2. LITERATURE REVIEW

2.1 Pulmonary Hypertension

Pulmonary hypertension (PH) is an uncommon lung disorder characterized by increased arterial pressure and vascular resistance within the pulmonary circulation. There are two types of PH i.e. precapillary PH and postcapillary PH which are differentiated from each other as depicted in **Figure 2.1**. Patients of post capillary PH, experience pulmonary venous congestion, which can lead to increased pulmonary capillary pressure (PCP) and pulmonary arterial pressure (PAP). Conversely, precapillary PH is accompanied by an isolated increase in PAP with normal PCP. Precapillary PH may lead to corpulmonale, right heart failure, and even death (1).

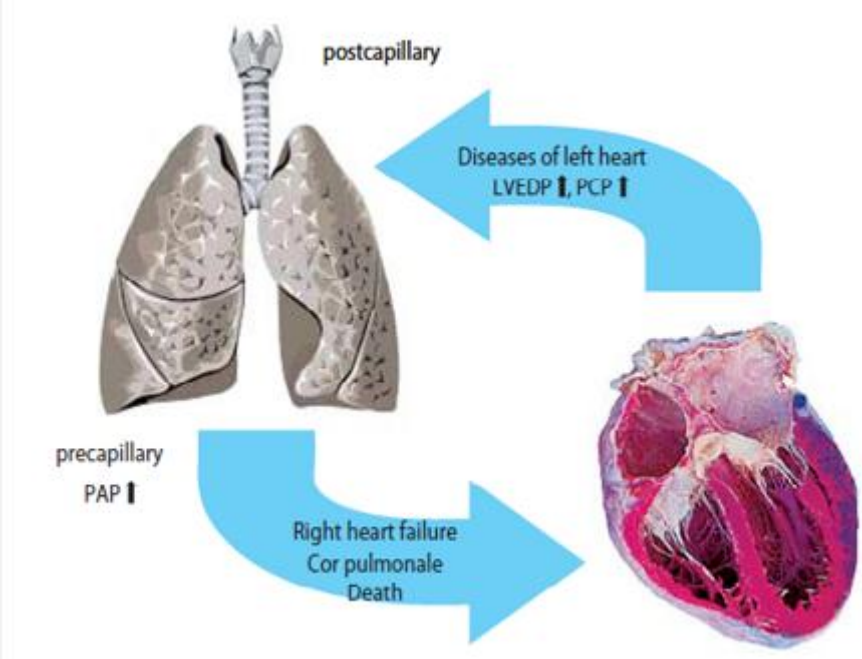

Figure 2.1 Pathogenesis of pulmonary hypertension.

In pathophysiological terms, PH is defined as a condition with multiple etiologies resulting in altered body's hemodynamics. As depicted in **Table 2.1,** a clinical condition wherein mean pulmonary arterial pressure (PAP) rises above 25 mmHg at rest, when measured by right heart catheterization (RHC), is referred to as PH (2, 3).

Table 2.1 Hemodynamic definitions of pulmonary hypertension

Definition	Characteristics	Clinical Group(s)
Pulmonary hypertension	Mean PAP ≥25 mmHg	All
Pre-capillary PH	Mean PAP ≥25 mmHg; PWP ≤15 mmHg; Cardiac Output (CO) normal or reduced[a]	1. PAH 2. PH due to lung diseases 3. Chronic thromboembolic PH 4. PH with unclear/ multifactorial mechanisms
Post-capillary PH	Mean PAP ≥25 mmHg; Pulmonary Wedge Pressure >15 mmHg; CO normal or reduced[a]	PH due to left heart disease

[a]High CO can be present in cases of hyperkinetic conditions such as systemic-to-pulmonary shunts (only in the pulmonary circulation), anemia, hyperthyroidism, etc.

2.2 Classification of Pulmonary Hypertension

Pulmonary hypertension is classified into various types depending on the cause and pathophysiology of disease. Good knowledge of this classification system can give idea of factors involved in the development of PH which can finally assist in deciding the treatment option for amelioration of PAH. The first classification for PH was conscripted in 1973 and from that time it has undergone several changes. In 2003, a classification system was drafted at Venice which later came to be known as Venice classification (4). This classification system was revised at fourth world symposium on pulmonary hypertension held at Dana Point, Silicon Valley, US, by the consensus of an international group of experts (5). In Dana point classification, several amendments were made in order to accurately reflect the information published over the past five years as well as to clarify certain areas that were ambiguous in the previous classification.

Dana Point Clinical Classification 2008

Group I - Pulmonary arterial hypertension (PAH)

1.1. Idiopathic (IPAH)

1.2. Heritable Familial (FPAH): BMPR II; ALK1, endoglin

1.3. Drug and toxin induced

1.4. Associated PAH (APAH): Collagen vascular disease (e.g. scleroderma); HIV infection; Portal hypertension; Congenital shunts between the systemic and pulmonary circulation; Schistosomiasis; Chronic hemolytic anemia

1.5. Persistent pulmonary hypertension of new born

Group II - Pulmonary hypertension associated with left heart disease

1.6. Systolic dysfunction

1.7. Diastolic dysfunction

1.8. Valvular disease (e.g. mitral stenosis)

Group III - Pulmonary hypertension associated with lung diseases

1.9. Chronic obstructive pulmonary disease (COPD),

1.10. Interstitial lung disease (ILD)

1.11. Other pulmonary disease with mixed restrictive and obstructive pattern

1.12. Sleep-disordered breathing

1.13. Alveolar hypoventilation

1.14. Chronic exposure to high altitude

1.15. Developmental lung abnormalities

Group IV - Pulmonary hypertension due to chronic thrombotic and/or embolic disease

1.16. Pulmonary embolism in the proximal or distal pulmonary arteries

1.17. Embolization of other matter, such as tumor cells or parasites

Group V – PH with unclear and/or multifactorial mechanisms

1.18. Hematological disorders

1.19. Systemic disorders

1.20. Metabolic disorders

1.21. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure

(ALK-1=activin receptor-like kinase 1 gene; BMPR II=bone morphogenetic protein receptor, type II; HIV=human immunodeficiency virus)

2.3 Epidemiology of PAH

PAH is a disease that affects about 15 to 50 subjects/ million inhabitants in the western world (6). A national registry in US in 1980 found about 187 patients of PAH corresponding to idiopathic and familial origin which was observed for five years along with their natural history (7). The French PAH Registry has gathered data on PAH patients in the modern management era (7, 8) and studied survival during a three-year follow-up. In this registry established in 2002 to 2003, 674 patients were incorporated with a strict catheter diagnosis of PAH in a network of about 20 university pulmonary vascular centers spread throughout the country (2).

About 53% of enrolled patients presented with idiopathic (39.2%), familial (3.9%), or anorexigen-associated PAH (9.5%). The remaining patients had PAH associated with other diseases such as connective tissue disease, congenital heart diseases, portal hypertension, or HIV infection (**Figure 2.2**). Particularly of interest is the fact that 29 patients (4.3%) displayed HIV infection and portal hypertension which are coexisting conditions known to be associated with PAH (**Figure 2.2**). Idiopathic PAH corresponds to sporadic disease, without any previous familial history or any other known trigger (9). As per the French registry, women are commonly affected from idiopathic PAH with a female to male ratio of 1.6:1.

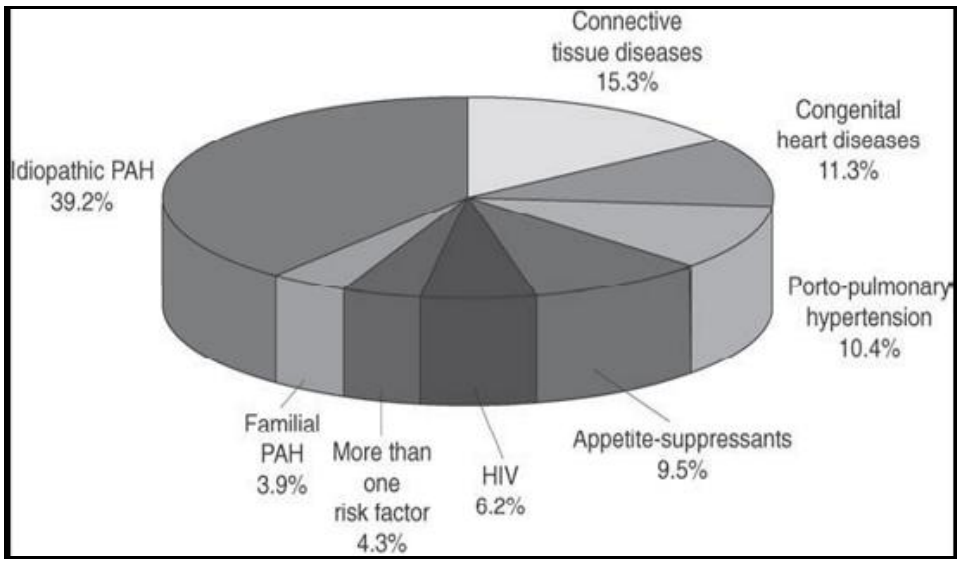

Figure 2.2 Distribution of patients with PAH in the 2002 to 2003 French Registry.

In developing countries, where prevalence of diseases such as chistosomiasis, sickle cell disease, HIV infection, liver cirrhosis, autoimmune diseases and congenital heart diseases is high, PH is much more prevalent than reported, as such diseases may trigger pulmonary vascular disease (6). In addition, hypoxia is a major risk factor for pulmonary hypertension affecting more than 140 million individuals living above 2500 million worldwide, including 80 millions in Asia and 35 millions in South America (10). Particularly, inhabitants of Andean mountains and other mountainous regions may show chronic mountain sickness which has become public health problem in such regions around the world (6, 10). In China and Brazil, PH is now being formally studied (11, 12). WHO program of the Global Alliance Against Chronic Respiratory Diseases (GARD) preaches in order to improve the awareness for diagnosis, prevention and treatment of pulmonary hypertension in developing countries (11). However, the global burden of pulmonary hypertension as a whole is currently unknown and largely underestimated (2, 6, 7).

Additionally, approximately 4% of patients with acute pulmonary embolism may develop chronic thromboembolic disease and pulmonary hypertension (13, 14). Altogether pulmonary hypertension is surely underrated both in developing and in developed countries, and further well-designed studies are mandatory in order to cope up with spread of the disease in populations exposed to different risk factors.

2.4 Pathogenesis

PH is a multifactorial heterogeneous disease characterized by increased pulmonary vascular resistance (PVR) and pulmonary artery pressure (PAP) either due to cardiac and pulmonary diseases or pathological changes observed in pulmonary vasculature. Augmented pulmonary vascular resistance may be due to vasoconstriction, vascular remodeling or thrombosis. As apparent from recent genetic and pathophysiological studies, PH is manifested by a combination of genetic factors, associated diseases and/or several other trigger mechanisms. During earlier stages of PAH, there are slight functional alterations of the pulmonary vasculature, but with progression of the disease morphological changes become apparent, especially vascular remodeling in small pulmonary arteries. Pulmonary vascular remodeling involves all layers of the vessel wall and is further complicated by cellular heterogeneity within

each compartment (15). The pathogenesis of PAH is outlined along with possible targets for treatment of such disorder in **Figure 2.3**. Indeed, endothelial cells, smooth muscle cells, fibroblasts as well as inflammatory cells and platelets play an important role in this condition. Endothelin-1, prostacyclin, nitric oxide-mediated signals and other such mediators play a vital role in the pathogenesis of PAH and such factors can be used as therapeutic targets for the effective management of this disease (16).

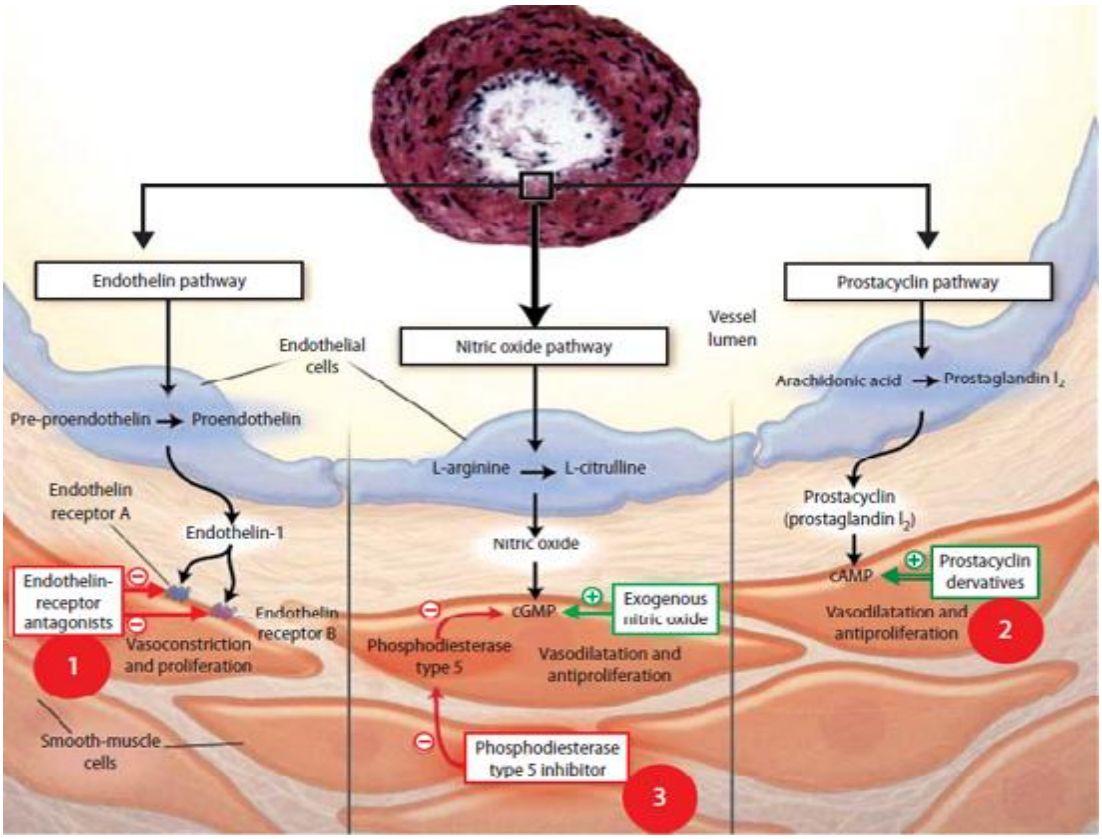

Figure 2.3 Potential targets for treatment of pulmonary hypertension.

2.5 Diagnosis

Diagnosis of "pulmonary hypertension" poses a formidable challenge in patients with dyspnea which may lead to delayed diagnosis, often by several months or years (17). Hence, the awareness for this disease is required along with improvements in its diagnosis. There are no specific signs and symptoms which can surely confirm the existence of this disease. Several non-specific symptoms like exertional dyspnea, decline of physical performance, fatigue, weakness, angina, syncope, peripheral edema, and abdominal distension may be used

as a marker for this disease. During diagnosis of patients, physical examination should focus on further signs and symptoms, because this may be the clue to possible linked diseases that represent the underlying cause of pulmonary hypertension. If physical examination (e.g., accentuated pulmonary component of S2, pansystolic murmur of tricuspid regurgitation, jugular vein distension, peripheral edema, hepatomegaly, ascites), clinical presentation (dyspnea on exertion/at rest, fatigue, weakness), and non-specific tests such as ECG (e.g., right ventricular hypertrophy and strain) or chest X-ray (e.g., central pulmonary arterial and/or right ventricular enlargement) indicates pulmonary hypertension, both non-invasive and invasive tests may be applied to confirm the diagnosis.

2.6 Current Therapy for Treatment of PAH

Many patients with PAH receive background or conventional therapy of warfarin, diuretics, digoxin and oxygen. Diuretics and digoxin provide symptomatic relief but are not thought to affect the course of the disease. Warfarin might provide a survival advantage but its contribution is difficult to estimate and its use is based on retrospective analysis (18). Calcium channel blockers, such as nifedipine, offer considerable benefit to the small number of patients that respond to them, although these account for only around 6% of patients (19).

Currently there are three main classes of drugs in use for the treatment of this disease; prostanoids, endothelin-1 receptor antagonists, and phosphodiesterase-type V (PDE V) inhibitors. Unfortunately there remains no cure for PAH and clinical worsening is unavoidable (20). L-type calcium channel blockers (eg. nifedipine) can be effective but are only safe for use in patients who respond to a one time vasodilator challenge with a 20% fall in mean PAP and no decline in cardiac output (21).

In last 25 years, numbers of treatments have been introduced in market to directly target at the pathology of PAH. Epoprostenol was the first of these to be studied, and in addition to symptomatic improvement, it is the only treatment that has been shown to offer a survival advantage (22). However, its administration (intravenous, via an indwelling catheter) is complex and it causes adverse effects such as headache, nausea and diarrhoea caused by systemic vasodilation and carries the risk of line sepsis and rebound PAH from inadvertent

interruption of infusion (23). Other prostanoid analogues such as iloprost (inhalation), treprostinil (subcutaneous) and beraprost (oral) can provide only short-term benefits which was confirmed from clinical trials (24).

A recent meta-analysis (25) investigated the efficacy of pharmacological treatments for PAH which created doubt regarding the true benefit of the current treatments. Although current therapies can offer some hope for PAH patients, they are accompanied with several adverse effects and still do not provide complete cure. Whereas the time to clinical worsening of symptoms is increased, the progression of the disease is inevitable and improved treatments must be developed. Definitely, in clinical practice, combination therapy has become the default position even though evidence to support this strategy is limited. Small scale clinical evaluation of combinations of prostanoids, Endothelin-1 receptor antagonists and PDE-V inhibitors have been tried with some success (26), with additional studies currently recruiting [eg. COMPASS-2 (sildenafil plus bosentan), STEP (iloprost plus bosentan)]; however, validation of these combination therapies will require further larger scale trials.

The advances in understanding of PAH pathophysiology has dramatically changed current approaches to experimental therapies of PAH. Better knowledge of role of endothelial pulmonary cells and pulmonary arterial smooth muscle cells in repair of damaged vessels can help to design novel and cell based therapies for treatment of PAH. Recent, data suggest that PECs may modulate immune functions in experimental models of PAH. Over expression of anti-inflammatory cytokine with vasculo-protective properties prevented MCT-induced PAH. Hence, future cell-based therapies will likely be triple-targeted to positively affect pulmonary vascular hemodynamics, vascular apoptosis/proliferation, and inflammation in treatment of PAH.

As we have gone through all research work and review articles, the current treatment available in market for PAH is expensive and/or difficult to deliver and are more palliative than curative. These treatments may slow the progression of the disease but do not afford a cure. In this regard, the treatment with cell based therapy (i.e. therapeutic genomics) may target more directly the vascular changes like proliferation, apoptosis process, immune mechanism and

pulmonary blood flow impairing inflammation would treat pulmonary arterial hypertension in a very significant way.

2.7 Gene Therapies for Pulmonary Hypertension

Pulmonary hypertension (PH) is a disease characterized by a marked and sustained elevation of pulmonary vascular resistance (PVR) with consequent progressive right ventricular failure and death (9). Endothelial dysfunction characterized by altered homeostasis between vasoconstrictors and vasodilators, i.e. reduced production of vasodilators like prostacyclin and nitric oxide and augmented production of vasoconstrictors like thromboxane and endothelin-1, mark the onset of this disease (27). Thus, the main objective for the development of therapeutic agents used in this disease is to restore the homeostasis between pulmonary endothelium-derived vasodilators and vasoconstrictors and this was the main stimulus for the development of prostacyclin and endothelin receptor antagonists. But the patients refractory to this agents have to undergo heart—lung or lung transplantation. Furthermore, systemic side effects and inconvenient drug administration routes may restrict the widespread use of vasodilator agents in the clinical management of PH (28). There is now a shift in the interest of the scientific community, focusing on therapies aiming to reverse the proliferative remodeling in PH (29).

A group of susceptible genes, including bone morphogenic protein receptor type II (BMPR-II) (30) and vasoactive intestinal peptide (VIP) (31) were found to be involved in the pathogenesis of primary pulmonary hypertension (PPH). Furthermore, a signature set of 106 genes discriminating patients with PH and normal individuals with high certainty, was identified. All this findings confirmed the importance of genetic predisposition in the development of PH. Simultaneously, literature indicates that gene transfer into lungs, aiming at correcting the deficiency or mutation of these genes, opens a new window for the treatment of this devastating disease. Number of genes have been involved in the development of pulmonary hypertension and are discussed in brief in **Table 2.2**.

Table 2.2 Involvement of different genes in pulmonary hypertension

Gene	Comment
eNOS	Endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) is believed to play an important role in maintaining physiological levels of PAP and PVR by inhibiting platelet aggregation and proliferation of vascular smooth muscle cells (VSMCs) (28, 32, 33).
CGRP	Calcitonin-gene-related peptide (CGRP) is a 37-amino acid endogenous peptide, formed as an alternative splice product of the calcitonin gene (34), with potent vasodilating effect and anti-proliferative effects on VSMCs (35, 36).
VEGF	Vascular endothelial growth factor (VEGF) is a mitogen specific peptide for ECs which fulfills its function by binding to two highly specific tyrosine kinase receptors (flt-1 and KDR/flk-1) expressed almost exclusively on ECs (34, 37, 38).
PGIS	Prostacyclin is a major arachidonic acid metabolite synthesized by prostaglandin I_2 synthase (PGIS) in the vascular wall having several vasoprotective effects like vasodilation, anti-platelet aggregation and inhibition of PASMCs proliferation *in vitro* (29, 39, 40).
AM	Adrenomedullin (AM), a potent long-lasting vasodilator peptide originally isolated from human pheochromocytoma (41) has the ability to induce tube formation in human umbilical vein endothelial cells-HUVECs, drive sprouting in porcine pulmonary arterial endothelial cells and promote new vessel formation in a mouse Matrigel plug assay (42).
BMPR-II	Many cases of familial and sporadic PPH are associated with heterozygous mutations in BMPR-II, a member of the transforming growth factor superfamily of receptors (43). In addition to these mutations, marked reduction of BMPR-II expression in the lung was also observed in patients with PPH in whom no mutation was identified in the BMPR-II gene and also among patients with secondary PH (44).

HGF	Hepatocyte growth factor (HGF) was originally purified and cloned as a potent mitogen for hepatocytes. HGF has a pulmotrophic effect on the regeneration and protection of the lungs besides having mitogenic, mutogenic and morphogenic activities in various cell types (43, 45, 46).
Kv	There is monopoly on expression of O_2 -sensitive Kv channels, especially K v1.5 and Kv 2.1 to some lesser extent in resistance pulmonary arteries, which is responsible for the heterogeneity of Hypoxic pulmonary vasoconstriction (HPV) localization in pulmonary circulation (47, 48).
Survivin	Recently, various studies reported that imbalance in the level of mediators regulating the proliferation and apoptosis of the cells within vascular walls might result in pulmonary arteries remodeling (49).
FGF2	It was found that FGF2 was released excessively by PECs of patients with IPH compared to other factors like PDGF, TGF-β, and EGF etc. FGF2 is a member of heparin-binding growth factors' family (50) and is synthesized by tumor cells, fibroblasts, ECs, and macrophages (51).

2.8 Fibroblast Growth Factor 2 (FGF2)

We focused on FGF2 because, among the main growth factors expressed by ECs, FGF2 is released in excessive amounts by P-ECs of patients with PAH. FGF2, a member of a large family of heparin-binding growth factors, is synthesized by several cell types including tumor cells, fibroblasts, ECs, and macrophages. Also, it was found that FGF2 can be sequestered and stored as a complex in the extracellular matrix and then released by proteolytic processes to bind and activate cell targets promoting mitogenesis (52). FGF2 binds to high affinity tyrosine kinase FGF receptors (FGFRs) expressed on the surface of vascular cells and thus exerts its biological activity (53). Increased levels of lung and circulating FGF2 have been reported in both experimental and human PH. In IPH approximately 51% of patients showed higher levels of FGF2 in blood, while 21% patients had increased FGF2 levels in urine. A lamb model of PH developed by inserting an aorto-pulmonary vascular bypass graft and the rat model of monocrotaline-induced (MCT-induced) PH also showed overexpression of FGF2 (54).

FGF2 has some unique features which can be consider during development of siRNA targeted to FGF2. Four cysteine residues at amino acids 26, 70, 88 and 93 are present in FGF2. While the cysteines at 26 and 93 are conserved, those at 70 and 88 are absent or located elsewhere in other type of fibroblast growth factor (55). Mutation of all four cysteines to serines results in a protein with the same secondary structure and equally mitogenic for 3T3 cells as the wild-type FGF-2 (56), suggesting that the formation of disulfide bridges is not important for the secondary structure and mitogenic activity of FGF-2 (55). It has also been observed that FGF-2 is a substrate for phosphorylation by protein kinase C (PKC) and protein kinase A (PKA). PKC phosphorylates FGF-2 at Ser64; however this has no effect on biological activity, heparin-binding capacity or receptor-binding capacity. On the other hand, PKA phosphorylates FGF-2 at Thr112 in the FGF receptor binding domain, resulting in 3- to 8-fold better binding (57).

The role of FGF_2 in MCT induced PH rat models was evaluated to find out the *in vivo* effect of FGF2 on the development of PH. Subcutaneously administered MCT is converted to its pneumotoxic metabolites in the liver, which are transported to the lungs, causing early injury to the pulmonary arterial endothelium and an inflammatory response before the onset of PA-SMC proliferation and the development of PH (58). Alterations in the structure of the pulmonary vessel walls (pulmonary vascular remodeling) include thickening of the medial layer of the SMCs, increased extracellular matrix synthesis and deposition, and mononuclear inflammatory cell infiltration resulting in increased thickness of the adventitial layer which ultimately leads to right ventricle (RV) hypertrophy. Accentuated expression of FGF2 has been documented in malignancies, whereby FGF2 acts on tumor cells via autocrine or paracrine mechanisms (59). Moreover, FGF2 binds heparin sulfate, proteoglycans and heparin, thereby establishing a biological reservoir that can be released locally in a regulated manner. This suggests that, in addition to the paracrine effect recently demonstrated in the PA-SMCs, the excess FGF2 observed in PAH might modulate the P-EC phenotype via separate autocrine effects.

Vascular disease in MCT-treated rats may cause morbidity and mortality (60). From studies, the role of FGF2 as a link between altered EC signaling and pulmonary vascular remodeling was confirmed. Additionally, elevation in FGF2 expression was found during the

development of MCT-induced PH within 12 hours after injection even when PA-SMC proliferation was not detectable. It was also reported that administration of FGFR inhibitor SU5402 resulted in considerable attenuation of PH development, as determined on 21st day based on the basis of reduction in PAP, RV hypertrophy, and distal pulmonary artery muscularization, compared with control rats. siRNA delivery targeted to FGF2 gene can be a good genomic approach in treatment of PAH.

2.9 RNA Interference

In the last decade, 'RNA Interference' (RNAi) was considered as one of the most important innovations in biology which is used to control gene expression within cells for the management of various diseases and also useful in study of gene function. In 1998, researchers innovated a new concept of RNAi for target-specific gene silencing in *Caenorhabditis elegans* (61). Additionally, small RNAs regulating genetic expression and controlling cellular function were discovered in the last decade. Various types of RNA like microRNAs (miRNA) (endogenous), short interfering RNAs (siRNAs, endo-siRNAs) and piwi interacting RNAs (piRNAs) were found to be the basic controllers of endogenous processes like apoptosis, stem cell self-renewal, differentiation and maintenance of cell integrity (62, 63). In recent times, microRNA (miRNA) therapy by administering synthetic version of exhausted miRNA has evolved as an innovative approach for cancer and neurodegenerative disease therapy (64). For effective silencing of gene responsible for disease, knowledge of target mRNA sequence and designing its complementary anti-sense sequence is essential which may be applied for personalized treatment of cancer, HIV and other viral diseases. This technique can also be applied to overcome the resistance of chemotherapeutic agents (65). In contrast to other available gene therapies, miRNA technique down-regulates disease specific protein or gene rather than acting on faulty gene and thus help in cure of the disorder. Diverse information is available on genes from the massive human genome projects which can be applied for development of antisense drugs. Various therapeutics based on this approach used for therapy of various disorders like cancer, HIV, age-related macular degeneration, respiratory syncytial virus infection as well as rare diseases like pachyonychia congenital is under clinical trials, while some are in queue to enter clinical trials. Unfortunately, Phase II trial of Sirna-027 from Sirna Therapeutics has been terminated.

2.9.1 Targets of RNAi

Gene silencing can be achieved by various approaches as follows:

1) Blocking synthesis of complementary mRNA from the targeted DNA molecule (transcription) which can be achieved by two different strategies - strand invasion and triple-strand formation. Amongst these strategies, triple-strand formation which includes formation hydrogen bonds between the third strand and the complementary strand of dsDNA molecule is most commonly used e.g. Homopyrimidine oligonucleotides (66).

2) Blocking of post transcriptional gene silencing (PTGS) phenomenon that includes inhibition of protein synthesis by the knock down or knock out of transcribed target mRNA.

Basis for gene therapy deals with targeting a particular gene which either gets knocked out or knocked down by antisense molecules such as RNAi therapeutics but there is a huge challenge in developing RNAi therapeutics to achieve effective knock-down or knock-out. More depth in this field and better knowledge and understanding can give more pronounced results by RNAi therapy.

2.9.2 Cellular Mechanisms of RNAi

RNAi therapy causes knock down of target gene or mRNA and thus give pharmacological effect serving the primary objective of the therapy. RNAi molecules exert their therapeutic effect by acting on mRNA molecules produced from DNA, which finally guides protein synthesis by translation process (**Figure 2.4**).

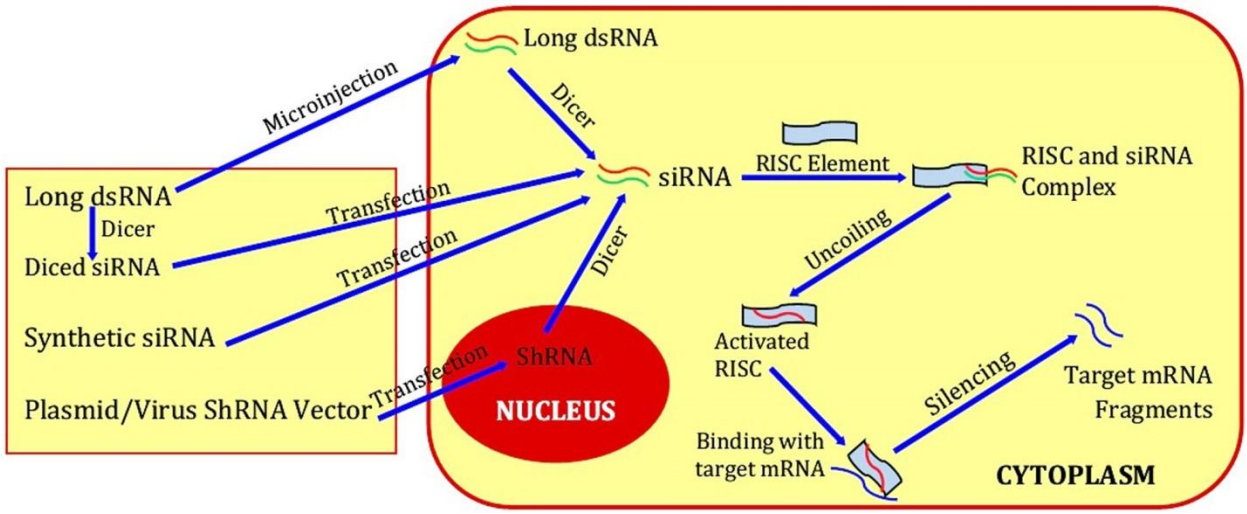

Figure 2.4 Cellular mechanism of RNAi.

RNAi, monitoring the activity and potency of genes within mortal cells, was also referred to as co-suppression, post transcriptional gene silencing (PTGS) or quelling. The RNAi pathway is divided into two phases such as initiation phase and execution phase. Presence of dsDNA precursor triggers the initiation phase which results in formation of short fragments of 20-23 nucleotides with over-hanging 3' ends known as siRNA (67). Enzyme dicer, having C-terminal dsRNA binding domain, an N-terminal RNA helicase as well as two RNaseIII like domains cleaves the dsDNA and forms siRNA (68). siRNA so formed is uncoiled into two single strands i.e. passenger strand and guide strand in execution phase. Amongst the two strands, the passenger strand gets degraded while guide strand couples with RNA induced silencing complex (RISC) leading to formation of a large multiprotein complex which brings out the post transcriptional gene silencing (PTGS). PTGS refers to a sequence specific base coupling between the guide strand of the siRNA and the target mRNA resulting in endonucleolytic cleavage of the mRNA strand across the middle of the siRNA strand (69, 70) and later degradation of the targeted unprotected mRNA. Due to the potency, maximal effectiveness, duration of action, and sequence specificity of small interfering RNA (siRNA), it has become an important tool of RNAi therapy both *in vitro* and *in vivo* (71-73).

2.10 Small Interfering RNA (siRNA)

Gene silencing by siRNA includes its binding to corresponding mRNA and degradation of target mRNA. Small interfering RNA (siRNA) belonging to a class of double stranded RNA

can be used for knockdown or silencing of targeted genes in most of the cells. siRNA is 19–23 base pair (bp) in length with molecular weight of about 13 to 15 kDa and negative charge of about 38 to 46 mV. The structure of siRNA consist of a two-nucleotide overhang and a hydroxyl group on the 3′ end of both strands and a phosphate group on the 5′ end (**Figure 2.5**) (69). Before getting assembled into a RISC, siRNAs must unwind and form single stranded structures. RISC is guided to the appropriate target mRNA molecule by the sense strand of siRNA where it destroys the complementary mRNA. The broken mRNA is rapidly degraded and protein expression is reduced or abolished (**Figure 2.6**) (69, 74).

siRNAs are divided into two classes depending on the thermodynamic stabilities at the two ends-symmetric siRNAs and asymmetric siRNAs. A symmetric siRNA consists of two equally stable ends and thus both the strands of the siRNA are assembled into the RISC with equivalent efficiency. An asymmetric siRNA contains one end with less stability than the other and after unwinding from the less stable end one strand of the siRNA can show the asymmetric assembly of RISCs (75). In mammalian cells, synthetic siRNA duplexes can activate RNAi which can cause knock-down of target mRNA sequence and hence can halt corresponding protein production. Bioinformatics can be applied to predict specific siRNA sequences for many target mRNAs. These artificial siRNAs are capable of silencing their complementary mRNAs by mechanisms similar to those of endogenous siRNA. siRNA can either be synthesized chemically as oligos (siRNAs) or cloned into a plasmid or virus vectors like adenovirus, retrovirus or lentivirus as short hairpin RNAs (shRNAs).

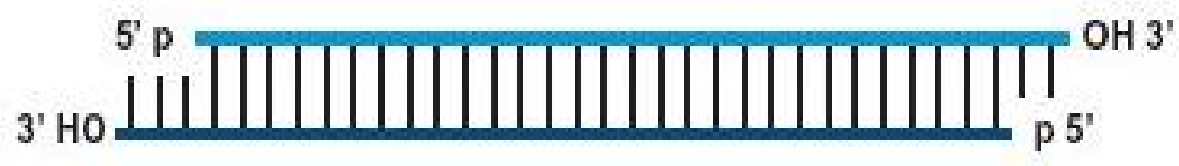

Figure 2.5 Basic structure of siRNA.

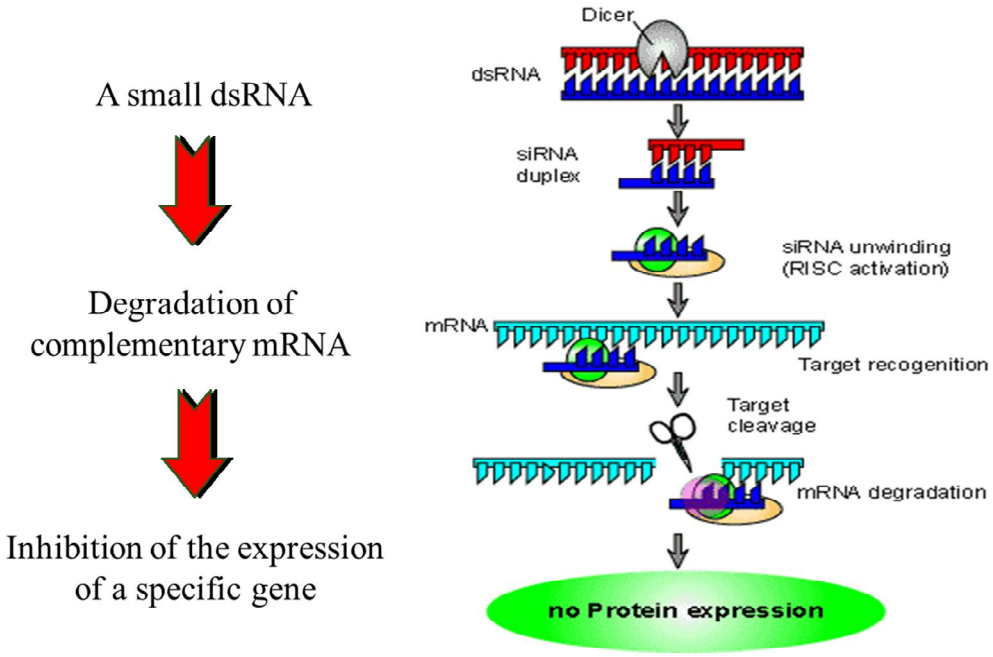

Figure 2.6 Mechanism of siRNA.

siRNA therapy is favorable over other methods like chemical inhibitors and dominant negative mutants as it has several advantages like specificity, reduced toxicity, ease of synthesis and cloning into expression vectors. Additionally, a number of chemical inhibitors are limited and most of them are non-specific and thus siRNA therapy is excellent treatment option for gene silencing. Even the effectiveness of siRNA therapy in genetic disease is demonstrated through preclinical studies. Additionally, carrier system protects siRNA from degradation by serum nucleases. Although siRNAs are exposed on the surface of recombinant Ab-fusion molecules, such complexes can be used to target specific cells as they also protect siRNA from nuclease degradation (76, 77). siRNA can be targeted to specific target site by conjugating them electrostatically with Abs, ligands or aptamers which can reduce its dose, achieve effective gene silencing and avoid off-targeting. Various non-viral delivery vectors like nanoparticles or liposomes can increase the quantity of siRNA delivered and thus improve its efficacy (77). There are various regulatory constraints in approval of such therapeutics which can be overcome by designing the delivery systems involving either chemical modifications like structural changes or using nanocarriers or surface modification by specific ligand attachment targeting at particular receptors. But still, there is a long way to travel before the application of RNAi therapy in the clinics.

2.11 Challenges to RNAi Delivery

Attaining the concentration above minimum effective concentration at target site is the main objective of RNAi therapy. There is a huge barrier of large size and ionic charge of RNAi therapeutics that may affect effective transfection of such carriers in the target site (78). Also various other factors including physiological, cellular and immunological factors (**Figure 2.7**) may become obstacles in the path from the administration site to the target site which is discussed in detail in later section.

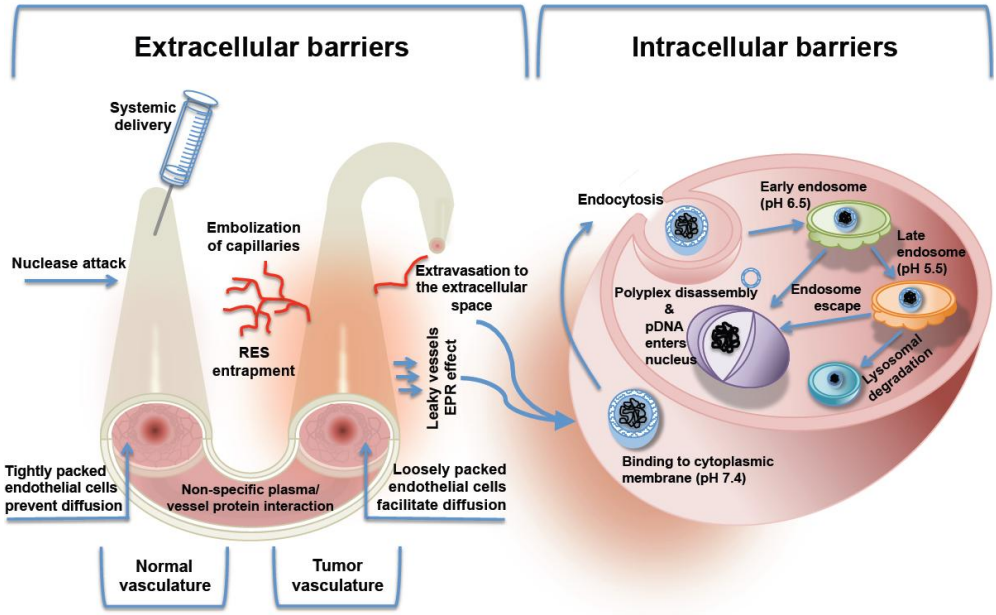

Figure 2.7 Various extracellular and intracellular barriers for siRNA.

2.11.1 Physiological Barriers

This is first and foremost barrier restricting the effective delivery of RNAi molecules. It consists of many check points like glomerular filtration, hepatic metabolism, RES uptake, endothelial barrier and degradation by nucleases. RNAi molecules are degraded by nucleases within a minute after their administration and thus making the therapy ineffective by approximately 70% (79). siRNA duplex have average size below 10 nm so naked siRNA when administered systemically will cause excretion through renal filtration (80). Some diseases require systemic administration of siRNA which results in higher therapeutic dose ultimately

leading to higher costs and side effects like activation of innate immunity and off-targeting ultimately leading to silencing of transcripts other than target site. Such process can lead to toxicity which can restrict the clinical development of siRNAs. Local alignment algorithms like BLAST and Smith-Waterman should be used for siRNA designing to minimize its off-targeting potential. Additionally, higher dosing of siRNAs may negatively affect endogenous miRNA function due to competition for the RNAi machinery. Furthermore, naked siRNA poses several difficulties to overcome multiple tissue barriers including liver, kidney and lymphoid organs when injected systemically (81, 82). Endothelial cells that form blood vessel lining regulate exchanges between the blood stream and the surrounding tissues by serving as semi-selective barrier and also control blood supply to various tissues. Such barriers and filters restricts cellular uptake of negatively charged nucleic acids.

Approaches like chemical modification or use of non-viral carriers can be employed to overcome this barrier and to prevent the cleavage of the RNAi agents. The approaches that can improve the RNAi agents' stability against nucleases (83) are stated below:

1) Alteration in pentose sugars at the 2'-OH position and 3' half of the siRNA molecule.
2) Formation of phosphorothioate oligonucleotides by replacing the oxygen with sulphur.
3) Modification of Hexitol nucleic acids (HNAs), morpholino compounds, locked nucleic acids (LNAs) and peptide nucleic acids (PNAs) at 2'-OH position.
4) Substituting 6-carbon sugar for ribose, 2'-F and 2'-OMe group along with the gapmers sustain the therapeutic activity of these molecules.
5) Formulating the anionic molecules into cationic nanoparticles, liposomes, lipoplex or polyplex prevents the cleavage from nucleases by virtue of the electrostatic interaction (84, 85).

The rate and extent of clearance of RNAi nanoconstructs from the systemic circulation depends on size and charge of the complex formed between RNAi nanoconstructs and serum proteins. RNAi therapeutic delivery using carriers like nanoparticles, liposomes, lipoplex or polyplex having particle size more than 200 nm are susceptible to phagocytosis by the reticuloendothelial system (RES) (86) while, carriers having particle size less than 100 nm get entrapped in the hepatic Kupffer cells. This clearance of nanocarriers can be lowered by coating nanoconstructs with hydrophilic agents like polyethylene glycol (PEG), polyvinyl

alcohol (PVA), polyvinyl pyrollidone (PVP) etc. which mask the surface charge and make them long circulating (87, 88). Hence, improvement of RNAi therapeutics may be obtained by controlling particle size and surface charge of the final formulation. Also opsonization of RNAi nanoconstructs favors its targeted delivery to liver and spleen (81).

Antisense molecules stimulate the pharmacological action after breaching the endothelial cell layer. Various adhering molecules like integrins are associated with these endothelial cells in the extracellular matrix and thus only small molecules can get through this paracellular route (89). The space at the junction in certain organs like liver and spleen allows the larger molecule to travel across the barrier. Additionally, caveolae mediated transcytosis also facilitate transport of RNAi molecules to the target site (90).

2.11.2 Cellular Barriers

It comprises of different check points like cell entry, endosomal escape and knock-down of protein expression. Some non-viral carriers are nonspecific in their targeting and thus can enter non-target cells resulting in toxicity. Cationic carriers interact with negatively charged cell membranes and thus results in non-specific uptake (91) which can be minimized either by coating with hydrophilic molecules like polyethylene glycol or conjugating with ligand motifs such as transferrin, folate, surface receptor-specific antibodies etc. resulting in their specific cellular entry.

Rate of cellular internalization and endosomal escape are the two main determinant factors that determine the transfection efficacy of carrier system (92, 93). Efficient RNAi activity of siRNA formulations can be achieved by their endosomal release of the internalized molecules. And in order to achieve this, several strategies have been designed. For example, use of cationic polymers (PEI) or polycationic dendrimer can improve transfection efficiency of siRNAs as per the principle of "proton-sponge" effect. Basically, the "proton-sponge" effect leads to high protonation of amine groups in the PEI or dendrimer vehicles, which causes osmotic swelling and vacuole disruption along with cytoplasmic release of the entrapped molecules. Additionally, acid-sensitive components (e.g. endosome-disruptive fusogenic

peptides and acid-responsive disulfide bonds) or osmotic agents like glycerol, sucrose, PVP etc. can also be used to facilitate endosomal escape of siRNA and to avoid this barrier.

Alternatively, endosomal escape is achieved using several approaches like rupturing lysosomal membranes by the use of fusogenic lipids or peptides and by forming pores in membranes (94, 95). Literature review suggests that the formulation of pH-sensitive liposomes encapsulating antisense agent consist of lipofectin and DOPE (1, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) (96). DOPE present in the liposomes forms pores inside the endosomal membrane resulting in its destabilization (97, 98).

Protein synthesis can be knocked down by nuclear localization of RNAi molecules which can be enhanced by conjugating them with cationic polymers like polyethylenimine or poly-l-lysine. Conversely, the anionic lipid competes with anionic RNAi molecules and displaces it from the complex of cationic lipid/polymer-antisense molecules (99, 100). Strength of interaction between RNAi agent and its complementary mRNA governs transfection efficiency of antisense carrier resulting in inhibition or down regulation of the protein or gene expression. Transfection efficiency is mainly dependent on the lipid/polymer to oligonucleotide ratio (92). Thus, knowledge and conceptual understanding of these barriers and carriers and proper understanding of bio-distribution and physiochemical properties of RNAi molecules lead to the efficient delivery of RNAi molecules to the target site and thus improve the success of therapy.

2.12 Potential of RNAi as Therapeutics

RNAi technology is currently being estimated as a potentially beneficial method to develop highly specific RNA-based gene-silencing therapeutics. RNAi is specific enough to allow the use of multiple RNAi targets at the same time, without any toxic effects that are frequent during chemotherapy or the sequence-independent toxic effects of antisense therapy. Overexpression of pathological proteins and genes is silenced by enzymatic cleavage of target which is modulated by RNAi and is applicable to all classes of molecular targets including the ones difficult to modulate selectively with traditional pharmaceutical approaches. RNAi therapeutics can exert potential transformational effect on modern medicine (101). RNAi is

also used to analyze biological function genes associated with diseases (102). RNAi is currently considered as a developing field for basic and biomedical research that may lead to a number of clinical applications. Various studies demonstrated the efficacy of RNAi therapy in silencing of genes involved in various disease like viral infection (respiratory and vaginal), ocular disease, disorders of the nervous system, cancer and inflammatory bowel disease in animal models. Both exogenous and endogenous genes have been silenced, and promising *in vivo* results have been obtained across multiple organs and tissues. Various potential targets, therapeutic intervention for siRNA have been summarized in **Table 2.3** and **Table 2.4** respectively.

Table 2.3 Modes of RNAi delivery and potential targets in various diseases

Sr. No.	Route of Administration	Potential organ target	Disease Target
1.	Local/Direct	Eye	Macular degeneration, Diabetic macular oedema
		Skin	Atopic dermatitis
		Vagina	Herpes simplex virus
		Rectum	Inflammatory Bowel disease
		Lung	SARS, RSV, Flu
		Brain	Huntington's disease, Depression, Alzheimer's disease, Spinocerebral ataxia, Encephalitis, Neuropathic pain
		Spinal cord	Chronic pain
		Vagina	HSV
		Isolated tumour	Glioblastoma multiforme, Prostate, Adenocarcinoma, Human papillomavirus
		Digestive system	Irritable Bowel disease
2.	Systemic	Liver	Hypercholesterolemia, HBV
		Heart	Myocardial infarction
		Kidney	Acute kidney injury
		Metastasized tumors	Ewing's sarcoma
		Joints	Rheumatoid arthritis

Table 2.4 Therapeutic intervention using siRNA

Sr. No.	Disease	Type	Target
1.	Viral	HIV-1	LTR, *vif, nef, Tat, Rev,* Gag, CD4, CCR5, p24, Pol
		Poliovirus	Capsid, viral polymerase
		Hepatitis B	Core region (3.5 kb RNA), Pregenomic RNA
		Rous sarcoma virus	Gag
		Hepatitis C	EMCV-IRE5, NS3, NS5B, NA, Core, NS4B, 5' UTR, NS5A
		Respiratory Syncytial Virus	Phosphoprotein (P), Fusion protein (F)
		Influenza A	NP, PA, PB1, PB2, M, NS
		Rotavirus	VP4
		Adenovirus (group B)	CD46 (cellular coreceptor)
		γ herpes virus	Rta, ORF45
2.	Cancer	Leukemia	c-raf, bcl-2
		Cervical carcinoma	E6, E7 (HPV)
		Pancreatic carcinoma	K-RAS^{V112}
		Melanoma	ATF2, BRAF^{V599E}
		Ovarian carcinoma	H-Ras, mVEGF, COX-2
		Prostate cancer	P110a, p110B of PI 3 kinase
		Wilms' tumor	Wt1, Pax2, Wnt4
3.	Angiogenesis	Tumor angiogenesis	VEGF
		Ocular neovascularization	VEGF, VEGFR1 and VEGFR2
		Rheumatoid arthrirtis	Akt, GG2-1, ASC
4.	Neurological Disorders	Alzheimer's disease	β -, γ –Secretase, Protein kinases (GSK-3, Cdk-5)
		Parkinson 's disease	α –Synuclein, LRRK2
		Huntington 's disease	Huntington
		Familiala amyotrophic lateral sclerosis	SOD-1
		Spinocerebral ataxia	SCA-1, SCA-2
		DYT1 dystonia	TOR 1A

2.13 siRNA Delivery: Role of Vectors

Gene therapy utilizes the delivery of DNA into cells, which can be accomplished by several methods. The two major classes of methods are those that use recombinant viruses (sometimes called biological nanoparticles or viral vectors) and those that use naked DNA or DNA complexes (non-viral methods). All viruses bind to their hosts and introduce their genetic material into the host cell as part of their replication cycle. This genetic material contains basic 'instructions' of how to produce more copies of these viruses, hacking the body's normal production machinery to serve the needs of the virus. The host cell will carry out these instructions and produce additional copies of the virus, leading to more and more cells becoming infected. Some types of viruses insert their genome into the host's cytoplasm, but do not actually enter the cell. Others penetrate the cell membrane disguised as protein molecules and enter the cell. There are two main types of virus infection: lytic and lysogenic. Shortly after inserting its DNA, viruses of the lytic cycle quickly produce more viruses, burst from the cell and infect more cells. Lysogenic viruses integrate their DNA into the DNA of the host cell and may live in the body for many years before responding to a trigger. The virus reproduces as the cell does and does not inflict bodily harm until it is triggered. The trigger releases the DNA from that of the host and employs it to create new viruses.

But, on other hand, transient expression (not good for genetic diseases), high immunogenicity, high titers of virus can be toxic, gene transcription with E1-negative virus is "leaky": many genes expressed at low level are the disadvantages related with these viral vectors which limits their use. Therefore, non-viral methods present certain advantages over viral methods, with simple large scale production and low host immunogenicity being just two. Commonly used non-viral siRNA delivery vectors include lipids, polymers and peptides. Various lipids like *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethyl ammonium chloride (DOTAP); 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE); 2′-(1″,2″-dioleoyloxypropyl dimethyl-ammonium bromide)-*N*-ethyl-6-amidospermine tetratrifluoro acetic acid salt (DOSPA); 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC); lipofectamine, and biodegradable polymers like chitosan, polyethylenimine (PEI), poly(D,L-lactide-co-glycolide) (PLGA), poly-L-lysine (PLL) are proved to a useful carrier for delivery of siRNA (103). Previously, low levels of transfection and expression of the gene held non-viral methods

are at a disadvantage; however, recent advances in vector technology are being developing molecules and techniques with good transfection efficiencies and low cytotoxicity.

2.14 Polyethylenimine

PEI exists in two forms, either as a branched polymer, or in linear form with molecular weight ranging from <1000 Da to 1600 kDa. The molecular weight of PEI most suitable for gene transfer ranges between 5 and 25 kDa. Chemical structure of PEIs is differ from other polymers, such as polylysine, in that only a fraction of the amino groups are protonated at physiological pH. When the pH of the endosomal compartment becomes acidic, the capacity of PEIs to capture protons causes osmotic swelling and subsequent endosome disruption. This leads to the release of endocytosed DNA into the cytosol. Some polycations can show high transfection efficiency without guidance of endosomolytic agents. Polyamidoamine (PAMAM) cascade polymer or Starburst dendrimer was first such molecule. Strong buffer capacity of PEI results in highest charge density and a high intrinsic endosomolytic activity. At physiological pH, PEI is partially protonated but when it is exposed to acidic conditions within endosomes or endolysosomes, it triggers passive chloride ion influx by proton sponge effect which finally leads to endosome rupture and escape. Additionally, structural flexibility and the molecular weight of PEI are important for efficient DNA delivery. From several studies it was found that although low molecular weight PEIs (800 Da, 2 kDa) have capacity to condense DNA they were not able to show efficient transfection; while PEIs with above 10 kDa molecular weight showed high transfection efficiency. Additionally, 800 kDa PEI shows high toxicity and erythrocyte aggregation after systemic application *in vivo* as compared to lower molecular weight PEIs and thus 20–25 kDa PEIs are favored for *in vivo* applications.

The positive surface charge of polycation/DNA complexes usually also serves to bind cells via electrostatic interactions with the negatively charged cell membrane followed by pinocytosis or endocytosis. This process is also referred to as non-specific adsorptive endocytosis. The binding of positively charged DNA complexes to sulfated proteoglycans on the cell membrane has been demonstrated. However, since the exact mechanism of PEI-mediated transfection remains to be elucidated, it is possible that additional properties are required to obtain high transfection efficiencies. **Figure 2.8** demonstrates the synthesis

pathways of linear and branched PEI. As proposed by Dick and Ham, based on the acid-catalyzed polymerization mechanism of aziridine, theoretical ratio of primary, secondary and tertiary nitrogen atoms in branched PEI was about 1 : 2 : 1 (104). The need for an excess positive charge for efficient DNA complexation and cell binding can, however, pose major problems, particularly for many *in vivo* applications, as discussed below. High molecular weight PEI can cause aggregation of huge clusters of the cationic polymer inducing necrosis (105) and thereby cause cytotoxicity (106). Contrastingly, low molecular weight PEI has demonstrated low cytotoxicity in cell culture study (107, 108). Linear PEI based transfection reagents are already commercially available (e.g. ExGen500, jetPEI) (109). Therefore, the current research is focused on furnishing safe and biocompatible derivatives of PEI while retaining the transfection efficacy.

PEIs exist in a linear and branched topology and are available in a wide range of molecular weights. The reason for synthesizing new derivatives may be considered as: Firstly because the efficiency of PEIs has still to be improved in order to become an alternative to viral vectors. Secondly, the solubility, biodegradability, toxicity and chemical homogeneity of these polymers are not satisfying. Finally, because the synthesis of PEI derivatives allows one to gain better insights on which properties of the polymers are essential for efficient gene transfer, this in turn should allow an improved vector designing.

Various chemical modifications to the basic PEI can be made in order to get various physicochemical properties and subsequent alteration of safety profile. Forrest et al. have made conjugates of 14–30 kDa by coupling low molecular weight 800 Da PEIs through short diacrylate linkages which resulted in the favorable properties like low toxicity, higher transfection efficiency in the developed formulation. *In vitro* cytotoxicity and ester bond hydrolysis could be correlated to the degradation behavior and it was found that the polymer having the smallest degradation half-life showed lowest toxicity and also the degradation products did not possess any cytotoxic effects (110). According to Kramer et al. PEI showed lowest cytotoxicity at degree of branching of about 60% (111) while further increase in degree of branching increases the *in vitro* cytotoxic effects, as well as the haemolysis of erythrocytes (112). Thus, for establishing structure-function relationships, nullifying cytotoxicity and improving biocompatibility, detailed knowledge of the polymer structure is required.

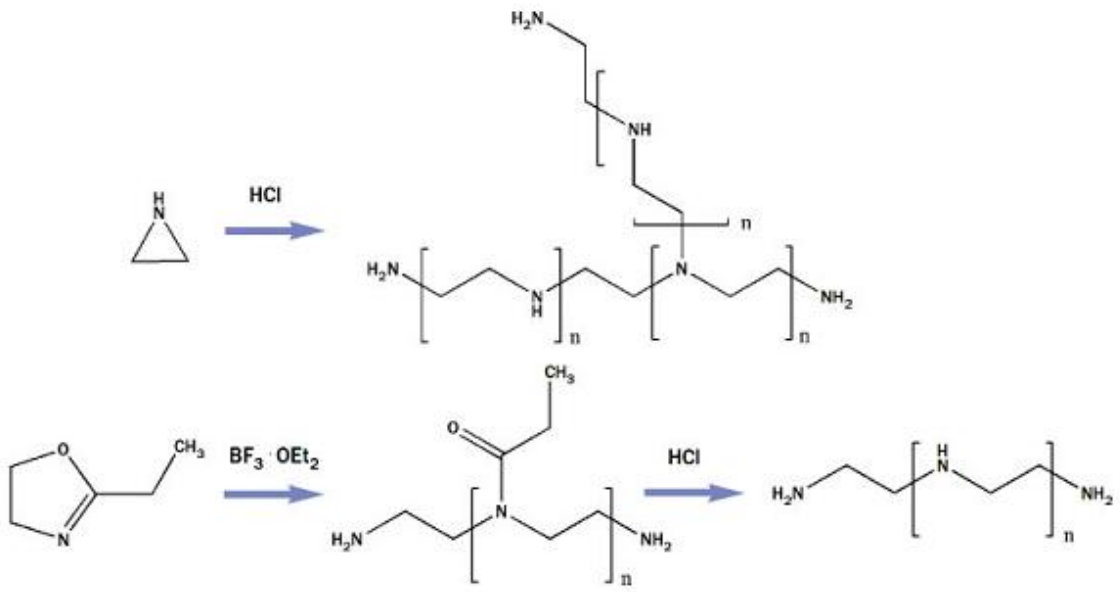

Figure 2.8 Acid-catalyzed polymerization of aziridine leads to branched PEI, whereas ring opening polymerization of 2-ethyl-2-oxazoline leads to the N -substituted polymer, which can be transformed via hydrolysis into linear PEI.

The most characteristic notable feature of PEI is its capacity to generate high cationic charge density of 20–25 micro-equivalents per gram as the nitrogen atoms which are found in high number in PEI are capable of protonation. Such creation of cationic charge is dependent on environmental pH due to absence of quaternary amines. For e.g. protonation of PEI at pH 7.4 is about 20% while at pH 5 it is about 45% (113). PEI can show highest buffer capacity between pH 8 and 10 due to presence of secondary amines (114), typical for polyamines (115)), which along with its charge density can affect cytotoxicity and endosomal release (112). Molecular weight and degree of branching of PEI can have an impact on basicity and protonation. Various studies proved that higher pKa values corresponds to higher protonation of the primary and secondary amines present, thus producing a higher number and density of charges (112). PEIs can also show buffer capacity in the pKa range between 4 and 6 by which it can buffer the interior of endosomes resulting in their osmotic swelling and rupture of the endosomal membrane (116). In recent years 'proton sponge' hypothesis has gained widespread approval (107). Funhoff et al. suggested that for polymers having buffer capacity at low pH values of

approximately 5, the proton sponge hypothesis may not be applicable (108). Still various evidences like living cell confocal microscopy are available to validate the proton sponge hypothesis (117). Additionally, decelerated acidification, elevated chloride accumulation, 140% increase in the relative volume of PEI-containing endosomes and the concept of reduction in transfection efficiency by removal of protonable amine groups by quarternization, supports the proton sponge hypothesis(118, 119)

2.14.1 PEI Complexes

For the efficient delivery of nucleic acids into cells, it is prerequisite to condense nucleic acid into small particles. Along with efficient endocytosis with particles <150–200 nm, the velocity of cytoplasmic movement was also found to be related to the particle size. Nucleic acid complexation with PEI can protect them against cleavage by nucleases as PEI can form stable complexes by condensation of plasmid DNA and RNA via electrostatic interaction which is dependent on polymer characteristics such as molecular weight, number and the density of charges and composition of the complexes. It was found that lower charge density or lower molecular weight can worsen the condensation capability (120). DNA-PEI condensates belong to the class of polyelectrolyte interpenetration complexes which can form under the influence of polycations resulting in formation of spherical, globular or rod-like structures (121). Electrostatic interactions can markedly influence this process as the binding ratio of DNA to cationic polymer was approximately 1:1 (122). The electrostatic interaction between plasmid DNA and PEI like polymers can be confirmed by observing the FTIR spectra of DNA in which there is marked reduction in frequency of the asymmetric phosphate stretching vibration (106). Additional evidence can be obtained by micro-calorimetric measurements (123). Various studies found that by increasing the salt concentration, there is a marked reduction in the binding affinity which indicates the presence of charge shielding effect at higher salt concentration (124). Polyelectrolyte complexes of PEI-DNA/RNA may experience polyion exchange and substitution reactions after formation which may be driven by entropic forces developing from the release of counter ions, forming more stable complexes (123). But still other interactions including hydrogen bonds, Van der Waals' forces or the removal of hydrating water molecules, may also contribute to complex formation. DNA-polycations complexation was found to be dependent on tertiary structure of DNA as the polymer especially complexed

with supercoiled DNA, at the pH value of around 5 (123). Independent of the molecular weight and N/P ratio (ratio of nitrogen-containing groups of the polymer to phosphate groups of the nucleic acid), pDNA remained in its β-form even after complexation suggesting that it has no effect on the overall helical form of the DNA (114).

DNA vector characterization can be used to propose its biological state as it is the main determinant governing the effectiveness of the DNA transported by the carrier molecule which in turn decides its therapeutic application (125). Still, there is no non-destructive method for the characterization of the PEI-DNA complexes and thus, there is limited information regarding this subject. Spectroscopic techniques can be utilized to estimate the amount of PEI in the presence of DNA (126); yet it cannot distinguish the fraction of bound polymer from the free polymer. Recent investigations by fluorescence correlation spectroscopy showed presence of 3.5 plasmid (5800 base pairs) and 30 PEI (25 kDa) molecules when prepared at N/P ratios of 6 and 10 in the polyplexes assuming that the DNA showed complete complexation (127). After DNA complexation, approximately 86% of the PEI was found in free form (127) which may be the main reason for the cytotoxic effect of the formulation (105). The resulting cytotoxicity was reduced by purification of PEI complexes, resulting in removal of excess PEI but such purified formulation was faced with another problem of reduced transfection efficiency because free polymer helps to propagate endosomal release. This assumption was approved when externally added free PEI restored the transfection efficiency (128). Additionally, free PEI is in equilibrium with complex and it generates a hydrophilic cationic corona around the complex for its sufficient solubilization, so its removal might create stability issues (129).

Along with solubility enhancement, the cationic surface charge is also essential for efficient cell transfection (130) which occurs only after interaction with anionic cell-surface proteoglycans (131) especially with transmembrane protein syndecan (132). Primarily, positively charged polyplexes (N/P ratios of approximately 5) are used in transfection experiments (133, 134), . Such contradiction concludes that still there is a long way to travel before complete and in-depth understanding of PEI/DNA polyplex structures, even though if the primary aim of such complexes i.e. cell surface binding is served (135). Compared to naked DNA (136) or other cationic polymers such as PLL (poly-L-lysine) (137), PEI was found to be highly effective in preventing RNA and DNA degradation by enzymes (138). For example,

naked DNA is degraded within 2 min on DNase I exposure, while DNA complexed to PEI 25 kDa was only marginally degraded after 15 and 30 min incubation (139), or after exposure to 25 units of DNase I for 24 h (107). Godbey et al. suggested that such protection was mainly due to physical or electrostatic barrier to enzymatic degradation or by inactivation of the enzymes (107).

2.14.2 Polymer Structure Influences Polyplex Characteristics

Condensation behavior and complex size were found to be dependent on the molecular weight of PEI in the case branched, as well as linear PEIs (140). Generally, there is inverse correlation between molecular weight of the PEI and complex size upto 25 kDa beyond which there was no increase in the size while the lower limit for such correlation was found out to about 2 kDa (141). It was estimated that PEI with molecular weight of 800 Da or lower gave huge aggregates of about 900 nm (142) indicating low condensation capacity of lower molecular weight PEIs (142). This was confirmed by observing the effect of covalently coupled low molecular weight substructures forming higher molecular weight conjugates on condensation capacity and complexation efficiency and it led to increase in both condensation and conjugation efficiency (110). Primary amines can cause effective condensation of DNA as compared to other amines due to their higher protonation at a given pH (143) which can improve the complex stability and thus give higher transfection efficiency (144). Not only the PEI's molecular weight, but also the degree of branching plays an important role in biological properties of complexes with nucleic acids. Furthermore, the increase in number of primary amines in the structure can improve binding capability (145). In another study, the content of primary amines exhibiting degrees of branching between 0 and 23% in 2000 Da PEI-N-(2-hydroxyethylethyleneimine)-copolymers was reduced to one half concluding that twice the N/P ratio was needed to form small condensates (112). Less branched PEI compared to highly branched derivatives require higher N/P ratios for a complete DNA condensation (105). Also by reducing the proportion of tertiary amines indirectly by increasing secondary amine fraction gave higher complexation efficiency (140).

Similarly degree of branching can have effect on gene transfer efficiency and *in vitro* toxicity i.e. highly branched PEIs achieve higher transfection efficiencies yet they possess

higher toxicity. According to a study carried out by Michelakis et al., it was found that flexible hyper branched PEI derivatives with additional secondary and tertiary amine groups showed enhanced transfection efficiency along with lower toxicity (146). Linear PEI also possesses a lower condensation capacity, as compared to the branched forms (121). The branched PEI (25 kDa) is able to retain pDNA up to 24 h in the condensed state in cytoplasm as compared to 4 h for linear PEI (22 kDa) (147).

Physicochemical properties of the polymer such as the molecular weight and branching ratio can significantly affect both the transfection efficiency and cytotoxicity which concludes that, polymer structure significantly influences the efficacy of PEI-based vectors (148). Therefore, proper selection of polymer based on the carrier systems like plasmids, oligonucleotides or siRNA is a very sophisticated task. Finally with application of all the available knowledge proper system must be designed which possess all the desired properties.

2.14.3 Modification to Achieve Tissue Specificity and Enhance Cellular Uptake

Along with intact delivery of the nucleic acid and reduction of side effects, ideal gene delivery system should also have capability for cell or tissue-specific targeting. Passive targeting of specific PEI or its modifications is one of the simplest approaches to achieve this purpose. This passive targeting approach was applied in the development of JetPEI to achieve tumor targeting based upon the EPR (enhanced vascular permeability and retention) effect (149). PEI grafted with Pluronic 123 or Pluronic 85 targeted hepatocytes while eight PEG chains grafted onto 2 kDa PEI targeted the kidneys (150). Active targeting is another approach which relies on receptor-mediated uptake of modified polyplexes to specific target tissues, such as hepatocytes and dendritic cells via carbohydrates; tumor tissue via folate receptor, integrin or transferrin targeting; and to tissues expressing specific receptors with antibodies or their fragments. For example, the conjugation of galactose to PEI is used for liver-specific targeting which acts on asialoglycoprotein receptor expressed on hepatocytes. The transfection efficiencies of such galactose-modified PEI increase with increase in grafting ratio upto 5% but further increase upto 31% lead to reduction in the transfection efficiency which may be due to steric shielding effect impairing complete DNA condensation (151, 152). Low extent of galactose grafting on PEI of about upto 1.7% resulted in only partially compacted structures with no hepatocyte

targeting effect, (133). Sagara et al. reported less improvement in transfection efficiency comparable to PEI-gal conjugates, depending on the grafting ratio (153).

It has recently been demonstrated that some peptide sequences known as transduction domains or membrane translocation signals contain positively charged amino acid residues such as arginine and lysine, which have been reported to have a high cell-penetrating ability (154-156). Arginine (Arg) conjugated polyamidoamine (PAMAM), poly-propylenimine, and poly(l-lysine) polymers improves the transfection efficiency compared to native polymers *in vitro*. Positively charged amino acids like Arg and lysine (Lys) have been reported to have enhanced transfection ability; however, this also appeared to be the case for hydrophobic moieties such as leucine (Leu). Kono et al. demonstrated that PAMAM dendrimers conjugated with phenylalanine (Phe) or Leu residues achieved efficient gene transfection of cells (157). They also demonstrated that dendrons with hydrophobic moieties led to a much increased transfection activity compared to PAMAM dendrimers of the same generation. Moreover, buffer capacity of the polymers with free amino groups in the side chain is much lower than that of the polymers with the Boc-protected amino groups, reflecting the higher degree of protonation of the former polymers at physiological pH. For example, comparing the polymers containing 10% of nicotinamide moieties, the buffer capacity decreases from 70% for p(Nic10-NHBoc) with Boc protected amino groups to 33% for p(Nic10-NH2) – for no Boc protection. Acetylation of the free amino groups results in a decreased amount of protonated amino groups at pH 7.4, similar to the Boc-protected polymers, and therefore an increase of the buffer capacity in the pH range 7.4–5.1 is observed. Polymer p(Nic10-NHAc) shows a buffer capacity of 57%, which is much higher than that of p(Nic10-NH2), but lower than that of p(Nic10-NHBoc). As p(Nic10-NHAc)-acetylated nocotanamide modified PEI and p(Nic10-NHBoc)- Boc protected niotinamide modified PEI have the same amount of basic nitrogens, the difference may be explained by the stronger hydrophobic environment of p(Nic10-NHBoc), giving rise to a lower degree of protonation of this polymer at pH 7.4 (158).

The use of antibodies or their fragments to target tissues expressing specific receptors has led to similarly inconsistent results. Chimeric antiGD2 antibody when coupled to PEI gave complexes with sizes of approximately 50–100 nm, but these complexes did not match the transfection capacity of unmodified PEI (133). Anti-CD3 antibody fragments improved

receptor-mediated uptake in human peripheral blood mononuclear cells (159). Recently, PEI conjugates coupled to OV-TL16 Fab fragment were formulated having complex size <200 nm and zeta potential approximately 0 mV which showed negligible hindrance to complex formation by antibody conjugation and also transfection efficiency was greatly improved compared to PEG-PEI (160).

The coupling strategies which contain a linker between the polyplex core and the ligand provide better effectiveness in contrast to ligands directly coupled to the polyplex core, as it provides better accessibility of the grafted ligand to its receptor. Presently very few work is done on the site-specific coupling of targeting moieties to PEI-based vectors but there are proof for such possible effect for polyplexes (161) and thus active targeting seems to be possible in principle but huge amount of research has to be carried out to optimize the targeted vector systems. Protein transduction domains (PTDs) are composed of a high number of basic amino acids which are suggested to interact non-specifically with negative cell-surface constituents due to arginine-rich motifs (162, 163). But belief that PTDs facilitate an endocytosis-independent means of cell entry was lately challenged (164). Later, macropinocytosis has been suggested to be possible entry mechanism for PTDs but still the complete mechanism regarding PTD translocation remains to be determined (165). However, PTDs can provide promising technique for cellular entry and may even circumvent endosomal release problems. Recently, successful application of PTDs in PEI-based gene delivery was witnessed. Another approach to improve transfection efficiency deals with pre-compaction of plasmid DNA using oligomers of the HIV-1 TAT peptide which was further complexed with transfection agents like PEI resulting in a 3-fold higher transfection in non-proliferating cells compared to PEI transfection in proliferating cells (166). Further improvement of the natural PTD sequences may expand this approach.

2.14.4 Linking Physico-chemistry to Biology

Detailed knowledge of physicochemical data helps to formulate gene delivery systems that can surpass various barriers for *in vivo* application of polyplexes which are challenged in various ways. In order to overcome these challenges several factors including stability in the extracellular environment, interaction with target cell surfaces, cell uptake, release from

endolysosomal vesicles, nuclear uptake as well as vector unpacking should be considered in detail (**Table 2.5**). The knowledge regarding these processes is still limited but PEI polyplexes offer upper edge that can subside such problems.

The complexation of nucleic acids and PEI provides several advantages that include enhanced stability against degradation in extracellular environment, improved cellular uptake by electrostatic interactions of the polycations with the negatively charged cell surface, and augmented endosomal release according to the proton sponge hypothesis.

Table 2.5 Strategies to overcome barriers with PEI-based gene delivery

Barrier	**Strategies**
Extracellular Stability	▪ Complexation with PEI homopolymer ▪ Steric shielding by copolymerization ▪ Crosslinking polyplex surface
Cell surface interaction and cell uptake	▪ Complexation with PEI homopolymer ▪ Active or passive targeting ▪ Protein transduction domains
Endo/lysozomal release	▪ Complexation with PEI homopolymer
Unpacking and nuclear uptake	▪ Physicochemical characteristic of PEI and PEI copolymers ▪ Controlled degradation by environmental stimuli ▪ Nuclear localization sequences

Polyplexes formulated by copolymerization have reduced interactions with blood components because of reduced surface charge and steric shielding capabilities, and also improved stability against shear stress by cross-linking of polyplex surface. Even using certain copolymers, directed biodistribution can be achieved. But still PEI-copolymers show reduced cell-surface interaction and thus thereby limits cellular uptake. The effect of copolymerization on vesicular release and degree of protection from degradation in the cytoplasm still remains mysterious. Generally unpacking of the vector occurs as a result of reduction in complexation ability of most copolymers yet the site where unpacking of the nucleic acid from the polyplex occurs is

not clear so release is controlled by physical and/or chemical characteristics of the polymer or by environmentally controlled degradation.

Recently targeted polyplexes are formulated that promote their biodistribution to specific tissues and cells via cell-surface interaction and cellular uptake and thus overcome barriers of cellular uptake due to steric shielding. Also targeting moieties have negligible influence on intracellular processing, but when it consist of nuclear localization sequences (NLS) it may break the nuclear envelope and stimulate nuclear entry (167). Conclusively recent works has suggested that direct membrane transduction can be used as an alternative approach to endocytic uptake which, if possible, would help to surpass barriers in endosomal release.

2.15 Pulmonary Drug Delivery

Numerous drugs are being administered via injection or other routes to treat PAH, but these deliveries can attain high systemic drug concentration instead of localization into the lung. Elevation in dose-related side effects is the result of administration of high doses of drug via oral or parenteral routes with only a small percentage of drugs actually reaching the affected pulmonary region. Pulmonary delivery of the drugs to the lung airway; primarily provides the local effect with negligible side effects as compared with the other routes. To date, enormous efforts have been invested to obtain effective treatment for pulmonary hypertension (PH). Local delivery of drugs has advantage over systemic delivery for the reason of site specificity and prevention of side effects. For the treatment of PAH, direct delivery to the lungs through inhalation therapy may prove to be very effective and useful and needs attention of the researchers for their useful contribution.

The delivery of siRNA through inhalation route can offer several advantaged including bypassing of systemic physiologic barrier, local targeting, immediate availability, decreased systemic side effects, and noninvasive application. Moreover, due to local targeting the efficacious dose of siRNA can be reduced considerably. Finally lung epithelial cells, which are considered to be the most affected region in lung diseases, can be efficiently targeted by pulmonary delivery of siRNA.

2.15.1 Human Pulmonary System

Human respiratory/pulmonary system is a complex organ system consisting mainly of (i) the conducting airway region consisting of nasal cavity, pharynx, trachea, bronchi and bronchioles; and (ii) the respiratory region which consists of the respiratory bronchioles and alveoli (where gas exchange occurs) across thin walls (**Figure 2.9**). High degree of branching is one of the most prominent features of the respiratory tract. The trachea is a flexible tube extending from the larynx which later on bifurcates into the left and right bronchi which further subdivides into the bronchioles. The tissue walls of the bronchi and the bronchioles are similar to that of the trachea and the presence of alveoli marks the respiratory zone. The respiratory zone consists of 300 million alveoli which provide a huge surface area for gaseous exchange. Such a large surface area of approximately about 100 m^2 is mainly due to bifurcation of airways more than 17 times (168). About 93% of the alveolar surface consists of epithelial type I cells which are basically involved in gas exchange functions and serve as a permeability barrier. Alveolar epithelial type II cells represent about 16% of the total cells in the lung and play basic role in synthesis, secretion and recycling of lung surfactant. The averages lung capacities in men and women are 6.7 and 4.9 L, respectively (169).

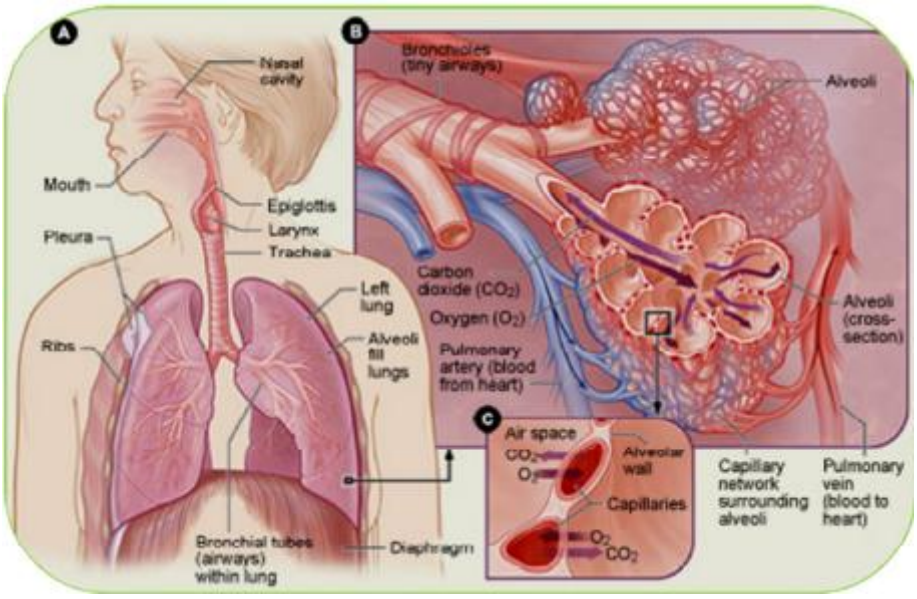

Figure 2.9 Anatomy and physiology of pulmonary system.

The alveolar blood barrier consists mainly of a single epithelial cell layer, a basement membrane, and a single endothelial cell layer. Such an arrangement can offer a major obstacle to larger molecules. Solutes must traverse a thin layer of the epithelial lining fluid before entering the systemic circulation, which gets collected at the corners of alveoli and is surrounded by an attenuated layer of lung surfactant consisting of phospholipids (mainly phosphatidylcholine and phosphatidylglycerol) and several key apoproteins. Recent studies indicate that the rate of diffusion out of the alveoli may be slowed down in presence of surfactants.

The respiratory airways, from the upper airways to the terminal bronchioles, are lined with 0.5–5.0 mm thick viscoelastic, gel-like mucus layer. The secretion lining consists of two layers: a low viscosity fluid layer surrounding the cilia (periciliary fluid layer), and a more viscous layer of mucus on top. The mucus is a protective layer comprising of glycoprotein mixture released primarily by the goblet cells and local glands and helps in removing the inhaled particles. The rate of mucociliary transport mainly depends on viscosity and elasticity. The main factor limiting pulmonary targeting is high vascularization of lung tissue, because it causes fast absorption of most drugs.

2.15.2 Challenges in Pulmonary Delivery of siRNA

Pulmonary drug delivery systems presents barrier to large, hydrophilic molecules like nucleic acids. Even though siRNA is comparatively smaller than plasmid DNA, it is still 50 times larger than typical small-molecule drugs (170). This is accompanied by strong negative charge which impedes its cellular uptake and thus limits its use. Other barriers like active clearance through cilia or cough, immune response due macrophages and polymorphonuclear cells (PMNs) limits the efficiency of siRNA therapy (171). Furthermore, mucus and other respiratory fluid can trap siRNA loaded carriers and reduce their velocity (172). Thus efficacious carrier system that can direct siRNA to target site should be developed to improve cellular uptake and overcome other limitations in siRNA therapy.

2.15.3 Delivery Strategies for Pulmonary Delivery of siRNA

Instabilities such as aggregation tendency and loss of transfection efficiency of aqueous suspensions of non-viral vector complexes is major barrier restricting clinical application of non-viral vectors (173). Thus to overcome these barriers DPIs were developed for gene therapy which may provide several advantages like increased stability, reduced drug loss during administration, improved portability and efficient delivery to the pulmonary targeted region (174).

siRNA formulation would be effective only after reaching the target site in adequate dose. Thus the efficiency of siRNA formulation depends on the device used along with the formulation and transfection agents. Several limitation encountered during systemic delivery of siRNA include inefficient targeting to the desired organ and cell type, rapid degradation by nucleases, systemic toxicity and rapid excretion which can be overcome by local targeted delivery. Till date little work has been done on inhalable siRNA but large amount of work has been done on inhalable DNA along with their testing in animal models (175, 176). The main factor responsible for successful transfection is to protect the DNA from the shear forces generated during nebulization for which viral vectors, cationic liposomes and polymers have been used (175). Stribling et al. demonstrated aerosol delivery of cationic liposomes complexed with plasmid DNA in 1992 (176). The main factor that can make siRNA therapy efficacious is its capability to show action at much lower concentrations which can provide upper edge during development of inhalation therapy. Special precaution should be observed to ensure siRNA stability during manufacturing and aerosolization. Early generation inhalers were designed for the delivery of small molecules, but they were not efficient for delivery of molecules like siRNA due to poor delivery efficiency. This limitation was overcome with the advent of the next generation of inhalers which were basically designed for protein delivery and thus could be used to deliver siRNA. Several companies have developed liquid-spray systems like Aeroneb (Aerogen) and the AERx (Aradigm) system having vibrating mesh plate systems which can aerosolize liquids through precision-controlled orifices. Till date siRNA therapies are at preclinical stage and so robust animal model should be designed to assess *in vivo* efficacy of siRNA. The method of intranasal instillation for siRNA delivery to lungs has shown some success in *in vivo* animal studies (177, 178). But this success cannot be extrapolated to humans

as animal models have different lung anatomies which necessitate careful choice of inhalation device. Various options are available for pulmonary siRNA delivery in human trials. One such trial named Alnylam's clinical trial used nebulizers for delivery which could effectively assess siRNA therapies in humans. It is hoped that several new devices would provide patient friendly and noninvasive delivery of therapeutic siRNA. But to achieve success with such devices for siRNA delivery, several factors like dose required, choice of transfection agent, stability, drug aerosolition and therapy duration have to be considered that can have great impact on the efficacy of therapy. siRNA have a good stability in dry powder form. Dry powder can easily reach upto deep lung and can give better therapeutic activity in treatment of various lung disorders and that's why preferably use for delivery of therapeutic genomics.

Currently DPIs of proteins and gene vector complexes are formulated using Freeze-drying approach. The suitability of lyophilization in stabilization of various delivery systems like lipid-pDNA (plasmid DNA) complexes (179), PEGylated lipid-pDNA vehicles (180) and PEI-pDNA complexes (181) have been proved by various studies. Additionally, lyoprotectants assist in preserving particle size and transfection efficiency (182), but still their mechanism is unknown. Allison et al. hypothesized particle isolation mechanism to explain role of disaccharides in maintaining particle size of lipid-pDNA complexes (183). Furthermore pDNA condensation by cationic agents like branched 25 kDa PEI may nullify damage of pDNA by shear-related forces during powderization. Till date, there is rare literature regarding the *in vivo* studies of DPIs. But as a ray of hope, chitosan/pDNA complexes as dry powder aerosol produced by spray-freeze-drying was efficient in gene delivery to lungs and when comparison was carried out between PEI and chitosan, PEI gave efficient gene expression in lungs. But still for better understanding of their activity in vivo studies of DPI has to be carried out.

2.16 References

1. Badesch DB, Abman SH, Ahearn GS, Barst RJ, McCrory DC, Simonneau G, et al. Medical therapy for pulmonary arterial hypertension: ACCP evidence-based clinical practice guidelines. Chest. 2004;126(1 Suppl):35S-62S.

2. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, et al. Pulmonary arterial hypertension in France: results from a national registry. Am J Respir Crit Care Med. 2006;173(9):1023-30.

3. Peacock AJ, Murphy NF, McMurray JJ, Caballero L, Stewart S. An epidemiological study of pulmonary arterial hypertension. Eur Respir J. 2007;30(1):104-9.

4. Rosenkranz S. Pulmonary hypertension: current diagnosis and treatment. Clin Res Cardiol. 2007;96(8):527-41.

5. Simonneau G, Galie N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, et al. Clinical classification of pulmonary hypertension. J Am Coll Cardiol. 2004;43(12 Suppl S):5S-12S.

6. Humbert M, Khaltaev N, Bousquet J, Souza R. Pulmonary hypertension: from an orphan disease to a public health problem. Chest. 2007;132(2):365-7.

7. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al. Primary pulmonary hypertension. A national prospective study. Ann Intern Med. 1987;107(2):216-23.

8. Barst RJ, McGoon M, Torbicki A, Sitbon O, Krowka MJ, Olschewski H, et al. Diagnosis and differential assessment of pulmonary arterial hypertension. J Am Coll Cardiol. 2004;43(12 Suppl S):40S-7S.

9. Runo JR, Loyd JE. Primary pulmonary hypertension. Lancet. 2003;361(9368):1533-44.

10. Penaloza D, Arias-Stella J. The heart and pulmonary circulation at high altitudes: healthy highlanders and chronic mountain sickness. Circulation. 2007;115(9):1132-46.

11. Bousquet J, Dahl R, Khaltaev N. Global alliance against chronic respiratory diseases. Allergy. 2007;62(3):216-23.

12. Jing ZC, Xu XQ, Han ZY, Wu Y, Deng KW, Wang H, et al. Registry and survival study in chinese patients with idiopathic and familial pulmonary arterial hypertension. Chest. 2007;132(2):373-9.

13. Pengo V, Lensing AW, Prins MH, Marchiori A, Davidson BL, Tiozzo F, et al. Incidence of chronic thromboembolic pulmonary hypertension after pulmonary embolism. N Engl J Med. 2004;350(22):2257-64.

14. Hoeper MM, Mayer E, Simonneau G, Rubin LJ. Chronic thromboembolic pulmonary hypertension. Circulation. 2006;113(16):2011-20.

15. Kaul S, Tei C, Hopkins JM, Shah PM. Assessment of right ventricular function using two-dimensional echocardiography. American heart journal. 1984;107(3):526-31.

16. McLaughlin VV, Gaine SP, Barst RJ, Oudiz RJ, Bourge RC, Frost A, et al. Efficacy and safety of treprostinil: an epoprostenol analog for primary pulmonary hypertension. J Cardiovasc Pharmacol. 2003;41(2):293-9.

17. Clarke JC, Aragam JR, Bhatt DL, Brown JD, Ferrazzani S, Pietro DA, et al. An unusual cause of dyspnea diagnosed late in life: severe pulmonary hypertension resulting from isolated anomalous pulmonary venous connection. Circ Cardiovasc Imaging. 2013;6(2):349-51.

18. Johnson SR, Mehta S, Granton JT. Anticoagulation in pulmonary arterial hypertension: a qualitative systematic review. Eur Respir J. 2006;28(5):999-1004.

19. Rubin LJ. Calcium channel blockers in primary pulmonary hypertension. Chest. 1985;88(4 Suppl):257S-60S.

20. Rhodes CJ, Davidson A, Gibbs JS, Wharton J, Wilkins MR. Therapeutic targets in pulmonary arterial hypertension. Pharmacol Ther. 2009;121(1):69-88.

21. Sitbon O, Humbert M, Jais X, Ioos V, Hamid AM, Provencher S, et al. Long-term response to calcium channel blockers in idiopathic pulmonary arterial hypertension. Circulation. 2005;111(23):3105-11.

22. Rubin LJ, Mendoza J, Hood M, McGoon M, Barst R, Williams WB, et al. Treatment of primary pulmonary hypertension with continuous intravenous prostacyclin (epoprostenol). Results of a randomized trial. Ann Intern Med. 1990;112(7):485-91.

23. Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, et al. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. N Engl J Med. 1996;334(5):296-301.

24. Olschewski H, Simonneau G, Galie N, Higenbottam T, Naeije R, Rubin LJ, et al. Inhaled iloprost for severe pulmonary hypertension. N Engl J Med. 2002;347(5):322-9.

25. Macchia A, Marchioli R, Marfisi R, Scarano M, Levantesi G, Tavazzi L, et al. A meta-analysis of trials of pulmonary hypertension: a clinical condition looking for drugs and research methodology. American heart journal. 2007;153(6):1037-47.

26. Ghofrani HA, Wiedemann R, Rose F, Olschewski H, Schermuly RT, Weissmann N, et al. Combination therapy with oral sildenafil and inhaled iloprost for severe pulmonary hypertension. Ann Intern Med. 2002;136(7):515-22.

27. Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, et al. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. Faseb J. 2001;15(2):427-38.

28. Michelakis ED. The role of the NO axis and its therapeutic implications in pulmonary arterial hypertension. Heart Fail Rev. 2003;8(1):5-21.

29. Clapp LH, Finney P, Turcato S, Tran S, Rubin LJ, Tinker A. Differential effects of stable prostacyclin analogs on smooth muscle proliferation and cyclic AMP generation in human pulmonary artery. Am J Respir Cell Mol Biol. 2002;26(2):194-201.

30. Machado RD, Rudarakanchana N, Atkinson C, Flanagan JA, Harrison R, Morrell NW, et al. Functional interaction between BMPR-II and Tctex-1, a light chain of Dynein, is isoform-specific and disrupted by mutations underlying primary pulmonary hypertension. Hum Mol Genet. 2003;12(24):3277-86.

31. Petkov V, Mosgoeller W, Ziesche R, Raderer M, Stiebellehner L, Vonbank K, et al. Vasoactive intestinal peptide as a new drug for treatment of primary pulmonary hypertension. J Clin Invest. 2003;111(9):1339-46.

32. Archer S, Rich S. Primary pulmonary hypertension: a vascular biology and translational research "Work in progress". Circulation. 2000;102(22):2781-91.

33. Rubin LJ. Therapy of pulmonary hypertension: the evolution from vasodilators to antiproliferative agents. Am J Respir Crit Care Med. 2002;166(10):1308-9.

34. Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature. 1982;298(5871):240-4.

35. Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N Engl J Med. 1995;333(4):214-21.

36. Xue C, Johns RA. Endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N Engl J Med. 1995;333(24):1642-4.

37. Ferrara N. VEGF: an update on biological and therapeutic aspects. Curr Opin Biotechnol. 2000;11(6):617-24.

38. Quinlan TR, Li D, Laubach VE, Shesely EG, Zhou N, Johns RA. eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. Am J Physiol Lung Cell Mol Physiol. 2000;279(4):L641-50.

39. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc Res. 2001;49(3):568-81.

40. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev. 2004;25(4):581-611.

41. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun. 1993;192(2):553-60.

42. Kim W, Moon SO, Sung MJ, Kim SH, Lee S, So JN, et al. Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. Faseb J. 2003;17(13):1937-9.

43. Machado RD, Pauciulo MW, Thomson JR, Lane KB, Morgan NV, Wheeler L, et al. BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. Am J Hum Genet. 2001;68(1):92-102.

44. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. 1993. Biochem Biophys Res Commun. 2012;425(3):548-55.

45. Matsumoto K, Nakamura T. Emerging multipotent aspects of hepatocyte growth factor. J Biochem. 1996;119(4):591-600.

46. Sakata J, Shimokubo T, Kitamura K, Nishizono M, Iehiki Y, Kangawa K, et al. Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. FEBS Lett. 1994;352(2):105-8.

47. Liu F, Schaphorst KL, Verin AD, Jacobs K, Birukova A, Day RM, et al. Hepatocyte growth factor enhances endothelial cell barrier function and cortical cytoskeletal rearrangement: potential role of glycogen synthase kinase-3beta. Faseb J. 2002;16(9):950-62.

48. Yasuda S, Noguchi T, Gohda M, Arai T, Tsutsui N, Matsuda T, et al. Single low-dose administration of human recombinant hepatocyte growth factor attenuates intimal hyperplasia in a balloon-injured rabbit iliac artery model. Circulation. 2000;101(21):2546-9.

49. Ono M, Sawa Y, Matsumoto K, Nakamura T, Kaneda Y, Matsuda H. In vivo gene transfection with hepatocyte growth factor via the pulmonary artery induces angiogenesis in the rat lung. Circulation. 2002;106(12 Suppl 1):I264-9.

50. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev. 2005;16(2):159-78.

51. Smith K, Fox SB, Whitehouse R, Taylor M, Greenall M, Clarke J, et al. Upregulation of basic fibroblast growth factor in breast carcinoma and its relationship to vascular density, oestrogen receptor, epidermal growth factor receptor and survival. Ann Oncol. 1999;10(6):707-13.

52. Benezra M, Vlodavsky I, Ishai-Michaeli R, Neufeld G, Bar-Shavit R. Thrombin-induced release of active basic fibroblast growth factor-heparan sulfate complexes from subendothelial extracellular matrix. Blood. 1993;81(12):3324-31.

53. Dionne CA, Jaye M, Schlessinger J. Structural diversity and binding of FGF receptors. Ann N Y Acad Sci. 1991;638:161-6.

54. Arcot SS, Fagerland JA, Lipke DW, Gillespie MN, Olson JW. Basic fibroblast growth factor alterations during development of monocrotaline-induced pulmonary hypertension in rats. Growth Factors. 1995;12(2):121-30.

55. Arakawa T, Hsu YR, Schiffer SG, Tsai LB, Curless C, Fox GM. Characterization of a cysteine-free analog of recombinant human basic fibroblast growth factor. Biochem Biophys Res Commun. 1989;161(1):335-41.

56. Fox G, Schiffer S, Rohde M, Tsai L, Banks A, Arakawa T. Production, biological activity, and structure of recombinant basic fibroblast growth factor and an analog with cysteine replaced by serine. Journal of Biological Chemistry. 1988;263(34):18452-8.

57. Feige J-J, Baird A. Basic fibroblast growth factor is a substrate for protein phosphorylation and is phosphorylated by capillary endothelial cells in culture. Proceedings of the National Academy of Sciences. 1989;86(9):3174-8.

58. Molteni A, Ward WF, Ts'ao CH, Hinz JM. Monocrotaline-induced cardiopulmonary injury in rats. Modification by the neutrophil elastase inhibitor SC39026. Biochem Pharmacol. 1989;38(15):2411-9.

59. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer. 2010;10(2):116-29.

60. Pan LC, Wilson DW, Lame MW, Jones AD, Segall HJ. COR pulmonale is caused by monocrotaline and dehydromonocrotaline, but not by glutathione or cysteine conjugates of dihydropyrrolizine. Toxicol Appl Pharmacol. 1993;118(1):87-97.

61. Jana S, Chakraborty C, Nandi S, Deb JK. RNA interference: potential therapeutic targets. Appl Microbiol Biotechnol. 2004;65(6):649-57.

62. Carninci P, Yasuda J, Hayashizaki Y. Multifaceted mammalian transcriptome. Curr Opin Cell Biol. 2008;20(3):274-80.

63. Costa FF. Non-coding RNAs: Meet thy masters. Bioessays. 2010;32(7):599-608.

64. Roshan R, Ghosh T, Scaria V, Pillai B. MicroRNAs: novel therapeutic targets in neurodegenerative diseases. Drug Discov Today. 2009;14(23-24):1123-9.

65. MacDiarmid JA, Amaro-Mugridge NB, Madrid-Weiss J, Sedliarou I, Wetzel S, Kochar K, et al. Sequential treatment of drug-resistant tumors with targeted minicells containing siRNA or a cytotoxic drug. Nat Biotechnol. 2009;27(7):643-51.

66. Helene C, Thuong NT, Harel-Bellan A. Control of gene expression by triple helix-forming oligonucleotides. The antigene strategy. Ann N Y Acad Sci. 1992;660:27-36.

67. Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL, et al. Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure. 2001;9(12):1225-36.

68. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409(6818):363-6.

69. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 2001;15(2):188-200.

70. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell. 2002;110(5):563-74.

71. Kher G, Trehan S, Misra A. 7 - Antisense Oligonucleotides and RNA Interference. In: Ambikanandan M, editor. Challenges in Delivery of Therapeutic Genomics and Proteomics. London: Elsevier; 2011. p. 325-86.

72. Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature. 2004;431(7006):343-9.

73. Vickers TA, Koo S, Bennett CF, Crooke ST, Dean NM, Baker BF. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. J Biol Chem. 2003;278(9):7108-18.

74. Khatri NI, Rathi MN, Kolte AA, Kore GG, Lalan MS, Trehan S, et al. Patents Review in siRNA Delivery for Pulmonary Disorders. Recent Pat Drug Deliv Formul. 2012;6(1):45-65.

75. Tang G. siRNA and miRNA: an insight into RISCs. Trends Biochem Sci. 2005;30(2):106-14.

76. Kumar P, Ban HS, Kim SS, Wu H, Pearson T, Greiner DL, et al. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. Cell. 2008;134(4):577-86.

77. Peer D, Park EJ, Morishita Y, Carman CV, Shimaoka M. Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. Science. 2008;319(5863):627-30.

78. Juliano R, Bauman J, Kang H, Ming X. Biological barriers to therapy with antisense and siRNA oligonucleotides. Mol Pharm. 2009;6(3):686-95.

79. Mahato RI, Kawabata K, Takakura Y, Hashida M. In vivo disposition characteristics of plasmid DNA complexed with cationic liposomes. J Drug Target. 1995;3(2):149-57.

80. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov. 2009;8(2):129-38.

81. Juliano R, Alam MR, Dixit V, Kang H. Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Res. 2008;36(12):4158-71.

82. Perez-Martinez FC, Guerra J, Posadas I, Cena V. Barriers to non-viral vector-mediated gene delivery in the nervous system. Pharm Res. 2011;28(8):1843-58.

83. Crooke ST. Progress in antisense technology. Annu Rev Med. 2004;55:61-95.

84. Sternberg B, Sorgi FL, Huang L. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. FEBS Lett. 1994;356(2-3):361-6.

85. Yi SW, Yune TY, Kim TW, Chung H, Choi YW, Kwon IC, et al. A cationic lipid emulsion/DNA complex as a physically stable and serum-resistant gene delivery system. Pharm Res. 2000;17(3):314-20.

86. Alexis F, Pridgen E, Molnar LK, Farokhzad OC. Factors affecting the clearance and biodistribution of polymeric nanoparticles. Mol Pharm. 2008;5(4):505-15.

87. Bailon P, Won CY. PEG-modified biopharmaceuticals. Expert Opin Drug Deliv. 2009;6(1):1-16.

88. Martin C. Woodle FJM, Annie Yau-Young, Carl T. Redemann, inventor; Liposome Technology, Inc., assignee. Liposomes with enhanced circulation time patent 5013556. May 7, 1991.

89. Rippe B, Rosengren BI, Carlsson O, Venturoli D. Transendothelial transport: the vesicle controversy. J Vasc Res. 2002;39(5):375-90.

90. Schubert W, Frank PG, Razani B, Park DS, Chow CW, Lisanti MP. Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin in vivo. J Biol Chem. 2001;276(52):48619-22.

91. Uyechi LS, Gagne L, Thurston G, Szoka FC, Jr. Mechanism of lipoplex gene delivery in mouse lung: binding and internalization of fluorescent lipid and DNA components. Gene Ther. 2001;8(11):828-36.

92. Lechardeur D, Lukacs GL. Intracellular barriers to non-viral gene transfer. Curr Gene Ther. 2002;2(2):183-94.

93. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem. 1995;270(32):18997-9007.

94. de Lima MC, Simoes S, Pires P, Gaspar R, Slepushkin V, Duzgunes N. Gene delivery mediated by cationic liposomes: from biophysical aspects to enhancement of transfection. Mol Membr Biol. 1999;16(1):103-9.

95. Pereira FB, Goni FM, Nieva JL. Liposome destabilization induced by the HIV-1 fusion peptide effect of a single amino acid substitution. FEBS Lett. 1995;362(2):243-6.

96. Kaneda Y, Uchida T, Kim J, Ishiura M, Okada Y. The improved efficient method for introducing macromolecules into cells using HVJ (Sendai virus) liposomes with gangliosides. Exp Cell Res. 1987;173(1):56-69.

97. Farhood H, Serbina N, Huang L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim Biophys Acta. 1995;1235(2):289-95.

98. Litzinger DC, Huang L. Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. Biochim Biophys Acta. 1992;1113(2):201-27.

99. Johnson-Saliba M, Jans DA. Gene therapy: optimising DNA delivery to the nucleus. Curr Drug Targets. 2001;2(4):371-99.

100. Xu Y, Szoka FC, Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry. 1996;35(18):5616-23.

101. Bumcrot D, Manoharan M, Koteliansky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol. 2006;2(12):711-9.

102. Seyhan AA. RNAi: a potential new class of therapeutic for human genetic disease. Hum Genet. 2011;130(5):583-605.

103. Khatri N, Rathi M, Baradia D, Trehan S, Misra A. In vivo delivery aspects of miRNA, shRNA and siRNA. Crit Rev Ther Drug Carrier Syst. 2012;29(6):487-527.

104. Dick CR, Ham GE. Characterization of Polyethylenimine. Journal of Macromolecular Science: Part A - Chemistry. 1970;4(6):1301-14.

105. Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res. 1999;16(8):1273-9.

106. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials. 2003;24(7):1121-31.

107. Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. Journal of biomedical materials research. 2000;51(3):321-8.

108. Funhoff AM, van Nostrum CF, Koning GA, Schuurmans-Nieuwenbroek NM, Crommelin DJ, Hennink WE. Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH. Biomacromolecules. 2004;5(1):32-9.

109. Ferrari S, Moro E, Pettenazzo A, Behr JP, Zacchello F, Scarpa M. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. Gene Ther. 1997;4(10):1100-6.

110. Forrest ML, Koerber JT, Pack DW. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. Bioconjugate chemistry. 2003;14(5):934-40.

111. Kramer M, Stumbe JF, Grimm G, Kaufmann B, Kruger U, Weber M, et al. Dendritic polyamines: simple access to new materials with defined treelike structures for application in nonviral gene delivery. Chembiochem : a European journal of chemical biology. 2004;5(8):1081-7.

112. Fischer D, von Harpe A, Kunath K, Petersen H, Li Y, Kissel T. Copolymers of ethylene imine and N-(2-hydroxyethyl)-ethylene imine as tools to study effects of polymer structure on physicochemical and biological properties of DNA complexes. Bioconjugate chemistry. 2002;13(5):1124-33.

113. Suh J. Ionization of Poly(ethylenimine) and Poly(allylamine) at Various pH′s. Bioorganic Chemistry. 1994;22(3):318-27.

114. Choosakoonkriang S, Lobo BA, Koe GS, Koe JG, Middaugh CR. Biophysical characterization of PEI/DNA complexes. Journal of pharmaceutical sciences. 2003;92(8):1710-22.

115. von Harpe A, Petersen H, Li Y, Kissel T. Characterization of commercially available and synthesized polyethylenimines for gene delivery. Journal of controlled release : official journal of the Controlled Release Society. 2000;69(2):309-22.

116. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(16):7297-301.

117. Merdan T, Kunath K, Fischer D, Kopecek J, Kissel T. Intracellular Processing of Poly(Ethylene Imine)/Ribozyme Complexes Can Be Observed in Living Cells by Using Confocal Laser Scanning Microscopy and Inhibitor Experiments. Pharm Res. 2002;19(2):140-6.

118. Sonawane ND, Szoka FC, Jr., Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem. 2003;278(45):44826-31.

119. Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(23):14640-5.

120. Wolfert MA, Seymour LW. Atomic force microscopic analysis of the influence of the molecular weight of poly(L)lysine on the size of polyelectrolyte complexes formed with DNA. Gene Ther. 1996;3(3):269-73.

121. Dunlap DD, Maggi A, Soria MR, Monaco L. Nanoscopic structure of DNA condensed for gene delivery. Nucleic Acids Res. 1997;25(15):3095-101.

122. Bloomfield VA. DNA condensation. Current opinion in structural biology. 1996;6(3):334-41.

123. Bronich T, Kabanov AV, Marky LA. A Thermodynamic Characterization of the Interaction of a Cationic Copolymer with DNA. The Journal of Physical Chemistry B. 2001;105(25):6042-50.

124. Kabanov VA, Kabanov AV. Interpolyelectrolyte and block ionomer complexes for gene delivery: physico-chemical aspects. Advanced drug delivery reviews. 1998;30(1-3):49-60.

125. Cherng JY, Schuurmans-Nieuwenbroek NM, Jiskoot W, Talsma H, Zuidam NJ, Hennink WE, et al. Effect of DNA topology on the transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes. Journal of controlled release : official journal of the Controlled Release Society. 1999;60(2-3):343-53.

126. Ungaro F, De Rosa G, Miro A, Quaglia F. Spectrophotometric determination of polyethylenimine in the presence of an oligonucleotide for the characterization of controlled release formulations. Journal of pharmaceutical and biomedical analysis. 2003;31(1):143-9.

127. Clamme JP, Azoulay J, Mely Y. Monitoring of the formation and dissociation of polyethylenimine/DNA complexes by two photon fluorescence correlation spectroscopy. Biophysical journal. 2003;84(3):1960-8.

128. Boeckle S, von Gersdorff K, van der Piepen S, Culmsee C, Wagner E, Ogris M. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. The journal of gene medicine. 2004;6(10):1102-11.

129. Vinogradov SV, Bronich TK, Kabanov AV. Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. Bioconjugate chemistry. 1998;9(6):805-12.

130. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(18):6982-6.

131. Ruponen M, Ronkko S, Honkakoski P, Pelkonen J, Tammi M, Urtti A. Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. J Biol Chem. 2001;276(36):33875-80.

132. Kopatz I, Remy JS, Behr JP. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. The journal of gene medicine. 2004;6(7):769-76.

133. Erbacher P, Bettinger T, Belguise-Valladier P, Zou S, Coll JL, Behr JP, et al. Transfection and physical properties of various saccharide, poly(ethylene glycol), and antibody-derivatized polyethylenimines (PEI). The journal of gene medicine. 1999;1(3):210-22.

134. Kunath K, von Harpe A, Petersen H, Fischer D, Voigt K, Kissel T, et al. The structure of PEG-modified poly(ethylene imines) influences biodistribution and pharmacokinetics of their complexes with NF-kappaB decoy in mice. Pharm Res. 2002;19(6):810-7.

135. Schaffer DV, Lauffenburger DA. Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. J Biol Chem. 1998;273(43):28004-9.

136. Oh YK, Suh D, Kim JM, Choi HG, Shin K, Ko JJ. Polyethylenimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA. Gene Ther. 2002;9(23):1627-32.

137. Marschall P, Malik N, Larin Z. Transfer of YACs up to 2.3 Mb intact into human cells with polyethylenimine. Gene Ther. 1999;6(9):1634-7.

138. Brus C, Petersen H, Aigner A, Czubayko F, Kissel T. Efficiency of polyethylenimines and polyethylenimine-graft-poly (ethylene glycol) block copolymers to protect oligonucleotides against enzymatic degradation. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2004;57(3):427-30.

139. Moret I, Esteban Peris J, Guillem VM, Benet M, Revert F, Dasi F, et al. Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum. Journal of controlled release : official journal of the Controlled Release Society. 2001;76(1-2):169-81.

140. Brissault B, Kichler A, Guis C, Leborgne C, Danos O, Cheradame H. Synthesis of linear polyethylenimine derivatives for DNA transfection. Bioconjugate chemistry. 2003;14(3):581-7.

141. Kunath K, von Harpe A, Fischer D, Petersen H, Bickel U, Voigt K, et al. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. Journal of controlled release : official journal of the Controlled Release Society. 2003;89(1):113-25.

142. Petersen H, Kunath K, Martin AL, Stolnik S, Roberts CJ, Davies MC, et al. Star-shaped poly(ethylene glycol)-block-polyethylenimine copolymers enhance DNA condensation of low molecular weight polyethylenimines. Biomacromolecules. 2002;3(5):926-36.

143. Wolfert MA, Dash PR, Nazarova O, Oupicky D, Seymour LW, Smart S, et al. Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA. Bioconjugate chemistry. 1999;10(6):993-1004.

144. Reschel T, Konak C, Oupicky D, Seymour LW, Ulbrich K. Physical properties and in vitro transfection efficiency of gene delivery vectors based on complexes of DNA with synthetic polycations. Journal of controlled release : official journal of the Controlled Release Society. 2002;81(1-2):201-17.

145. Tang MX, Szoka FC. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther. 1997;4(8):823-32.

146. Michelakis ED, Dyck JR, McMurtry MS, Wang S, Wu XC, Moudgil R, et al. Gene transfer and metabolic modulators as new therapies for pulmonary hypertension. Increasing expression and activity of potassium channels in rat and human models. Adv Exp Med Biol. 2001;502:401-18.

147. Itaka K, Harada A, Yamasaki Y, Nakamura K, Kawaguchi H, Kataoka K. In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. The journal of gene medicine. 2004;6(1):76-84.

148. Hunter AC, Moghimi SM. Therapeutic synthetic polymers: a game of Russian roulette? Drug Discov Today. 2002;7(19):998-1001.

149. Aoki K, Furuhata S, Hatanaka K, Maeda M, Remy JS, Behr JP, et al. Polyethylenimine-mediated gene transfer into pancreatic tumor dissemination in the murine peritoneal cavity. Gene Ther. 2001;8(7):508-14.

150. Ochietti B, Guerin N, Vinogradov SV, St-Pierre Y, Lemieux P, Kabanov AV, et al. Altered organ accumulation of oligonucleotides using polyethyleneimine grafted with poly(ethylene oxide) or pluronic as carriers. J Drug Target. 2002;10(2):113-21.

151. Zanta MA, Boussif O, Adib A, Behr JP. In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. Bioconjugate chemistry. 1997;8(6):839-44.

152. Kunath K, von Harpe A, Fischer D, Kissel T. Galactose-PEI-DNA complexes for targeted gene delivery: degree of substitution affects complex size and transfection efficiency. Journal of controlled release : official journal of the Controlled Release Society. 2003;88(1):159-72.

153. Sagara K, Kim SW. A new synthesis of galactose-poly(ethylene glycol)-polyethylenimine for gene delivery to hepatocytes. Journal of controlled release : official journal of the Controlled Release Society. 2002;79(1-3):271-81.

154. Futaki S. Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms. Int J Pharm. 2002;245(1-2):1-7.

155. Tung CH, Weissleder R. Arginine containing peptides as delivery vectors. Advanced drug delivery reviews. 2003;55(2):281-94.

156. Nakanishi M, Eguchi A, Akuta T, Nagoshi E, Fujita S, Okabe J, et al. Basic peptides as functional components of non-viral gene transfer vehicles. Curr Protein Pept Sci. 2003;4(2):141-50.

157. Kono K, Akiyama H, Takahashi T, Takagishi T, Harada A. Transfection activity of polyamidoamine dendrimers having hydrophobic amino acid residues in the periphery. Bioconjug Chem. 2005;16(1):208-14.

158. Mateos-Timoneda MA, Lok MC, Hennink WE, Feijen J, Engbersen JF. Poly(amido amine)s as gene delivery vectors: effects of quaternary nicotinamide moieties in the side chains. ChemMedChem. 2008;3(3):478-86.

159. O'Neill MM, Kennedy CA, Barton RW, Tatake RJ. Receptor-mediated gene delivery to human peripheral blood mononuclear cells using anti-CD3 antibody coupled to polyethylenimine. Gene Ther. 2001;8(5):362-8.

160. Merdan T, Callahan J, Petersen H, Kunath K, Bakowsky U, Kopeckova P, et al. Pegylated polyethylenimine-Fab' antibody fragment conjugates for targeted gene delivery to human ovarian carcinoma cells. Bioconjugate chemistry. 2003;14(5):989-96.

161. Blessing T, Kursa M, Holzhauser R, Kircheis R, Wagner E. Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. Bioconjugate chemistry. 2001;12(4):529-37.

162. Ziegler A, Seelig J. Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters. Biophysical journal. 2004;86(1 Pt 1):254-63.

163. Suzuki T, Futaki S, Niwa M, Tanaka S, Ueda K, Sugiura Y. Possible existence of common internalization mechanisms among arginine-rich peptides. J Biol Chem. 2002;277(4):2437-43.

164. Leifert JA, Harkins S, Whitton JL. Full-length proteins attached to the HIV tat protein transduction domain are neither transduced between cells, nor exhibit enhanced immunogenicity. Gene Ther. 2002;9(21):1422-8.

165. Kaplan IM, Wadia JS, Dowdy SF. Cationic TAT peptide transduction domain enters cells by macropinocytosis. Journal of controlled release : official journal of the Controlled Release Society. 2005;102(1):247-53.

166. Rudolph C, Plank C, Lausier J, Schillinger U, Muller RH, Rosenecker J. Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. J Biol Chem. 2003;278(13):11411-8.

167. Escriou V, Carriere M, Scherman D, Wils P. NLS bioconjugates for targeting therapeutic genes to the nucleus. Advanced drug delivery reviews. 2003;55(2):295-306.

168. Martin TR, Feldman HA, Fredberg JJ, Castile RG, Mead J, Wohl ME. Relationship between maximal expiratory flows and lung volumes in growing humans. J Appl Physiol (1985). 1988;65(2):822-8.

169. Thurlbeck WM. The internal surface area of nonemphysematous lungs. Am Rev Respir Dis. 1967;95(5):765-73.

170. Akhtar S, Benter IF. Nonviral delivery of synthetic siRNAs in vivo. J Clin Invest. 2007;117(12):3623-32.

171. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect. 2005;113(7):823-39.

172. Sanders N, Rudolph C, Braeckmans K, De Smedt SC, Demeester J. Extracellular barriers in respiratory gene therapy. Advanced drug delivery reviews. 2009;61(2):115-27.

173. Sioud M. RNA interference and innate immunity. Advanced drug delivery reviews. 2007;59(2-3):153-63.

174. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell. 2003;115(2):209-16.

175. Gautam A, Waldrep JC, Densmore CL. Aerosol gene therapy. Mol Biotechnol. 2003;23(1):51-60.

176. Stribling R, Brunette E, Liggitt D, Gaensler K, Debs R. Aerosol gene delivery in vivo. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(23):11277-81.

177. Tompkins SM, Lo C-Y, Tumpey TM, Epstein SL. Protection against lethal influenza virus challenge by RNA interference in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(23):8682-6.

178. Massaro D, Massaro GD, Clerch LB. Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice. Am J Physiol Lung Cell Mol Physiol. 2004;287(5):L1066-70.

179. del Pozo-Rodriguez A, Solinis MA, Gascon AR, Pedraz JL. Short- and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2009;71(2):181-9.

180. Armstrong TK, Girouard LG, Anchordoquy TJ. Effects of PEGylation on the preservation of cationic lipid/DNA complexes during freeze-thawing and lyophilization. Journal of pharmaceutical sciences. 2002;91(12):2549-58.

181. Brus C, Kleemann E, Aigner A, Czubayko F, Kissel T. Stabilization of oligonucleotide-polyethylenimine complexes by freeze-drying: physicochemical and biological characterization. Journal of controlled release : official journal of the Controlled Release Society. 2004;95(1):119-31.

182. Packhaeuser CB, Lahnstein K, Sitterberg J, Schmehl T, Gessler T, Bakowsky U, et al. Stabilization of aerosolizable nano-carriers by freeze-drying. Pharm Res. 2009;26(1):129-38.

183. Allison SD, Molina MC, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochimica et biophysica acta. 2000;1468(1-2):127-38.