CRISPR editing in the lung with novel lipids

**Uri Elia, Edo Kon & Dan Peer**

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CRISPR RNA is delivered to the mouse lung by inhalation using improved lipid nanoparticles.

Many patients with inherited lung diseases lack effective treatment and could derive immense benefit from gene-editing therapies that correct the causative mutation. Direct delivery of such therapies to the lung by inhalation would be transformative, but the respiratory route of administration faces special challenges arising from physiological mechanisms that protect the lung against foreign particles. In a new study in *Nature Biotechnology*, Li et al.[1] tackle this longstanding issue with a focus on RNA–lipid nanoparticles (LNPs) – a drug class that has been much in the news thanks to COVID-19 mRNA vaccines. By synthesizing and screening 720 novel lipids, the authors identified a nanoparticle formulation that achieves moderately efficient editing in the mouse lung after intratracheal delivery of CRISPR RNA–LNPs. This achievement paves the way for therapeutic CRISPR

applications in the lung that involve not only gene editing but also gene therapy.

The lung has evolved robust defenses against foreign pathogens and particles that rely on physical barriers and clearance mechanisms. The airway lumen is surrounded by a continuous barrier of epithelial cells of various subtypes bound together by tight junctions. These cells secrete mucus and other factors into the lumen, which, together with oscillating cilia, efficiently entrap and clear foreign particles. Additionally, alveolar macrophages internalize and clear foreign particles and pathogens that enter the airway[2].

Pulmonary drug delivery has been the subject of decades of research. Clinically approved inhaled therapeutics include aerosolized antibiotics and small-molecule drugs, such as β2-adrenergic agonists, antimuscarinic agents and corticosteroids for the treatment of asthma and chronic obstructive pulmonary disease. If pulmonary delivery of small molecules has been routine, inhaled nucleic acid therapies are just beginning to emerge. Several clinical trials have evaluated
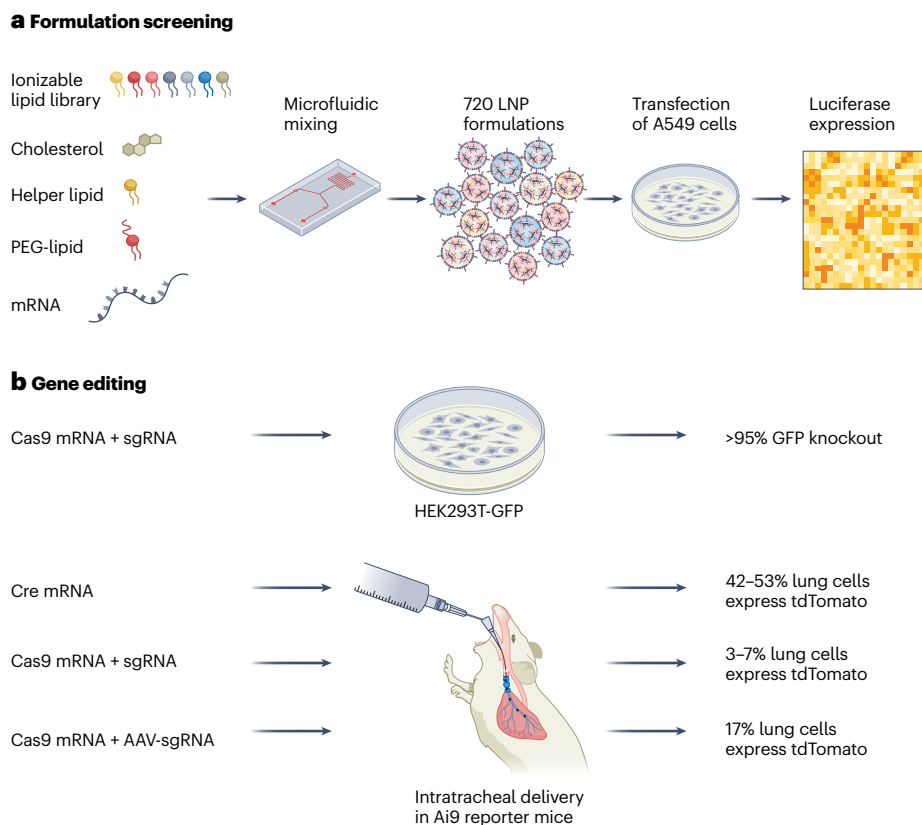
Fig. 1 | Overview of LNP formulation screening process. a, A library of 720 rationally designed LNP formulations was screened in vitro for luciferase expression in A549 human alveolar basal epithelial cells. **b**, Potent LNP formulations were first used for evaluation of Cas9-mediated gene editing in

vitro (GFP knockout in HEK293T cells). Next, lung gene editing was evaluated in *lox–3xSTOP–lox (LSL)–tdTomato* (Ai9) reporter mice following intratracheal administration of *Cre* mRNA alone, *Cas9* mRNA plus single-guide RNA (sgRNA) together in an LNP, or *Cas9* mRNA in an LNP plus sgRNA in an AAV vector.

pulmonary delivery of antisense oligonucleotide drugs to treat asthma, cystic fibrosis and chronic obstructive pulmonary disease. Gene editing in lungs, however, is still at the preclinical stage. For example, efficient editing in the mouse lung was achieved by intratracheal delivery of Cas9 and single-guide RNAs by adeno-associated virus serotype 5 (AAV5)[3].

CRISPR editing tools have been formatted as DNA, RNA or ribonucleoproteins. In their study, Li et al. focused on Cas9 nuclease and guide RNA provided in the form of RNA. When RNA–LNPs are injected into the circulation, most particles are taken up in the liver. Although some uptake by other organs has been reported[4], targeting the lung by respiratory delivery may enable greater efficacy, lower doses and less activity in off-target organs. Moreover, RNA–LNPs have several advantages over conventional drugs, including cell-free, rapid manufacturing; high versatility; and a favorable safety profile[5].

LNPs may also have unique advantages for overcoming the lung's physiological barriers. First, they protect the RNA cargo from degradation. Second, the use of biosimilar or naturally occurring phospholipids in the formulation could reduce toxicity, phagocytic clearance and the induction of lung immune responses. Third, the small size of LNPs allows them to reach deep lung tissues, which is often necessary for effective treatment. Finally, LNPs can adhere to the mucosal surface, extending residency time in lung tissue and improving pharmacokinetic profiles[2].

LNPs are typically composed of an amino-ionizable lipid, a helper lipid, a polyethylene glycol lipid and cholesterol. The ratio of these components strongly affects the particles' size, shape, charge, stability and distribution. Li et al. began by synthesizing biodegradable, ionizable lipids containing a nitro ricinoleic acrylate linker with one of 10 different tails and 72 headgroups, for a total of 720 new lipids (Fig. 1a). The lipids were incorporated into standard LNP formulations, which were evaluated in vitro and in vivo for RNA transfection efficiency. A lipid called RCB-4-8 emerged as the most potent for intratracheal delivery, with approximately a 100-fold improvement over LNPs formulated with DLin-MC3-DMA (MC3), a clinically approved lipid used for mRNA delivery. Notably, RCB-4-8 was cleared from lung tissues much faster than MC3, reducing the risk of toxicity.

Next, the authors studied the properties of RCB-4-8 for gene editing in the mouse lung (Fig. 1b). Intratracheal administration of LNPs loaded with Cre mRNA to lox–3xSTOP–lox (LSL)–tdTomato (Ai9) reporter mice led to gene editing in a large proportion of lung cells, which was further augmented by repetitive dosing. Replacing the Cre mRNA with Cas9 mRNA co-encapsulated with sgRNA appeared to substantially lower the editing efficiency, but the authors succeeded in boosting efficiency using a hybrid approach in which Cas9 mRNA was packaged in LNPs and the guide RNAs were delivered by the viral vector AAV5. Although the efficiency achieved with the hybrid system was similar to that of a previous all-AAV5 method for lung gene editing[3], encoding the Cas9 enzyme in mRNA has the advantage of avoiding safety concerns associated with AAV delivery of Cas9, such as long-term nuclease expression and higher risks of genomic integration and off-target activity.

Many congenital lung diseases, such as surfactant-protein deficiency disorders, cystic fibrosis and α-1 antitrypsin deficiency, are caused by known mutations that could serve as targets for gene editing. Intratracheal delivery of CRISPR components by viral vectors[3] has several potential drawbacks, including long-term expression of Cas9, which increases the likelihood of genome integration and off-target

activity, and antiviral immune responses, which limit repeated dosing. In contrast, LNPs offer a non-integrating, minimally immunogenic vehicle that allows transient protein expression, repetitive dosing and larger gene cargos.

RNA–LNPs have already enabled CRISPR editing in the liver both in preclinical studies[6] and in a first-in-human clinical trial for patients with hereditary transthyretin amyloidosis[7]. To our knowledge, Li et al. are the first to demonstrate the approach in the lung. Their tour de force work suggests several avenues for advancing the technology. Depending on the disease, it may be desirable to target specific lung cell types, perhaps by adapting existing approaches for cell type–specific delivery of LNPs[8,9]. Approaches to reduce LNP uptake by alveolar macrophages could also be beneficial. Progress in lung delivery can be expected to increase the percentage of gene-edited cells. In cancer settings, for example, in vivo gene editing has been demonstrated in 80% and 70% of tumor cells in metastatic ovarian and glioblastoma cancer models, respectively, after intraperitoneal (ovarian cancer model) or intracerebral (glioblastoma model) administration of LNPs co-encapsulating Cas9 mRNA and a single guide RNA targeting PLK1 (ref. 10). Lastly, some alterations to the lipid formulation may be necessary for LNPs to withstand nebulization in the clinic.

The method of Li et al. for respiratory delivery of RNA enables gene editing in mouse lung cell types that are currently less accessible through intravascular injection. The approach should aid the discovery of gene-editing drugs that are effective in treating lung diseases in animal models and, with further optimization, could provide an enabling technology for clinical applications.

Uri Elia[1,2,3,4,5,6], **Edo Kon**[1,2,3,4,6] **& Dan Peer** [1,2,3,4] ✉

[1]Laboratory of Precision NanoMedicine, Shmunis School for Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel. [2]Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv, Israel. [3]Department of Materials Sciences and Engineering, Iby and Aladar Fleischman Faculty of Engineering, Tel Aviv University, Tel Aviv, Israel. [4]Cancer Biology Research Center, Tel Aviv University, Tel Aviv, Israel. [5]Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness-Ziona, Israel. [6]These authors contributed equally: Uri Elia, Edo Kon
✉e-mail: peer@tauex.tau.ac.il

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