CCAM in a rat model. The striking similarity of these lesions to those seen in human CCAM strongly implicates mesenchymal overexpression of FGF10 in the initial events invoking CCAM formation. Our data thus far support FGF10 overexpression as the most important mechanistic component of CCAM formation. However, the identity of the primary mesenchymal perturbation that induces FGF10 overexpression remains to be determined.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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CHAPTER 4

DISCUSSION AND CONCLUSIONS

Discussion

Advances in prenatal diagnosis of genetic and congenital disorders with progressively more sensitive techniques may increase opportunities for consideration of prenatal gene therapy. There are a number of genetic and acquired disorders with peri or postnatal pulmonary manifestations. These include monogenetic diseases like cystic fibrosis or surfactant protein B deficiency that would presumably require long-term expression of the deficient or defective gene. However, there are also abnormalities of lung growth, such as congenital diaphragmatic hernia, or lung maturation, such as respiratory distress syndrome of prematurity, that could potentially benefit from strategies that achieve transient gene expression in specific pulmonary distributions. Considered an attractive target organ for fetal gene transfer, the developing lung, poses also some obstacles that would only be overcome with the development of a variety of gene transfer methodologies: different types of vector, optimal site, route and timing of gene delivery.

The goal of this thesis was to develop new strategies to specifically target the developing lung in early stages of development. The optimized model would constitute an important tool of *in utero* gene delivery. After this, the newly established gene transfer model was used to modulate lung growth and development, by inducing transient overexpression of a key factor in lung development, FGF10, in the fetal rat lung. This study, not only contributed for further characterization of FGF10 role in normal lung development, but also provided an unique insight into developmental mechanisms that may contribute to congenital cystic adenomatoid malformation (CCAM) etiology.

In chapter 2, we report the feasibility of using ultrasound biomicroscopy (UBM) to perform *in utero* intrapulmonary injections in rats at the pseudoglandular stage of lung development. Several routes of vector administration have been utilized to achieve prenatal gene transfer to the lung including intra-pulmonary. Intra-amniotic and systemic approaches share the same limitations of being nonspecific for fetal lung. Intratracheal and intrapulmonary delivery, although more specific to the lung, have previously only been performed in large animal models, such as ovine and non-human primates [Pitt BR, et al., 1995; Sylvester KG, et al 1997; David AL, et al. 2003, 2006; Luton D, et al. 2004; Peebles D, et al., 2004; Yu ZY, et al. 2007; Tarantal AF, et al. 2001, 2005]. One of the major concerns in gene transfer protocols is to target a specific organ/system, in our case, we aimed to specifically target the lung. This was only possible, by using a relatively new imaging technology: the ultrasound-guided biomicroscopy (UBM). UBM utilizes high-frequency (20–100 MHz), pulse-echo ultrasound for imaging live tissues and organs, allowing near microscopic resolution and has been previously

utilized for fetal brain injections [Turnbull DH, et al. 1995; Foster FS, et al. 2000]. Using UBM, we could successfully target the rat lung at E15.5, which corresponds to the mid-point of the pseudoglandular stage of lung development. In fact, we attempt to target the developing lung earlier, at E13.5 and E14.5, however the lung bud was very difficult to visualize and mortality was unacceptably high. Biodistribution of the viral vectors was evaluated and we concluded that gene delivery was localized to the injected lung with the only exceptions being the needle track through the thorax and a very low copy number in the heart. This could be due to inadvertent injection of the heart but is more likely due to the small amount of intravascular injection associated with this technique. A few animals had EGFP expression noted by stereoscopic fluorescent analysis in the skin or eye. However, in the animals selected for PCR analysis, these tissues did not have statistically higher gene copy numbers than PBS controls. This discrepancy likely represents inconsistent amounts of leakage of the vector into the amniotic space during removal of the micropipette. The lack of pulmonary epithelial transduction in these animals despite intra-amniotic leakage is most likely explained by the very minimal volume of leakage into the amniotic space relative to the volume of injectate in the intra-amniotic injection experiments as well as the timing of the injection.

The timing of vector administration was definitively one of the major challenges of this work. We aimed to establish a model of lung *in utero* gene transfer, envisaging the manipulation of normal and abnormal lung development during pseudoglandular stage. This stage, characterized by intense branching morphogenesis, is the period of greatest overall growth of the airways and vasculature of the fetal lung, and corresponds to a stage of immunologic immaturity. Therefore, gene transfer during this period has the potential to have major effects on the key elements of lung growth with minimal potential for detrimental immune responses. Also, stem-cell targeted gene transfer and intervention in rodent models for human diseases (e.g. nitrofen-induced congenital diaphragmatic hernia rat model and cystic fibrosis murine models), that requires pertinent therapeutic window periods, would benefit from gene delivery early in lung development.

Regarding the type of vectors we used first-generation adenoviral and EIAV-based lentiviral vectors. We chose to study first-generation adenoviral vectors rather than adeno-associated viral vectors because we wished to see rapid, high-level expression of the marker gene. EIAV-based owing to the fact that HIV-1-based lentiviral vectors have not shown high efficiency transduction [Hofmann W, et al. 1999, Romano G, et al. 2000] in many tissues and owing to the successful application of EAIV vectors in fetal rodent models by Waddington et al. [Waddington SN, et al. 2003]. Adenovirus has

been one of the most extensively studied recombinant viral systems because of its high transduction efficiency, rapid expression, accommodation of large transgene inserts, and high titers [Romano G, et al. 2000; Senoo M, et al. 2000; Breyer B, et al. 2001; Driskell RA and Engelhardt JF 2003]. The primary problem with adenovirus is its high immunogenicity and propensity to invoke strong immune responses. Previous studies documented this problem with intratracheal administration in late gestational fetal lambs; however, in the same model, no significant inflammation is observed with adenoviral administration during the preimmune phase of lamb immunologic development [Yang EY, et al. 1999b]. Similarly, as we would predict from the stage of rat immune development at E15.5, we saw no overt inflammatory response. In contrast to adenovirus, lentiviral vectors are relatively non-toxic and minimally immunogenic and can stably integrate transgene into dividing and non-dividing cells with subsequent long-term gene expression [Naldini L, et al. 1996, Zufferey R, et al. 1997]. The main disadvantages are the low titers that are usually obtained *in vitro* [Romano G, et al. 2000]. EIAV vector obtained titers were relatively low (10^7–10^8 infectious particles per mL), therefore we needed to inject the maximal tolerated volume of 350 nL of EIAV to achieve significant transduction. The rapid loss of EGFP expression in the adenoviral-transduced lungs was expected due to the episomal location of adenoviral gene expression, the small volume of vector administered, and the rapid proliferation of fetal lung tissue. We confirmed that EIAV to stably integrate transgene into the host genome. As expected, adenoviral vector expression appeared quickly and was transient, whereas lentiviral vector expression was relatively delayed and persisted through the time period of this study. Adenoviral would be the most appropriate vector to use when the goal is to induce rapid and transient overexpression of a gene in this model, whereas, lentivirus would be a more suitable vector to induce sustained and long-term gene expression.

Interestingly, both viral vectors efficiently transduced the interstitial compartment of developing lung, Unexpectedly, in contrast to intra-amniotic injections, both vectors selectively transduced interstitial cells and not alveolar or airway epithelial cells, or vascular endothelial cells. There was no expression of reporter gene in the surface epithelium of airways or in the vascular endothelium. We were not able to definitively identify the cells in this study owing to a lack of specific markers for cell types in the interstitium. Although we suspected that the cells were mesenchymal in origin, they do not stain with vimentin, a common mesenchymal and fibroblast marker. Attempts to colocalize EGFP staining with SP-B, a surfactant protein expressed in type II pneumocytes, confirmed that the transduced cells were not epithelial, and that they were located within the interstitial compartment of the lung. This is in distinct contrast to the epithelial restricted expression seen with intra-amniotic vector injections. Although the obvious explanation is the route of injection, in the two previous

studies of direct lung transduction in primates using human immunodeficiency virus-1 lentiviral vectors, only epithelial expression was described, so this appears to be a model-dependent observation. At the time of publication, this was the first observation of this pattern of parenchymal expression with any reports of prenatal lung gene transfer. Recently, Cohen and coworkers [Cohen JC, et al. 2008] were able to induce the expression of reporter gene in parenchymal cells surrounding small airways, following intraamniotic administration of adenoviral vector in mouse fetuses at E16. The adenoviral vectors contained full-length CFTR, a short anti-sense CFTR gene fragment, or a reporter gene as control were used in an intraamniotic gene therapy procedure to transiently modify CFTR expression in the fetal lung. The modulation of gene expression of lung parenchymal cells is, in this case, related with the use of BAT-gal transgenic reporter mouse line, expressing β-galactosidase under a canonical Wnt/β-catenin-responsive promoter. The observation of transduction of distinct cell populations within the lung with different routes of transduction raises the possibility of manipulating gene expression in specific and separate cell populations within the developing lung. This may have interesting applications toward understanding mesenchymal epithelial inductive interactions during the pseudoglandular phase of lung development.

The ability to achieve gene transfer by direct intrapulmonary injection in a rat model provides a novel tool for the exploration of potential therapeutic strategies for lung disorders and for biological studies examining the effects of specific genes on lung development. Obvious advantages of this technique include ease of manipulation, minimal expense, and, if translatable to the mouse, the availability of well-characterized murine models of human lung diseases [Boyd RL, et al. 1984; Muller RM, et al. 1985; Tenbrinck R, et al. 1990].

The established model of intrapulmonary *in utero* gene transfer would constitute an important tool in pursuing the studies of the mechanisms related to lung development in normal and hypoplastic lungs. We aimed to modulate lung development acting upon key players envisaging a potential induction of growth in hypoplastic lungs and/or maturation in abnormally immature lungs.

After long time, our lab has focused our research on the pathophysiology and development of potential prenatal therapeutics for CDH. The possibility to revert fetal lung hypoplasia, one of the major causes of mortality in CDH infants, by prenatal gene transfer, was appealing. Bearing that in

mind, we considered that rapid, high-level of expression of the transgene was preferable whether sustained long-term expression was not required. Therefore, we pursue our studies with modulation of lung development by intrapulmonary adenovirus-mediated gene transfer, as described in chapter 3.

After an extensive review of the main factors involved in the branching process and those implicated in CDH, we selected two strong candidates for transgene: FGF10 and TTF1. FGF10 null mice lack of lung development and die after birth [Sekine K, et al. 1999] and TTF-1 null mice have disruption in lung branching morphogenesis and also present a perinatal lethal phenotype [Kimura, et al. 1996]. In addition, both factors are reduced in experimental CDH [Chinoy MR, et al. 2001; Teramoto H, et al. 2003]. Also, Acosta and collaborators, using lung explants from animals exposed to nitrofen, demonstrated that this toxic induced an almost complete arrest of lung budding in the left lung and significantly decreased branching in the right lung [Acosta JM, et al. 2001]. Exogenous FGF-10 produced a significant lung growth by stimulating lung branching morphogenesis in both control and nitrofen exposed lungs in culture. Gains in branching with FGF-10 after nitrofen exposure were very impressive with the right lung restored to the same number of branches as the wild-type lungs grown without the benefit of FGF-10, whereas the number of branches in the left lung increased by 77% [Acosta JM, et al. 2001]. Besides these evidences about the potent role that FGF10 has in lung growth another theoretical advantage of FGF10 as compared to TTF-1 is that the former can induce directly cellular growth, whereas the latter need to bind to a promoter region and activate its target gene. Therefore, we decided to construct an adenoviral vector with FGF10 as the transgene and to analyze the effects of FGF10 transient overexpression by fetal lung gene transfer during the pseudoglandular stage.

FGF10 overexpression from the mesenchyme was focally and transiently induced at different locations and developmental stages in the fetal rat lung using our method of intraparenchymal gene transfer. In the area of forced FGF10 expression, pulmonary morphogenesis was markedly perturbed with the very rapid appearance of localized cystic lung malformations. The type of malformation observed was developmental stage and location dependent, with the spectrum ranging from macrocystic malformations lined by predominantly bronchial epithelium, to focal microcystic malformations lined by predominantly alveolar epithelium. The malformations were detectable by prenatal ultrasound examination within 24 hours after vector injection, and persisted until at least 1 week after birth. Whether examined grossly, by pre or postnatal imaging, or microscopically, the malformations appeared remarkably similar to the spectrum of human malformations characterized

as CCAM. Preliminary *in vitro* results, confirm that microinjection of adenoviral vector encoding rFGF10 induces focal cystic formations in fetal rat lung explant cultures (data not shown).

As expected, overexpression of FGF10 in this study was focal rather than diffuse, and was expressed in interstitial cells rather than ectopically from respiratory epithelium. The expression profile of FGF10 demonstrated that there is a peak approximately 24 hours after injection with rapid decrease of transgene expression. Preliminary results of *in vitro* study showed that fetal rat lung explants injected with Ad-rFGF10 exhibited an increase of phosphorylated ERK1/2, whereas no differences were observed in p38, Akt and Src phosphorylation, when compared with PBS injected explants. These data confirm the involvement of ERK-MAPK pathway in the formation of FGF10-induced cyst malformation.

Current models in branching morphogenesis propose that FGF10 and reciprocal influences such as Bmp4 and Spry2 regulate lung development. Highly localized expression of fgf10 in the mesenchyme adjacent to the tip of the nascent airway stimulates proliferation and growth outward. FGF10 signaling induces a steady increase in Bmp4, Spry2 or other reciprocal factors in epithelial cells. Bmp4 and Spry2 are inhibitory to epithelial cell proliferation/movement arresting the outward movement of the developing airway [Mailleux AA, et al. 2001; Weaver M, et al. 2000]. The distinct phenotypes for cystic formations, secondary to early versus late overexpression of FGF10, can be interpreted. During early stages of branching morphogenesis, high levels of mesenchymal FGF10 expression would be expected to induce a burst of proliferation and outward migration of the epithelium responsive to FGF10, with less proliferation at branch points where Bmp4 and other antagonists are expressed. Our results showed that BMP4 and for Spry2 inhibitory effect over FGF10 might not be effective in lungs. Thus, at the early time point, within the first 24 to 48 hours when FGF10 levels are high unopposed action of FGF10 allows the rapid formation of large cysts. In contrast, in late injected lungs, expression of these two factors increase in the epithelium surrounding cystic areas, leading to the formation of smaller cysts. Taking in consideration the spatial variable of our model: proximal versus distal injections, we suggest that, during pseudoglandular stage, lungs injected in the proximal airways, where fewer branch points exist, and high columnar epithelium predominates, unopposed FGF10 signaling would be predicted to result in large cysts lined by predominantly bronchial epithelium. Whereas, in distal airways, where multiple branch points are already present and cuboidal epithelium predominates, smaller and more numerous cysts would form lined by predominantly alveolar epithelium. During the canalicular stage of lung development only terminal branching occurs, so FGF10 overexpression might stimulate short

segments of responsive epithelium into small cyst and differentiated adenomas formation. Curiously, the binome Hoxb-FGF10 is a well established as the promoter of cystic like structure formation in the chick lung. Studies showed that the pattern difference between the branched airway (dorsal) and the air-sac (ventral cystic structure) in chick lung is due to the difference in the diffusion coefficient of FGF10 between these two regions [Miura T, et al. 2009], and that that the cyst-branch difference in this system is caused by region-specific mesenchymal properties related to Hoxb cluster nested gene expression [Sakiyama J, et al. 2000]. In our study, we observed rapid formation and persistence of the cystic lesions. These findings support the hypothesis that FGF10 is involved in the early inductive events in CCAM formation and that continued presence of FGF10 is not necessarily required in the natural evolution of CCAM malformations.

CCAMs are relatively rare developmental abnormalities of the lung that cause significant morbidity and mortality in infants due to associated respiratory distress, lung hypoplasia, fetal hydrops and pulmonary infections. The lesions are currently described as hamartomatous lesions, i.e. normal lung tissues in a disorganized spatial arrangement. In these lesions, the terminal bronchioles develop a nonsystematic overgrowth, with a consequent suppression of alveolar growth. The result of this process is a multicystic mass that replaces the normal lung structure. Human CCAMs are confined to a single lobe and can be classified as macrocystic or microcystic [Wilson RD, et al. 2006; Laje P and Liechty KW 2008].

Interestingly, the ultrasound and MRI appearance, lobar distribution, and ultrasound classification of the malformations observed were strikingly similar to human CCAM. FGF10 gene transfer during the pseudoglandular stage resulted in macrocystic lesions by ultrasound classification that were confirmed by MRI evaluation at Postnatal Day 7. A completely different ultrasound pattern was observed when gene transfer was performed during the canalicular stage—no cysts were visualized, but well-defined areas of increased echogenicity could be identified. Other classification of CCAM, based on clinical features, macroscopic and microscopic criteria, proposed by Stocker [Stocker JT 1994], classified CCAM into five 5 types. Depending on differences in cytodifferentiation, these five categories could be assembled into two major subtypes: CCAM types 1, 2, and 3, with a bronchiolar-type epithelial differentiation; and a second subtype consisting of CCAM type 4, which has an acinar-alveolar epithelial differentiation. Despite CCAM pathogenesis remains unknown, several authors have hypothesized that to originate at distinct stages of lung development the first subtype (CCAM types 1, 2, and 3) may develop at the pseudoglandular stage, and the second subtype (CCAM type 4) may be due to a late event that disrupts branching of the distal acinar structures in the saccular

period [Morotti RA, et al. 1999]. Our results support this pathophysiologic hypothesis: pseudoglandular stage injections induced cysts resembling bronchiolar-type epithelial differentiation, whereas canalicular stage injections induced cysts resembling acinar epithelial-type differentiation of human CCAMs.

Several studies, using human fetal resected CCAM tissue, tried to discern factors that could be responsible for this pathology. Volpe et al. demonstrated that Hoxb5, in human fetal resected CCAM tissue, was maintained in a higher level of expression, characteristic of early stages of lung development [Volpe MV, et al. 2003]. Recently, these authors have also demonstrated that specific cell adhesion molecules, such as α-2 integrin and E-cadherin, important to lung development and airway morphogenesis are altered. They also suggested that CCAM pathogenesis might be associated with potentially altered integrin cytoplasmic signaling [Volpe MV, et al. 2009]. Fgf 7 gene expression or protein production was evaluated and no differences were found when compared with normal lungs [Cass DL, et al. 1998]. PDGF-BB gene expression and protein production were found to be increased [Liechty KW, et al. 1999], and glial cell–derived neurotrophic factor presented abnormal expression in epithelial cells lining CCAM cysts. Jancelewicz et al., analyzed gene expression from laser dissected epithelium and mesenchyme of human fetal and postnatal CCAM. They demonstrated that markers of early lung development, such as Hoxb5 and TTF1, are overexpressed in fetal CCAM. They also demonstrated that FGF9 was overexpressed, however a decrease in FGF7 and no altered expression of FGF10 and FGFR2 [Jancelewicz T, et al. 2008]. Besides, increased proliferation and decreased apoptosis were also verified in CCAM specimens [Cass DL, et al. 1998]. All these data, strongly suggest a focal arrest in lung maturation during the fetal period. However, these studies have a common major limitation, which is that the analysis was performed in CCAM specimens that were surgically resected, analysis of which is unlikely to reveal the initial inciting events. Previous studies in transgenic murine models, where heterotopic overexpression of FGF7 [Simonet WS, et al. 1995] and FGF10 [Clark JC, et al. 2001], and orthotopic overexpression of FGF9 [White AC, et al. 2006] resulted in marked perturbations of lung morphogenesis, suggesting that these factors might be implicated in the development of adenomatoid malformations. Again, it is unknown whether anomalous overexpression of genes such as FGF9 represents causation or a global delay in differentiation.

In our study, we have been able to evaluate in a prospective fashion the formation of CCAM-like malformations in response to overexpression of FGF10 in the mesenchymal compartment of the developing lung. We have demonstrated that transient overexpression of a single gene in a focal area of the lung is sufficient to reproduce the spectrum of gross and histologic features of human CCAM in a rat model. The striking similarity of these lesions to those seen in human CCAM strongly implicates mesenchymal overexpression of FGF10 in the initial events invoking CCAM formation. Our data thus far support FGF10 overexpression as the most important mechanistic component of CCAM formation. However, the identity of the primary mesenchymal perturbation that induces FGF10 over-expression remains to be determined.

We have developed a powerfull tool for lung development modulation early in gestation that specifically targets the mesenchymal compartment. Despite not being able to induce lung growth, we contribute to unveil the mechanism underlying the formation of CCAM, and possibly to establish an experimental animal model for this disease. The selection of other growth or transcription factors and the application of the intrapulmonary fetal gene transfer methodology to murine models of human diseases, opens the perspectives for future pre-clinical studies.

Main Conclusions

1. We report for the first time, the feasibility of using ultrasound biomicroscopy to perform *in utero* intrapulmonary injections in rats during pseudoglandular stage of lung development. Both adenoviral and lentiviral vectors efficiently transduced lung parenchyma, although different expression patterns were observed following injection. Adenoviral vector expression was transient and started very quickly after its injection, whereas lentiviral vector expression was relatively delayed and persisted through seven days after birth, the time-point that we analyzed.

2. The possibility of targeting the lung during the pseudoglandular stage may present unique experimental and ultimately perhaps clinical opportunities. Gene transfer during this period has the potential to modulate major key elements of pulmonary branching with minimal effects on immune responses. The ability to achieve gene transfer by direct intrapulmonary injection in a rat model provides a novel tool for exploration of potential therapeutic strategies for lung diseases.

3. Pulmonary interstitial cells, rather than alveolar or airway epithelial cells, or vascular endothelial cells, were selectively transduced both by adenoviral and lentiviral vectors. This was the first observation of this pattern of parenchymal expression following prenatal lung gene transfer. The ability to target mesenchymal compartment raises the possibility to modulate mesenchymal-factors involved in lung development with a more discrete and physiological approach.

4. FGF10 over-expression in the lung mesenchyme during pseudoglandular stage by intraparenchymal gene transfer did not induced lung growth. Instead, there was a disturbance of pulmonary morphogenesis with appearance of localized cystic lung malformations. The type of malformation observed was developmental stage and location dependent, with the spectrum ranging from macrocystic malformations lined by predominantly bronchial epithelium, to microcystic malformations lined by predominantly alveolar epithelium.

5. We have demonstrated prospectively that transient over-expression of a single gene in a focal area of the lung is sufficient to reproduce the spectrum of gross and histological features of human CCAM in a rat model. The striking similarity of these lesions to those seen in human CCAM strongly implicates mesenchymal over-expression of FGF10 in the initial events invoking CCAM formation.

CHAPTER 5

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