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第３次対がん総合戦略研究事業

脳転移性エクソソームによる前転移ニッシェの解明

平成２５年度　総括・分担研究報告書

研究代表者　落谷　孝広

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目　次

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分担研究報告書

脳転移性エクソソームによる前転移ニッシェの解明および研究の総括
研究代表者　落谷孝広　国立がん研究センター研究所分子細胞治療研究分野・分野長

研究要旨

本研究の目的は、乳がん細胞の血液脳関門(BBB)通過の分子メカニズムや、その前転移ニッシェの分子機構の解明、脳転移機構におけるトロピズムを乳がん細胞の分泌する小胞顆粒であるエクソソームを中心に明らかにすることで、癌の脳転移を予防する新しい方策を開発することである。平成２５年度は、初年度に引き続き、脳転移における癌細胞がBBBを通過する仕組みを、細胞間の新たなコミュニケーションツールであるエクソソーム中のmicroRNA解明を中心に解析することで、この乳がん細胞株が分泌するエクソソームがBBB破綻を誘導するメカニズムを解析した。その結果，脳に高転移する乳がん細胞から分泌されたエクソソームのBBB破壊は，エクソソームにパッケージされた特定のmicroRNA (miR-18X)が脳血管内皮細胞のタイトジャンクションを司る分子を制御する事で，タイトジャンクション，あるいは接着ジャンクションの破綻を誘導する結果を得た。さらにこのBBB破綻のmiR-18Xは、実際の脳転移を有する患者血清中に高い値を示す事も明らかとなり、臨床での実証研究も基礎研究データを支持するものであった。

A．研究背景、目的

（背景）

癌の脳転移は近年増加傾向にある。とりわけ、乳がんでは、ある特定のサブタイプに脳転移を多く認め、その生物学的特性と転移臓器におけるトロピズムの存在が示唆される。近年の分子標的治療薬の進歩により生存期間が延長した癌患者に脳転移は今後頻発すると予測され、我が国において脳転移の治療と管理法の開発は緊急かつ重要な課題である。癌転移のメカニズムには、癌細胞が脳血管関門(BBB)を通過し、脳内で腫瘍を形成できるよう、あらかじめ血管内皮細胞や間質成分などがニッシェ（前転移ニッシェ）を形成することが癌細胞の定着や増殖の最初のプロセスに重要である。本研究の目的は、BBB通過の分子メカニズムや、その前転移ニッシェの分子機構の解明、脳転移機構におけるトロピズムを乳がん細胞の分泌する小胞顆粒であるエクソソームを中心に明らかにすることで、癌の脳転移を予防する新しい方策を開発することである。

B．研究方法

２年次となる平成25年度は、おもに脳転移における癌細胞がBBBを通過する仕組みを、細胞間の新たなコミュニケーションツールであるエクソソームの中のmicroRNAを中心に解析する目的で、初年度に樹立した脳に高転移性を示すヒト乳がん細胞株（落谷、小野）の分泌するエクソソームからRNAを抽出し，そのmicroRNAの網羅的発現解析を実施，そのデータを解析することで、BBB破綻を誘導するmicroRNAを同定し，その分子メカニズムを解明する。

（倫理面への配慮）

遺伝子組み換え生物等の使用等の規制による生物多様性の確保に関する法律（「バイオセーフティに関するカルタヘナ議定書」に基づくカルタヘナ法）」の定める細則と、文部科学省・厚生労働省・経済産業省の定める細則、ならびに施設内の組み換えDNA実験指針の基準に従って、定められた基準に適合することを確認し、指針に従ってDNA組み換え実験委員会等の倫理審査委員会の審査を経る手続きを適切に行う。動物実験は、国立がん研究センターの定める動物実験指針に従うとともに、動物倫理委員会の承認を得たうえで、動物の苦痛の低減に務め、動物愛護の精神に基づく実験を行う。ヒトの臨床サンプル解析に関しては、センターの倫理審査委員会の承認を得て実施する。ヒト臨床検体の使用は，所内の倫理委員会に計画書を提出し、審査を受けて承認が得られている。

C．研究結果

エクソソームのmicroRNA網羅的発現解析の結果，脳に高転移する乳がん細胞から分泌されたエクソソームのBBB破壊は，エクソソームにパッケージされた特定のmicroRNA (miR-18X)が脳血管内皮細胞のタイトジャンクションを司る分子を制御する事で，タイトジャンクション，あるいは接着ジャンクションの破綻を誘導する結果を得た。さらにこのBBB破綻のmiR-18Xは、実際の脳転移を有する患者血清中に高い値を示す事も明らかとなり、臨床での実証研究も基礎研究データを支持するものであった。

以上の成果は，高転移の細胞由来のエクソソームにはBBBを破綻させる能力が有る事が示唆された。さらに、血管内皮細胞のタイトジャンクションを形成するoccludin, claudin等，あるいは接着ジャンクションを形成するN-cadherin等の分子の免疫染色を実施した結果，高転移の細胞由来のエクソソーム処理によって，これらの分子の細胞表面の局在が失われることが判明した。従ってエクソソームに由来するmiR-18Xの標的分子群が，これらのジャンクションを制御する分子あるいは関連制御分子である可能性が浮上した。また、国立がん研究センター 中央病院の乳腺腫瘍科のコホート研究の一部の血清を用いて，ステージ３／４の転移患者のmiR-18Xの量を解析した結果，脳転移を有する患者群で、血清中のmiR-18Xの量が有意に高い事も判明した。

D．考察

エクソソームによって引き起こされるBBB破壊のメカニズムを明らかにするに至るとともに，BBBを破壊するmicroRNAの臨床的意義にまで研究を発展する事が出来た事は特筆に値する研究成果である。

E．結論

転移性乳癌細胞の分泌するエクソソームには、血液脳関門を破壊し，乳がん細胞を脳に転移するし機構が存在する事が示唆された。さらに、こうした現象の原因となりうるエクソソーム中のマイクロRNAの存在も，臨床検体の解析から示唆された事は意義の有る研究成果であり，今後診断，治療に向けた発展が期待される。

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G. 知的財産権の出願・登録状況（予定を含む。）

1.特許取得
特になし。
2.実用新案登録
特になし。
3.その他

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

脳転移を規定する non-coding RNA のエピゲノムプロファイリング

研究分担者　畑田出穂　群馬大学生体調節研究所

研究要旨

転移能、薬剤耐性を獲得した癌細胞でのエピゲノムの変化、特に DNA のメチル化の変化は癌細胞の性質を決定づける重要な因子である。脳転移の関連した miRNA の転写調節領域での DNA のメチル化の変化はエクソソーム内での miRNA の量的変化への関連が予想される。本研究では昨年、miRNA の転写調節領域での DNA のメチル化を次世代シーケンサーを用いて調べた。miRNA の転写調節領域の DNA のメチル化が癌における miRNA の転写制御に重要な役割を担っていることを明らかにしてきた。今年度はこのメチル化を制御する因子として miR-29 ファミリーをみいだした。miR-29 はメチル化酵素と脱メチル化酵素の両者を抑制することにより、メチルの変化を抑えエピジェネティク状態を維持して癌化を防いでいることがわかった。

A．研究背景、目的

（背景）

癌細胞におけるエピゲノムの変化、特にDNAメチル化の変化は癌化、癌の転移能、薬剤耐性など悪性化に関連した様々な性質において重要な働きをしていることが知られている。小分子RNAのひとつであるmiRNAの発現変化は癌化やその悪性化において重要な働きをしていることがわかってきている。これらmiRNAをコードする遺伝子においてもDNAのメチル化の変化は通常の遺伝子と同様、癌細胞で変化が見られる。

一方、細胞が分泌するエンドソーム由来の小胞顆粒であるエクソソームの中に、miRNAが安定して存在することが発見され、細胞間のメッセンジャーとして機能することが示唆されている。特にがん患者の血清中のエクソソームには健常人と異なる種類と量のmiRNAが含まれていることが報告されており、バイオマーカーとしても注目されている。このような変化は癌細胞中におけるmiRNAの発現量の変化とも関係しており、癌細胞におけるエピゲノムの研究が重要であることがわかる。

さて、どのようにしてメチル化は制御されているのだろうか？今回我々はmiR-29がその役割をなしていることを示唆する証拠をえたので報告する。

B．研究方法

miR-29 は miR-29a、miR-29b、miR-29c、のファミリーからなるが、癌抑制遺伝子として知られている。すなわちその発現と予後との関係が報告されていたり、癌細胞で強発現をすることで腫瘍形成が抑えられることが報告されているからである。従来 miR-29 は DNA メチル化酵素の DNMT3A, DNMT3B を抑制することでがん抑制遺伝子のメチル化を防ぐ働きがあるといわれているが、本当にそれだけかを検証するためにターゲット抑制ソフトの miRanda などを用いて miR-29 のターゲットの検索をおこなった。そして候補の遺伝子をレポーター実験などで検証した。

（倫理面への配慮）

今回の解析では該当しない。

C．研究結果

miR-29 のターゲットとして miRanda の候補で多くの候補が上がってきた。その中でヒトでもマウスでも保存されているものを上げると興味深いことに脱メチル化に関与する TET1 と TDG があった。そこでこれらの遺伝子のターゲットを含む配列を用いてレポーター実験をおこなったところ、いずれの遺伝子も miR-29 によって発現が抑制されることがわかった。さらに miR-29 が内在性の TET1 と TDG を抑制できるかを Realtime-PCR やウエスタンブロットで確認することができた。

D．考察

miR-29 はがん抑制遺伝子であるが、今回 DNA 脱メチル化に関与する TET1 と TDG を抑制することがわかった。従来 miR-29 はメチル化の酵素 DNMT3A と DNMT3B を抑制することが知られていた。そとことからがん抑制遺伝子のメチル化を防ぐことが mi-29 の働きと考えられていたが、今回脱メチル化も抑制していることがわかり、むし

ろ miR-29 はメチルの変化を抑えエピジェネティク状態を維持して癌化を防いでいると考えられる。

E．結論

miR-29 はメチル化酵素と脱メチル化酵素の両者を抑制することにより、メチルの変化を抑えエピジェネティク状態を維持して癌化を防いでいる。

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G．知的財産権の出願・登録状況
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特になし。
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特になし。
3.その他
特になし。

別紙４

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Fujita Y, Takeshita F, Mizutani T, Ohgi T, Kuwano K, Ochiya T.	A novel platform to enable inhaled naked RNAi medicine for lung cancer.	Sci Rep	3	3325	2013
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Review

RNAi Therapeutic Platforms for Lung Diseases

Yu Fujita [1,2], Fumitaka Takeshita [1], Kazuyoshi Kuwano [2] and Takahiro Ochiya [1,*]

[1] Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, 104-0045, Japan; E-Mails: yufujit2@ncc.go.jp (Y.F.); futakesh@ncc.go.jp (F.T.)

[2] Division of Respiratory Diseases, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, 105-8461, Japan; E-Mail: kkuwano@jikei.ac.jp (K.K.)

* Author to whom correspondence should be addressed; E-Mail: tochiya@ncc.go.jp; Tel.: +81-3-3542-2511; Fax: +81-3-5565-0727.

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Abstract: RNA interference (RNAi) is rapidly becoming an important method for analyzing gene functions in many eukaryotes and holds promise for the development of therapeutic gene silencing. The induction of RNAi relies on small silencing RNAs, which affect specific messenger RNA (mRNA) degradation. Two types of small RNA molecules, *i.e.* small interfering RNAs (siRNAs) and microRNAs (miRNAs), are central to RNAi. Drug discovery studies and novel treatments of siRNAs are currently targeting a wide range of diseases, including various viral infections and cancers. Lung diseases in general are attractive targets for siRNA therapeutics because of their lethality and prevalence. In addition, the lung is anatomically accessible to therapeutic agents via the intrapulmonary route. Recently, increasing evidence indicates that miRNAs play an important role in lung abnormalities, such as inflammation and oncogenesis. Therefore, miRNAs are being targeted for therapeutic purposes. In this review, we present strategies for RNAi delivery and discuss the current state-of-the-art RNAi-based therapeutics for various lung diseases.

Key words: RNAi; siRNA; miRNA; drug delivery system; lung diseases; lung cancer

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1. Introduction

RNA interference (RNAi) is a natural endogenous mechanism for silencing gene expression that, recently, has been the focus of considerable attention for its potential use in new drugs [1]. The expression of a specific gene can be regulated using different mediators, such as short hairpin RNA

(shRNA), microRNA (miRNA), and small interfering RNA (siRNA). Gene silencing can be induced by siRNAs through a sequence-specific cleavage of perfectly complementary messenger RNA (mRNA); in contrast, miRNAs mediate translational repression and transcript degradation for imperfectly complementary targets. RNAi-based therapy may provide several advantages over conventional therapeutic approaches using small molecules, proteins, and monoclonal antibodies. Unlike traditional drugs, RNAi-based therapeutics can inhibit all classes of gene targets with high selectivity and potency, can provide personalized therapy, can be easily synthesized, and can be conducted through rapid steps of lead identification and optimization [2]. Synthetic oligonucleotides have other potential advantages, such as drug-like properties, that can often be improved through the introduction of chemical modifications, and manufacturing processes are usually amenable to scaled-up production. Several *in vivo* studies in animal models have demonstrated that RNAi-based therapeutics are effective for the treatment of various diseases, such as viral hepatitis [3], Huntington's disease [4], and some cancers [5]. Furthermore, there are several RNAi therapeutic agents in clinical development. Nevertheless, previous investigations have shown that there are several obstacles that need to be overcome before routine clinical applications are made. RNAi-based therapeutics are promptly degraded by nucleases when they are administered systemically, and chemical modifications at specific positions or formulation with delivery vectors have been shown to improve stability, but they may attenuate the suppressive activity of oligonucleotides [6]. Their systemic administration may induce undesirable off-target effects by activating the innate immune system via toll-like receptor (TLR)-dependent or independent mechanisms, leading to an increased number of inflammatory cytokines [7]. Success of the delivery of RNAi-based therapeutics necessitates efficiency, convenience, and patient compliance of the delivery route. For this reason, direct administration of RNAi-based therapeutics into the target organs is a promising approach for overcoming the problems of systemic administration. So far, an approach for drug treatment has been developed that includes transdermal, rectal, vaginal, and pulmonary drug delivery systems.

The lung is susceptible to many diseases because of its location and physiological function. It is usually exposed to many environmental pollutants, including smoke and volatile organic compounds, which lead to diseases such as asthma, emphysema, and lung cancer. Furthermore, many of the lethal infectious diseases are airborne and use the lungs as their main entrance to the body. Therefore, lung diseases have received particular attention as targets of direct administration of RNAi-based therapeutics. As a direct route to the lung, pulmonary delivery has offered a new method for the treatment of various lung diseases, such as cancer [8–12], respiratory infectious diseases [13–17], asthma [18,19], and pulmonary fibrosis [20,21]. The approach could potentially enhance the retention of RNAi-based therapeutics in the lungs and reduce systemic toxic effects. However, the development of pulmonary delivery for clinical applications remains a challenge for research of drug delivery systems and development. This review focuses on the latest development of pulmonary delivery and future plans for the RNAi-based treatment of various lung diseases.

2. Delivery of RNAi-Based Therapeutics to the Lungs

The lung is emerging as an attractive target for the treatment of various pathogenic disorders using RNAi-based therapeutics because of the increasing incidence of lung diseases with high mortality and

morbidity. The primary obstacle to translating RNAi-based therapy from the laboratories into the clinics is delivery. Delivery of siRNAs to the lungs is often studied and described using different routes and delivery strategies [22]; therefore, the focus of this chapter is on the characteristics of siRNA delivery to the lung.

In general, lung targeting can be achieved by intravenous as well as intrapulmonary administration. Although multiple routes of administration using siRNAs have been used, ranging from direct injection into target tissues to systemic administration, the use of siRNAs for the treatment of respiratory diseases has tended to focus on direct intratracheal or intranasal delivery of siRNAs to the lungs. The direct route offers several important benefits over systemic delivery, including the requirement for lower doses of siRNAs, the reduction of undesirable systemic side effects, and improved siRNA stability due to lower nuclease activity in the airways than in the serum. Lastly, and most importantly, in the context of treating respiratory disease, local administration of siRNAs allows direct access to lung epithelial cells, which are important cell types in a variety of pulmonary disorders [23]. Since the lung is accessible to therapeutic agents via multiple intrapulmonary routes, it has been a convenient model for *in vivo* validation of siRNA-mediated therapeutic gene silencing.

2.1. Pulmonary Delivery Approaches

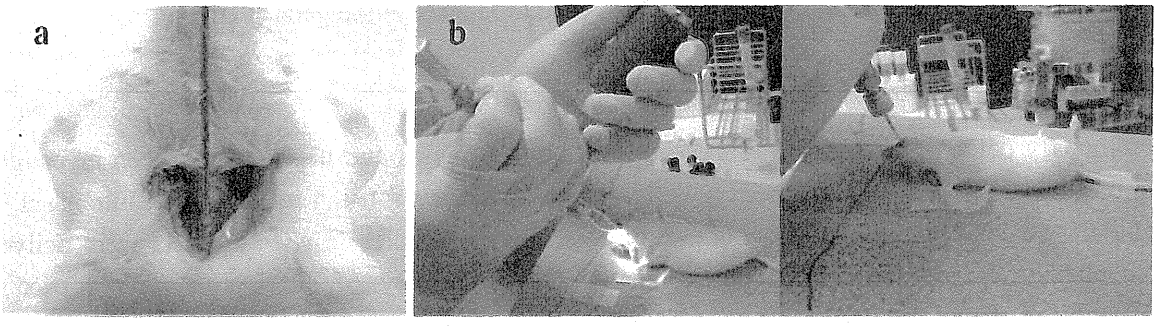
Pulmonary delivery of therapeutic molecules, such as proteins and peptides, has been investigated for more than 30 years [23]. Pulmonary delivery can be achieved using intratracheal, intranasal, and inhalation routes. In most of the pulmonary siRNA therapy studies *in vivo*, siRNAs were delivered intratracheally or intranasally. In particular, intranasal delivery of siRNAs is widely used for administration due to its simplicity and adaptability to the delivery of various siRNA formulations, such as nasal spray and droplets. Although administration by inhalation is clinically the most common and non-invasive method to deliver therapeutic agents into the lung, only a few animal studies have been conducted on the formulation of inhalation of siRNAs [24,25].

2.1.1. Intratracheal and Intranasal Delivery

Intratracheal administration is one common method of pulmonary drug application. The pulmonary application method can be useful for the study of drug and vaccine delivery to the airway and lungs. Many animal studies have relied on intratracheal delivery of siRNAs to the lungs [15,26–31]. Moreover, some of them have also reported successful delivery of unmodified siRNAs without delivery vectors. The advantages of the intratracheal route are that it ensures high delivery efficiency with minimal loss of the drug and the application itself is quick and relatively inexpensive. The disadvantage is that, because it requires a surgical procedure, such as a tracheotomy, it is not a comfortable delivery method from the patient's viewpoint. With this method, the trachea is exposed during the procedure, and an endotracheal tube or microsyringe is inserted through an incision between the tracheal rings [Figure 1(a)]. This method is not routinely used for drug administration in humans [32]. On the other hand, Bivas-Benita *et al.* reported a relatively non-invasive pulmonary delivery via the endotracheal route [33]. In this method, the formulation is sprayed under anesthesia from the mouth to the trachea of mice using a microsyringe (Figure 1b). The main benefit of the endotracheal application is the visualization of the trachea, which is important for reliable lung administration. Compared with

traditional surgery, Bivas-Benita *et al.* reported that no mortality occurred as a result of the use of the endotracheal technique. Endotracheal applications are currently being used by many practitioners in the pulmonary field [22,34]; this is useful for studying pulmonary drug delivery in mice. However, the approach is more complex in humans because an artificial path for the delivery of drugs into the lungs is used. Therefore, the method is being used in animal models to test and evaluate its reliability for possible clinical applications.

Figure 1. Intratracheal route of siRNA administration into the lungs *in vivo* studies. (**a**) Intratracheal route: under anesthesia, the trachea is exposed surgically, and a tube or needle is inserted through an incision made between the tracheal rings. Complications, such as vascular injury and air leakage, are possible due to the tracheotomy. (**b**) Endotracheal route: siRNAs are sprayed directly from the mouth into the lungs using a MicroSprayer® aerolizer (Penn-Century, Philadelphia, PA, USA) and a laryngoscope. It is important to maintain a clear view of the trachea during the procedure.



Intranasal delivery is another common method of pulmonary drug application in animal studies. In many studies, *in vivo* success has been demonstrated in delivering siRNAs to the lungs intranasally [22,35,36]. An experimental setup of intranasal delivery by spray or droplet is simple and painless for the animal. Although the success in delivering siRNAs intranasally in rodents cannot be completely extrapolated to human use because of the significant differences in lung anatomy [37], this approach has potential for the clinical application of siRNAs. Phase II clinical trials have been initiated for the treatment of respiratory syncytial virus (RSV) infection, making use of intranasal application of naked chemically modified siRNA molecules that target viral gene products [17,38] (see Section 3.1.1. for details).

Intranasal entry has long been used to administer small molecules, such as proteins, for systemic delivery. Because the nasal mucosa is highly vascularized, delivery of a thin epithelium of medication across the surface area can result in rapid absorption of the medication into the blood. Therefore, siRNAs administered intranasally might be deposited in the nose, and some of them may be unable to reach the lower respiratory tract. In fact, it has been reported that intranasal application of unformulated siRNAs resulted in lower delivery efficiency and homogeneous pulmonary distribution than that achieved with intratracheal application [31]. The intranasal method is suitable for some lung diseases, such as upper respiratory infection by RSV, and it also has potential for systemic delivery rather than pulmonary delivery of siRNAs. Therefore, it is important to consider the route of administration in animal studies when assessing the delivery and therapeutic efficacy of a formulation

for pulmonary delivery. Careful choice of efficient delivery in response to the condition of lung diseases is necessary.

2.1.2. Inhalation Delivery

The use of aerosols to deliver medication to the lungs has a long history. Administration by inhalation is a popular and non-invasive method of delivering agents into the lungs. There are several inhalation devices available for the delivery of drugs into the lungs. Metered dose inhalers (MDIs) and dry powder inhalers (DPIs) are the most common modes of inhaled delivery. MDIs are the most commonly used inhalers for several lung diseases, such as asthma, bronchitis, and chronic obstructive pulmonary disease (COPD), and a spacer is an external device that is attached to an MDI to allow for better drug delivery by enhanced actuation and inhalation coordination. For most MDIs, the propellant is one or more gases called chlorofluorocarbons (CFCs). Although CFCs in drugs are safe for patients to inhale, they are harmful to the environment. Therefore, further development of inhalable siRNAs may not be the best way forward. DPIs are devices that deliver medication to the lungs in the form of dry powder. The use of DPIs has already shown promise for the *in vivo* delivery of therapeutic macromolecules such as insulin [39] and low-molecular-weight heparin [40]; thus, it could be a better device for delivering siRNAs to the lungs. The advantages of DPIs are improved stability and sterility of biomolecules over liquid aerosols and propellant-free formation.

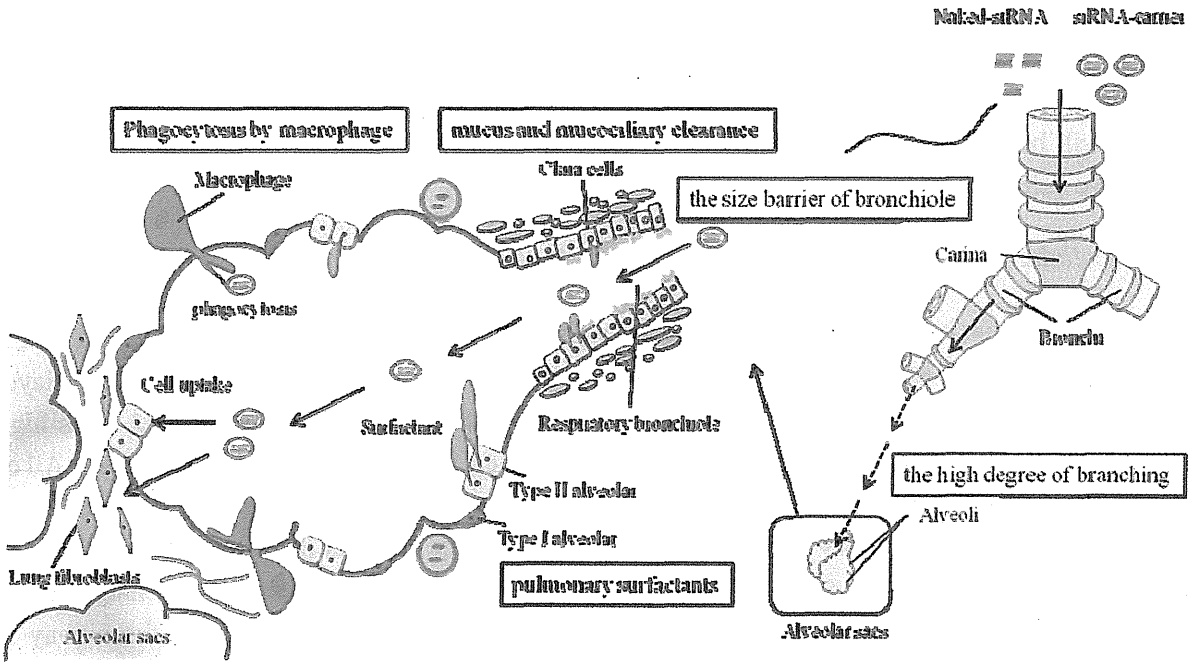
Although drugs are commonly delivered to the lungs by inhalation, most *in vivo* studies using siRNAs have relied on intratracheal or intranasal delivery. The reason could be the difficulty in formulating inhalable siRNAs and maintaining the stability during the delivery process. A suitable carrier is also needed to protect nucleic acids from degradation due to shear force and increased temperature during the drying process. The use of spray-drying as a technique for engineering dry powder formulations of siRNA nanoparticles, which might enable the local delivery of biologically active siRNA directly to the lung tissue, has been demonstrated [24,25]. In the future, the technique is desirable to estimate the *in vivo* study on siRNA therapy for inhalation. In the long term, we anticipate that there will be more sophisticated devices for clinical use and that those currently being developed will be more suitable.

2.2. Extracellular and Intracellular Barriers to siRNA Delivery

There are two main barriers to efficient pulmonary siRNA delivery to the cells of the lung. The first is the complex, branched anatomy of the lungs and biomechanical barriers, such as the mucus layer covering the airway cells [41,42] (Figure 2). A remarkable feature of the respiratory tract is its high degree of branching. Airway consists of respiratory bronchioles, alveolar ducts, and alveolar sacs. All of these structures bear alveoli, the tiny air sacs in which the gas exchange takes place. It is generally acknowledged that the critical factor for efficient siRNA delivery depends on the properties of RNAi drug particles in terms of size, charge, shape, velocity and density. For efficient pulmonary siRNA delivery, the particles must be deposited in the lower respiratory tract. Deposition in the airway is affected by the particle size and patient's pulmonary function. A particle size between 1–5 μm is found to be the most appropriate for deposition at the lower respiratory tract [23]. In addition, the presence of mucus and surfactant proteins, the mucociliary clearance actions, and phagocytosis by macrophages

present major barriers to targeted pulmonary delivery. Therefore, delivery systems usually require delivery vectors, and these vectors need to be designed in order to maximize the siRNA deposition to the diseased area of the respiratory tract. Besides, the extracellular barriers to siRNA delivery also depend on physiological features of the respiratory tract, which may change with the disease stage and characteristics of the patient. At the active stage of lung disease, the physiological conditions of the airways might change and have significant impact on the efficiency of the pulmonary delivery system. During infection, inflammation, and allergic reaction, there is an increase in mucus secretion along with the impaired mucociliary clearance [43]. Moreover, asthma and COPD are both chronic inflammatory conditions of the lung associated with structural "remodeling" that is inappropriate to the maintenance of normal lung function [44]. The airway wall thickness, the high viscosity, and the composition of the mucus layer might be altered in patients who have inflammatory lung diseases.

Figure 2. Extracellular barriers to pulmonary siRNA delivery. The anatomical feature of the respiratory tract is its high degree of branching. The mucus lines the respiratory epithelium from the nasal cavity to the terminal bronchioles. The deposited particles on the ciliated epithelial cells are rapidly cleared by the mucociliary clearance actions. Mucus and mucociliary clearance of mucus-trapped particles is a pulmonary defense mechanism as a physiological barrier. In the alveolar, clara cells and type II alveolar cells secrete on the surface of the alveolar epithelium, forming a thin layer of pulmonary surfactants. The surfactants act as the main barrier for siRNA delivery because they reduce the transfection efficiency. In addition, the macrophages located in the alveoli rapidly engulf the foreign particles by phagocytosis. The particles taken up into the macrophages are subsequently degraded inside the cells. These factors present major barriers to targeted pulmonary delivery.

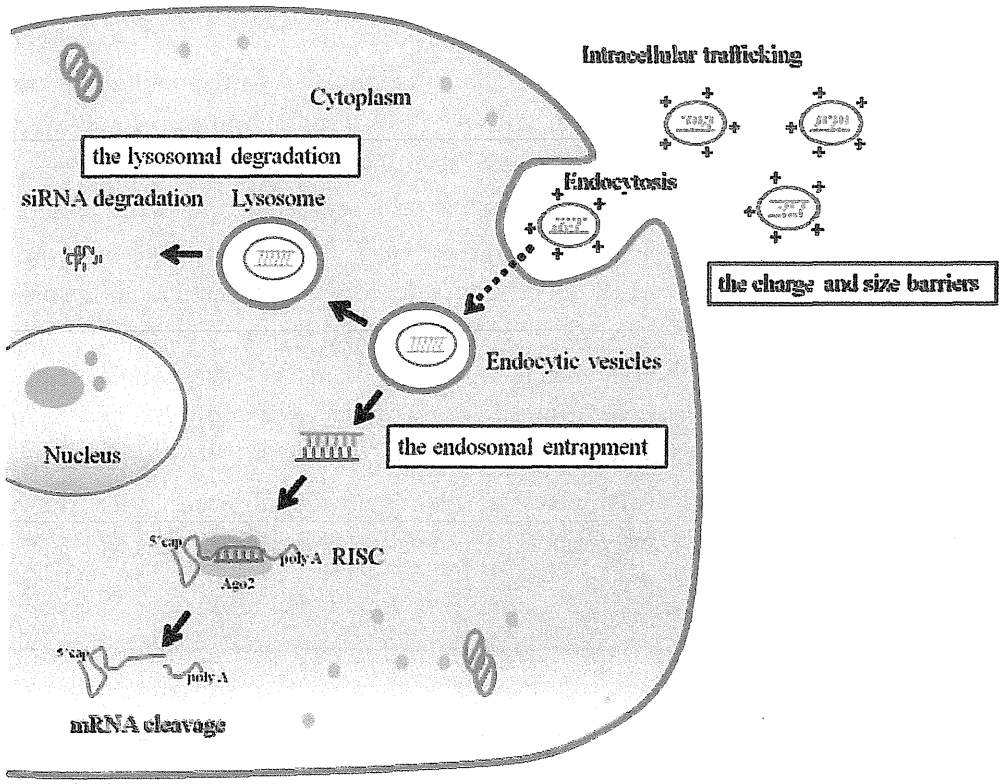


The second is the airway cell membrance and its intracellular barriers (Figure 3). For efficient gene silencing in the lungs, siRNAs must be delivered to their site of action, be stable, enter the target cells, and be present in the cytoplasm at sufficient concentration. Once the siRNAs reach the target cells, they must be trafficked into the cytoplasm and taken up by Argonaute (Ago)2/RNA-induced silencing complex (RISC), which degrades mRNAs and, subsequently, suppresses the sequence-specific gene expression. For efficient endocytosis to occur, particles should be under 150 nm in size. Particles within this size range could also avoid macrophage uptake and delayed lung clearance [45]. The physicochemical properties of siRNAs also play a significant role in crossing the biological membrane. Despite their small size, the negative charge and chemical degradability of siRNA molecules prevent them from readily crossing biological membranes. Therefore, efficient siRNA delivery approaches need to overcome this limitation by facilitating cellular uptake. One of the main functions of a delivery vector is to facilitate the cellular uptake of siRNAs [46]. The electrostatic complexation of siRNA molecules with cationic lipids and polymers helps to mask their net negative charge. The positively charged siRNA carrier complex interacts with anionic proteoglycans on the cell membrance, forms an endocytic vesicle, and enters the cells by endocytosis [47]. After cellular internalization, the siRNA carrier complex in endocytic vesicles is transported along microtubules to lysosomes that are co-localized with the microtubule-organizing center. To avoid lysosomal degradation, siRNAs must escape from the endosome into the cytoplasm, where they can associate with the RNAi machinery. Endosomal escape is a major barrier for efficient siRNA delivery [48,49]. The endosomal entrapment and lysosomal degradation of siRNA and carriers contribute to the low transfection efficiency and is a major difficulty for delivery vectors. An ideal delivery agent should protect siRNAs from enzymatic degradation, facilitate cellular uptake, and promote endosomal escape inside the cells with negligible toxicity.

2.3. Delivery Method of siRNA to the Lungs

Multiple approaches for the delivery of siRNAs have been reported, ranging from the relatively simple direct administration of saline-formulated siRNAs to lipid-based and polymer-based nanoparticle approaches and siRNA conjugation and complexation approaches [50]. The negative charge and chemical degradability of siRNAs under physiologically relevant conditions make its delivery a major challenge. Accordingly, the delivery of siRNAs usually requires a vector or carriers for their transfection into the target cells. In general, both viral and non-viral vectors are being assessed for siRNA delivery to lung cells. Some viral vectors, such as retroviruses and adenoviruses, have been demonstrated to mediate gene silencing in an *in vitro* lung model [51] and to induce RNAi in a range of animal tissues [52]. Recently, Guo *et al.* showed that lentivirus-mediated siRNA was used to specifically knock down the expression of nuclear protein 1 (NUPR1) *in vivo*, which resulted in inhibited tumor growth [53]. However, viral-based delivery has several disadvantages. The immune response to viruses not only impedes gene delivery but also has the potential to cause severe complications [54]. Recent well-documented cases, such as the death of Jesse Gelsinger due to complications related with an adenoviral delivery vector, highlight this problem [55]. In addition, some viral vectors may insert their genome at random positions in the host chromosome, which eventually restrict the gene function [56].

Figure 3. Intracellular barriers to pulmonary siRNA delivery. Barriers to cellular internalization are dependent on the surface properties of siRNA and carriers (e.g., charge and size). After siRNAs are successfully taken into the target cells by endocytosis, the main barriers for delivering siRNAs to its site of action are the endosomal entrapment and lysosomal degradation of siRNA and carriers. To direct target-gene silencing, the siRNAs need to escape from the endosome into the cytoplasm, where they associate with the Ago2/RNA-induced silencing complex (RISC) to direct the cleavage of mRNAs bearing complementary binding sites.



As an alternative to viral vectors, non-viral vectors, including lipid and polymer-based vectors, have been generally used for the delivery of siRNAs to the lungs due to their reduced toxicity [57]. Ongoing research into the transfection of primary cells and whole organisms with siRNA using non-viral transfection agents has produced some promising results. Lipid-based delivery vectors are successfully used to deliver siRNA *in vitro* and *in vivo* [58]. Cationic lipids are composed of positively charged head, a linker and hydrophobic. In general, lipid-based complexes are easy to formulate and good transfection efficacy is achieved due to interaction with negative charged cell membrance. Many commercial siRNA transfection agents are lipid-based delivery system, some of which are also employed for pulmonary delivery—DharmFECT [30], Oligofectamine [59], Lipofectamine [60] and TransIT-TKO [35]. Similarly, cationic polymers have also been assessed for siRNA delivery to lung cells. Cationic polymer polyethylenimine (PEI) is widely used for siRNA delivery [13,61]. PEI is considered as the gold standard for *in vitro* gene delivery and its transfection efficiency depends on the molecular weight and degree of branching.

On the other hand, lipid-based vectors can also induce toxicity and non-specific activation of inflammatory cytokine and interferon responses [62,63]. Although polymer-based vectors elicit a relatively less strong immune response than lipid-based vectors, effective siRNA delivery to a local area in lung diseases requires more attention to the development of non-toxic delivery vectors. An important point for siRNA-mediated inhibition of gene expression is whether the observed effects are specific rather than due to off-target effects and free from potential interferon responses [64,65]. Interestingly, some studies have shown that it was possible to administer "naked siRNAs" to mice and down-regulate an endogenous or exogenous target without inducing an interferon response [66].

The term "naked siRNAs" refers to the delivery of siRNAs without any delivery vectors. Naked siRNAs are degraded by serum endonucleases and are usually removed by glomerular filtration, resulting in a short plasma half-life of < 10 min. Thus, some studies of systemic delivery of naked siRNAs have failed to achieve the downregulation of the targeted gene [67,68]. In contrast, there have also been some successes of locally delivering naked siRNAs to the lungs [15,16,20,31]. A few of them reported that the use of delivery vectors showed no significant difference in gene silencing efficiency compared to that of naked siRNAs [16,35]. Indeed, in one clinical trial, the delivery of naked siRNAs for the treatment of RSV has been used [17,38]. This successful evidence can be because that naked siRNAs for clinical applications are highly chemically modified to prevent nuclease-induced degradation and presumably minimize immune stimulatory effects. Although it is unclear how the naked siRNAs cross the cell membrane, gain access to the cytoplasm, and remain intact to perform their biological action, both animal and human trials have been conducted successfully, showing the efficacy of naked siRNAs (ALN-RSV01) that were administered intranasally. This explanation has not been confirmed, but the physiological damage of respiratory epithelial cells caused by viral infection may have possibly influenced the mystery. The active change in airway epithelial cell membrance caused by infectious disease might affect cellular internalization. Naked siRNA delivery has some advantages, such as simple formation and the absence of toxicity or inflammatory responses that are usually associated with delivery vectors. Nevertheless, the advantage of naked siRNAs over delivery vectors in the treatment of lung diseases is controversial [69,70]. Further *in vivo* investigations about both naked siRNAs and non-viral vectors are required.

3. RNAi Medicine in Lung Diseases

Lung disease is a major cause of death, and diminished quality of life is responsible for the suffering of many patients. Various lung diseases make life extremely difficult for the patients, and severe cases of these lung diseases can result in death. The high death rates associated with lung cancer are partially due to the fact that it is unfortunately difficult to cure. Above all, COPD is the fourth-leading cause of death in most industrialized countries and is predicted to become third by 2020 [71]. Therefore, decisive action is needed to stem the rising health and economic burden this represents. Chronic lung diseases, such as COPD and asthma, are disorders of the airways largely related to the presence of persistent inflammation. The approval of inhaled corticosteroids pioneered a new generation of therapy in treating chronic inflammatory diseases. This was the first time that an anti-inflammatory product was available to reduce the characteristic lung inflammation in airways and the associated obstruction. Corticosteroids are still an important therapeutic intervention. However,

they are used with limitations in COPD and moderate to severe asthma. Likewise, the treatment of various refractory lung diseases also depends on systemic corticosteroid therapy. Many of these patients also suffered various side effects from systemic corticosteroid use, such as weight gain and uncontrolled hyperglycemia. Treatment of lung disease using cell-specific targeting as well as RNAi techniques represents a novel strategy and could possibly provide new opportunities in nanomedicine. Pulmonary applications of siRNA in *in vivo* conditions are frequently studied and often result in clinical trials [57,72]. The findings of recent clinical studies of pulmonary RNAi therapeutics are discussed.

3.1. Therapeutic siRNAs for Lung Diseases

Since the discovery of RNAi, the therapeutic potential of siRNAs has been rapidly recognized. In 2004, the first human clinical trial of RNAi-based therapy was initiated for the treatment of age-related macular degeneration with a siRNA targeting VEGF-receptor 1 delivered intravitreally [73]. Many studies have been conducted over the past few years that involve the delivery of siRNAs to the lungs for the treatment of various lung diseases. Delivery to the lungs will be most important to moving siRNA technology into the clinic. A number of siRNA-based therapies are being evaluated in clinical trials for the treatment of different conditions, including lung diseases such as asthma and RSV infection. Table 1 is a summary of clinical trials of siRNA-based therapeutics [74].

Table 1. Summary of siRNA-based therapeutics in clinical trials.

Drug	Route of Administration	Delivery Agent	Disease	Target	Stage of Clinical Trial
Excellair™	Inhalation	Unknown	Asthma	Syk kinase	II
ALN-RSV01	Intranasal spray	Naked siRNA	RSV infection	RSV nucleocapsid	IIb
Atu027	IV	Lipid nanoparticles	Advanced solid cancer (Metastatic lung cancer)	PKN3	I
TKM-ApoB	IV	Lipid nanoparticles	Hypercholesterolemia	ApoB	I
TKM-PLK1	IV	Lipid nanoparticles	Cancer	Polo-like-kinase1	I
ALN-VSP02	IV	Lipid nanoparticles	Solid cancers with liver involvement	KSP and VEGF	I
ALN-TTR01	IV	Lipid nanoparticles	Transthyretin-mediated amyloidosis (ATTR)	Transthyretin (TTR)	I
CALAA-01	IV	Cyclodextrin nanoparticles	Solid tumor	RRM2	I
siG12D LODER	EUS biopsy needle	Miniature biodegradable polymer matrix	Pancreatic ductal adenocarcinoma	KRAS	I
I5NP	IV	Naked siRNA	Acute kidney injury	p53	I/II

Table 1. *Cont.*

Drug	Route of Administration	Delivery Agent	Disease	Target	Stage of Clinical Trial
QPI-1007	IVT	Naked siRNA	Glaucoma and acute eye injury	Caspase-2	I
TD101	Intradermal injection	Naked siRNA	Pachyonychia congenita	Keratin 6a N171Kmutant mRNA	Ib
SYL040012	Ophthalmic drops	Naked siRNA	Ocular hypertension and glaucoma	Adrenergic receptor beta-2	I/II
AGN-745	IVT	Naked siRNA	AMD	VEGF-receptor1	II
PF-655	IVT	Naked siRNA	AMD and diabetic macular edema	RTP801 (pro-angiogenic factor)	II
Bevasiranib	IVT	Naked siRNA	AMD	VEGF	III

IV: Intravenous injection; IVT: Intravitreal injection; RSV: Respiratory syncytical virus; AMD: Age-related macular degeneration; Syk: spleen tyrosine kinase; PKN3: protein kinase N3; KSP: kinesin spindle protein; RRM2: M2 submit of ribonucleotidereductase; KRAS: V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog; VEGF: vascular endothelial growth factor.

3.1.1. Pulmonary Viral Infections

SiRNA shows potential for the treatment of various pulmonary viral infections, and it has been reported that siRNA-based therapeutics can also be used in the treatment of influenza [13], parainfluenza virus [35], severe acute respiratory syndrome (SARS) [14], and RSV [35]. Above all, RSV is the most promising therapeutic target of siRNAs.

RSV is a common cause of serious respiratory infections in infants and children. It also produces significant morbidity and mortality in adult immunocompromised or elderly populations [75]. An RSV vaccine is not available, and the only approved antiviral therapy for RSV is undesirable for pediatric patients due to its potential teratogenicity and limited effectiveness. Thus, a safe and efficacious RSV therapy has long been awaited for both pediatric and adult patients. RNAi-based therapy has shown promising effects in murine models of RSV infection [35]. The siRNA, ALN-RSV01, is directed against the mRNA encoding the N-protein of RSV that exhibits specific *in vitro* and *in vivo* anti-RSV activity. It is delivered without a delivery vector as a nasal spray and targets the upper respiratory tract instead of the lower lung area. ALN-RSV01 has undergone complete phase I intranasal and inhalation studies in healthy adults and has been found to be generally well tolerated [38]. Additionally, ALN-RSV01 has been evaluated in a randomized, double-blind, placebo-controlled phase II trial in lung transplant patients with RSV respiratory tract infection [76]. The administration of ALN-RSV01 to RSV infected lung transplant patients was safe and well tolerated and associated with a statistically significant improvement in symptoms. Based on these results, a larger multinational, randomized,