

Cystic fibrosis findings

Stephen Hart, Professor of Molecular Genetics at the University College London Institute of Child Health in the UK, shares his enthusiasm for new and vital research into gene-based therapies for cystic fibrosis



Respiratory disease is the main cause of premature death in cystic fibrosis (CF) patients, of which there are around 9,000 in the UK. What current treatments exist for this condition?

CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-activated chloride channel. The drug Kalydeco (Vertex Pharmaceuticals) restores the functionality of CFTR, but only in a rare group of patients with the G551D mutation. It was recently approved for clinical use and significantly improves lung function. However, for most patients the only treatment currently available is symptomatic relief.

Our improved understanding of CF pathology, genetics and immunology, as well as new technologies, demonstrate that it is time for a re-evaluation of the potential of new CF gene therapy strategies.

In a previous study, your group demonstrated that a receptor-targeted nanocomplex – comprising targeting peptides, cationic liposomes and plasmid DNA – is highly effective for airway epithelial transfection *in vitro* and *in vivo*. Could you provide some insight into how this technique works?

The peptide component, through its amino acid sequence, contains two functional domains:

one a lysine-rich, positively charged region that enables packaging of the negatively charged nucleic acids into nanoparticle structure; the other a receptor-targeting motif similar to the targeting molecules on rhinovirus, which infects lungs to cause colds.

The peptide-targeting properties and net positive charge of the nanocomplexes enable binding and uptake into cells by endocytosis. Endosomal enzymes can destroy the DNA but the lipid components of the nanocomplexes enable fusion with the endosomal bilayer and escape into the cytoplasm and subsequently the nucleus. Thus, the combination of lipids and peptides endows the nanocomplexes with multiple functionalities that are essential for high overall transfection efficiency.

Why have you chosen to develop a CpG-free CFTR gene plasmid in a minicircle vector for gene therapy?

Minicircles, expressing a codon-optimised, CpG-depleted *CFTR*, offer greater gene expression with lower immunogenic toxicity than conventional plasmid constructs. Plasmids contain elements that, although necessary for amplification in bacteria, are not required for gene expression in mammalian cells. These elements can be removed in minicircle DNA, reducing the size of the therapeutic molecule and increasing its potency.

Hypomethylated CpG motifs in plasmids occur more frequently in bacterial DNA than in eukaryotic genomes. Mammalian immune systems recognise unmethylated CpG motifs and induce an inflammatory response. This is highly undesirable, especially in CF lungs. Minicircles contain less CpG motifs and during gene synthesis most remaining CpG elements can be removed from the CF gene – *CFTR* – exploiting the redundancy of the genetic code to maintain the encoded protein sequence. Also during gene synthesis, codon optimisation can be performed to ensure highly efficient messenger RNA translation into protein, using codons for which transfer RNAs are most frequent.

RNA interference is a technique for switching off gene expression. What would be the effect of silencing the epithelial sodium channel (ENaC)?

Loss of CFTR causes hyperactivity of ENaC leading to increased salt and water uptake from the watery, periciliary liquid (PCL) layer lining the lung; which is essential for normal ciliary clearance of particles, including bacteria, from the lung. Thus, dehydration of the PCL and mucus above the epithelial cells may be a major pathogenic mechanism in CF. Silencing the *ENaC* gene by small interfering RNA (siRNA) could reduce salt and water uptake, restoring hydration of the lung surface and so restoring normal mucociliary clearance. This could help to protect the lung from the frequent, damaging bacterial infections in the CF lung.

Existing ENaC inhibitors are effective in reducing sodium uptake and restoring the PCL but their effects are too brief for practical benefit. How do you plan to improve this technique for CF treatment?

We aim to use siRNA which, once introduced and assembled into the enzymatic RNA-induced silencing complex (RISC) in long-lived epithelial cells, could potentially have a regulatory effect on ENaC for weeks before repeated administration is required.

Are there any new research developments that you think will have an impact on gene-based CF therapies in the future?

New genome editing technologies such as the CRISPR/Cas9 system provide opportunities for correction of the specific CF mutation at the chromosomal level with a high degree of efficiency. Research suggests that this can be performed at all stages of development from stem cells to adult. Hence, I think that we may see genome editing gathering momentum in the coming years for CF gene therapy as well as many other diseases. Viral vectors also remain of interest, with research particularly active in lentiviral and adeno-associated virus-based vectors.

Genetic therapy, **genuine relief**

Cystic fibrosis is a debilitating, life-threatening disease for which the majority of treatment is limited to symptomatic relief, which can be extremely gruelling. Novel research is developing gene therapies with the potential to hugely ameliorate both life expectancy and quality of life

CYSTIC FIBROSIS (CF) is one of the most common autosomal recessive genetic disorders. It occurs in approximately one in every 2,000 live births in the UK, and there are around 60,000 people with the condition worldwide. CF was first described in the 1930s; the name refers to the scarring (fibrosis) and cysts observed within the pancreas of sufferers. It is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-activated chloride channel.

The most severe effects of CF are in the lung, but the disorder also affects the pancreas, liver and intestines. It causes imbalanced water and ion movement across the epithelium of multiple organs which, in the periciliary liquid (PCL) layer of the lungs, produces a thick mucus. This is, in turn, associated with inflammation and bacterial infection, as well as life-threatening, progressive loss of lung function. Poor growth and infertility are other common symptoms, as are osteoporosis, liver problems, and ear, nose and throat disorders.

LIMITED RELIEF

Symptomatic relief is, for most CF patients, the only treatment currently available. Such treatments are invasive, as Professor Stephen Hart, who is currently researching gene-based therapies for CF in the Institute of Child Health at University College London, UK, explains: "Typically, patients require twice-daily regimens of physiotherapy and nebuliser treatments, supplemented with numerous orally delivered medications, often requiring more than three hours of treatment every day".

There are also drug treatments, such as bronchodilators which relax the airways and improve breathing, antibiotics to treat bacterial infections, steroids to reduce inflammation and mucolytics such as deoxyribonuclease (DNase) which reduce the thickness of the mucus in the lungs, making it easier to clear during physiotherapy. However, it is evident that these treatments leave CF patients with a dramatically decreased quality of life. The hope of ameliorating the long-term health prospects and quality of life of CF patients, especially children, is what drives Hart's search for an effective genetic therapy for the disease.

The trend towards symptomatic relief of CF has been influenced by unimpressive results from several genetic therapy trials in the 1990s. Since that time, many have been reluctant to pursue research in this area, but recent developments in small molecule drug therapies have rendered older liposome technologies and immunogenic

viral vectors, with which these past trials were performed, obsolete.

A RETURN TO GENETICS

New technologies, such as nanoparticles, nucleic acids, nebulisation and imaging, are proving vital aids in the development of novel genetic therapy strategies. The success of the oral drug Kalydeco, developed by Vertex Pharmaceuticals and approved by the US Food and Drug Administration in early 2012, has demonstrated the potential of such therapies.

Kalydeco is a small molecule drug which is genotype specific but not a nanoparticle or gene therapy. It has been effective in correcting CFTR activity, which is the same ultimate goal as gene therapy, hence providing proof of concept. However, Kalydeco is only able to target a very rare mutation found in approximately 4 per cent of CF sufferers. It is hoped that further research will produce a molecule which targets $\Delta F508$, occurring in 90 per cent of CF individuals.

Despite the proven efficacy of such genotype-specific therapies, their development and implementation may prove too expensive for UK National Health Service (NHS) provision. Thus, Hart's current focus is on developing gene replacement and small interfering RNA (siRNA) therapies that will, in the long term, be far more cost-effective as they can be administered to all CF patients, regardless of genotype.

NEBULISATION OF NANOCOMPLEXES

Hart is currently investigating whether inactivation of the *ENaC* gene – which causes dehydration of the PCL and the often fatal build up of mucus in the lungs of CF patients – is able to protect CF lungs from bacterial infection. Treatment would be administered by nebulisation, most probably for the duration of the patient's lifetime. There are challenges associated with this form of nanocomplex delivery; for instance, the output from the nebuliser relative to input and the size of aerosol particles produced by the nebuliser



INTELLIGENCE

GENE BASED THERAPIES FOR CYSTIC FIBROSIS

OBJECTIVES

To develop new therapeutic options for cystic fibrosis, specifically focusing on gene therapy, including vector development, cystic fibrosis transmembrane conductance regulator gene replacement therapy and small interfering RNA therapy by silencing of the epithelial sodium channel.

KEY COLLABORATORS

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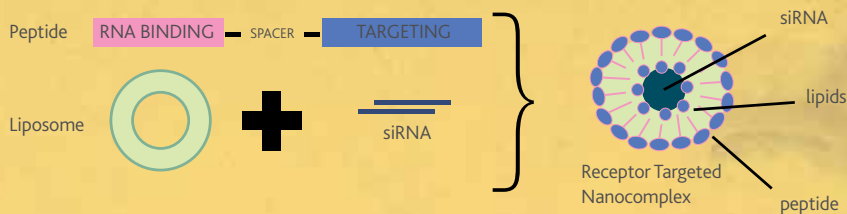
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STEPHEN HART is Professor in Molecular Genetics at the UCL Institute of Child Health.

He received his PhD from the University of Cape Town, South Africa. His research interests include the development of gene and small interfering RNA therapies for cystic fibrosis. He has more than 100 research publications and 10 patents.



Cartoon of the nanoparticle formulation.

will determine where in the lung the inhaled aerosol is deposited. In the CF lung, the target is the conducting airways.

In a project funded by Sparks, a leading children's medical research charity, Hart's team demonstrated the importance of imaging CF lungs prior to and during the administration of the gene therapy vector. This allows the distribution of the vector in the lungs to be monitored. In humans, clinicians will be able to predict the level of efficacy of treatment, as this was shown to be altered by build up of mucus and obstruction in the lungs. After testing *in vivo* in pigs – a suitable animal model due to their comparable lung size – Hart's team found the Trudell AeroEclipse* Breath Actuated Nebulizer to be best suited to their needs. Furthermore, histological and toxicological analyses reported no adverse affects from nebulisation.

MURINE MODELS

Various studies were also carried out in the lungs of mice. "Experiments included instillation of nanocomplexes encoding luciferase or beta galactosidase reporter genes into murine lungs by the technique of oropharyngeal instillation," outlines Hart. "Reporter gene expression was evaluated in homogenised lung extracts and in histological sections." One important discovery from these trials was that repeat dosing of DNA maintained the same level effectiveness as the first dose. Furthermore, a single dose was persistent in expression for up to 21 days. Were this to be replicated in human lungs, the improvement to the quality of life of CF patients compared with currently available treatments is evident.

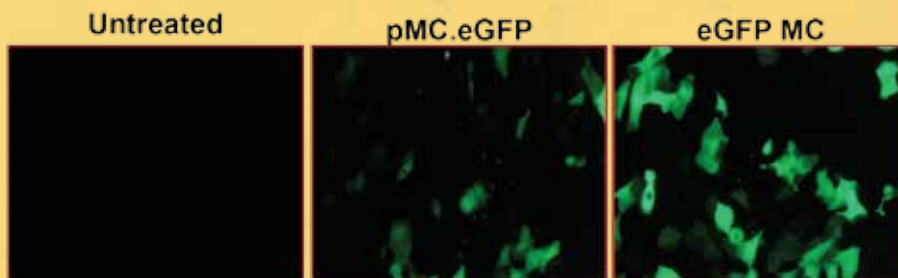
At present, Hart is working on further optimising CFTR transfection in the lungs of mice, for both

single and repeat dosing. The aim is to improve the vector formulation composition: "We are investigating anionic nanocomplexes using anionic lipid components as opposed to the current cationic formulations, as well as surface modification strategies, such as PEGylation, both of which could help to improve penetration through mucus to better access the epithelia cell surface," Hart explains. There will also be an improvement in vector formulation mixing through the use of microfluidic technologies, which facilitate the production of smaller, more consistent nanoparticles that can better penetrate mucus and therefore access the epithelial cell surface more effectively.

HUMAN IMPACT

Hart's research is not limited to animal models. By collecting human normal and CF epithelial cells by nasal brushings and maintaining these cells on a semi-permeable membrane at an air-liquid interface (ALI), a culture containing the full range of cell types can be developed. CF cells on an ALI display many of the same defects as CF lungs, even producing mucus. As the ALI allows the cells to be maintained over many weeks, they provide a valuable opportunity to learn more about the development of CF in humans and to test the efficacy of therapies at the cellular level.

Results from this ongoing research could have life-changing and life-saving consequences for thousands of CF individuals. The proven success of Kalydeco has given both the medical establishment and health services cause to believe in the potential of gene-based therapy for the treatment of CF, thus Hart can hope that, once his therapies are fully developed, they will be granted the opportunity to have a real impact.



Transfection of cells showing differences in enhanced green fluorescent protein (eGFP) expression from plasmid (pMC) and mini-circle (MC).

