Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Pharmacology

The suppression of premature termination codons and the repair of splicing mutations in CFTR

Yifat S Oren[1,2], Iwona M Pranke[3,5], Batsheva Kerem[1] and Isabelle Sermet-Gaudelus[3,4,5]

Premature termination codons (PTC) originate from nucleotide substitution introducing an in-frame PTC. They induce truncated, usually non-functional, proteins, degradation of the PTC containing transcripts by the nonsense-mediated decay (NMD) pathway and abnormal exon skipping. Readthrough compounds facilitate near cognate amino-acyl-tRNA incorporation, leading potentially to restoration of a functional full-length protein.Splicing mutations can lead to aberrantly spliced transcripts by creating a cryptic splice site or destroying a normal site. Most mutations result in disruption of the open reading frame and activation of NMD. Antisense oligonucleotides are single stranded short synthetic RNA-like molecules chemically modified to improve their stability and ability to recognize their target RNAs and modify the splice site. This review focuses on recent developments in therapies aiming to improve the health of CF patients carrying nonsense or splicing mutations.

Addresses
[1] Department of Genetics, The Life Sciences Institute, The Hebrew University, Jerusalem, Israel
[2] SpliSense Therapeutics, Givat Ram Campus, Hebrew University, Jerusalem, Israel
[3] INSERM U 1151, Institut Necker Enfants Malades, Paris, France
[4] Unité de Pneumo-Allergologie Pédiatrique, Centre de Ressources et de Compétence de la Mucoviscidose, Hôpital Necker Enfants Malades, Paris, France
[5] Université Paris Sorbonne, France

Corresponding authors: Kerem, Batsheva (batshevak@savion.huji.ac.il), Sermet-Gaudelus, Isabelle (isabelle.sermet@aphp.fr)

Current Opinion in Pharmacology 2017, **34**:125–131

This review comes from a themed issue on **Respiratory**

Edited by **David N Sheppard**, **Hugo R de Jonge** and **Christine E Bear**

For a complete overview see the Issue and the Editorial

Available online 10th November 2017

https://doi.org/10.1016/j.coph.2017.09.017

Cystic fibrosis (CF), is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, leading to impaired anion secretion and ultimately infected obstructive lung disease, among other symptoms [1,2]. Novel insights and hopes have arisen from recent proof of concept studies demonstrating that recovery of 10–25% of the wild-type (WT) CFTR activity is sufficient to achieve significant clinical improvement [3,4••].

This review discusses recent developments in therapies to improve CFTR function in patients carrying nonsense or splicing mutations to achieve clinical benefit.

Nonsense mutations

Premature termination codons (PTCs) (UAG, UGA, or UAA) (encoded by nonsense mutations) account for ~20% of gene defects in human diseases and are often associated with severe phenotypes. PTCs usually originate from nucleotide substitutions introducing an in-frame PTC. These mutations induce three distinct molecular defects: firstly, production of a truncated, usually non-functional, protein; secondly, degradation of the PTC containing transcripts by the nonsense-mediated decay (NMD) pathway and thirdly, exon skipping, due to the use of alternative cryptic acceptor or donor sites within the exon encompassing the stop codon [5].

The 165 PTCs, described in the CFTR gene account for ~5 to 10% of all CF mutations (cystic fibrosis mutation database; //http://www.genet.sickkids.on.ca/app). They are mostly associated with severe forms of CF [6]. However, genotype–phenotype studies revealed wide variability in disease severity among patients and even among sibs indicating that factors other than the mutation itself affect CFTR function and disease severity [7].

Homeostatic cellular mechanisms affecting RNA stability

Transcripts carrying nonsense mutations are subjected to Nonsense Mediated Decay (NMD), a surveillance mechanism which detects and degrades transcripts carrying nonsense mutations, preventing the synthesis of truncated proteins [8] and protecting the cell from potentially deleterious truncated proteins [9]. In vertebrates, the NMD mechanism occurs following pre-mRNA splicing and is mostly mediated by the exon junction complex (EJC) loaded on mRNA upstream of exon–exon junctions [10]. A stop codon located >50–55 nucleotides upstream of an exon–exon junction is recognized as a PTC during the pioneer round of translation [11]. The NMD pathway is therefore a crucial cellular post-transcriptional regulatory mechanism that affects the expression of broad classes of physiologic transcripts [12]. As NMD

modulates the level of nonsense transcripts available for the readthrough treatment, this pathway also regulates the response to read-though treatment [13].

Variability in NMD efficiency is an inherent feature of cells [14] which can be modulated by genetic variations [15], environmental conditions [16], or aberrant cellular regulation [17]. Importantly, inefficient NMD increases the number of transcripts carrying nonsense mutations, resulting in generation of truncated proteins that cannot be folded correctly and therefore accumulate in the endoplamic reticulum (ER). This in turn activates the Unfolded Protein Response (UPR) to restore cellular homeostasis by activating ER chaperones and foldases and inhibiting translation of new substrates [18]. This translation attenuation also inhibits the NMD mechanism by a feedback mechanism. Hence, the NMD and UPR mechanisms and the interplay between them, may modulate the response to read-through treatment. Indeed substantial differences in UPR activation were found between patients correlating with their response to readthrough [19••].

Potential therapies for nonsense mutations

PTCs are susceptible to limited (~0.1%) constitutive readthrough, which can be stimulated up to ~2 to 6% by readthrough compounds (e.g. gentamicin, G418) facilitating near cognate amino-acyl-tRNA incorporation [20], leading potentially to restoration of a functional full-length protein. If enough PTCs are converted to sense codons, sufficient full-length protein might be produced to provide a therapeutic benefit if the protein is functional (illustrated in Figure 1). The best characterized readthrough drugs are aminoglycosides, demonstrated to restore the CFTR-dependent Cl^- secretion and protein expression with improved clinical end-points [21,22]. Unfortunately, gentamicin cannot be used as a readthrough drug, due to serious renal toxicity and ototoxicity when used for prolonged periods. High throughput screening for readthrough drugs identified PTC124 (Ataluren), which has no antibiotic properties and is hypothetized to promote readthrough of PTCs without affecting the termination at normal stop codons and is without severe side effects [23].

Variable responses to Gentamicin and Ataluren were found among CF patients carrying the same nonsense mutation [21,22,24–26]. This variability may reflect differences in the efficiency of the treatment and can depend on the stop codon itself and the near cognate tRNA paired [27••], the nucleotide sequence neighboring the PTC [28], the number of transcripts affected by exon skipping [29••] and finally by the efficiency of NMD. Indeed, responding patients had higher levels of CFTR mRNA which could serve as templates for the readthrough processes, while non-responders had significantly lower levels [13].

Although a recent double-blind, placebo-controlled Phase III study using Ataluren was unsuccessful, (PTC Therapeutics Announces Results from Pivotal Phase 3 Clinical Trial of Ataluren in Patients Living with Nonsense Mutation Cystic Fibrosis; http://ir.ptcbio.com/releasedetail.cfm?ReleaseID=1015471 2017), novel readthrough molecules are being identified, such as RTC13 and RTC14 [30], NB124 and derivatives [31] and recently Escin [32••]. Moreover, as some of the new synthesized proteins are misfolded or possess defective activity, increasing their function with CFTR correctors and potentiators is likely to have therapeutic benefit [33••,34••]. Importantly, the development of such novel drugs should consider the impact on normal termination codons to avoid off target effects.

Therefore, additional strategies to impede NMD efficiency have the potential to improve the response to readthrough. One of the possible ways to reduce NMD efficiency is to inhibit the binding of the EJCs downstream of the considered PTC. This can be achieved with gene-specific antisense oligonucleotides (AONs) for PTCs that are susceptible to NMD [35]. Interestingly, an NMD inhibitor, Amlexanox, has also been shown to induce readthrough [36]. Such drugs with dual activities might increase readthrough efficiency by increasing the amount of target transcripts.

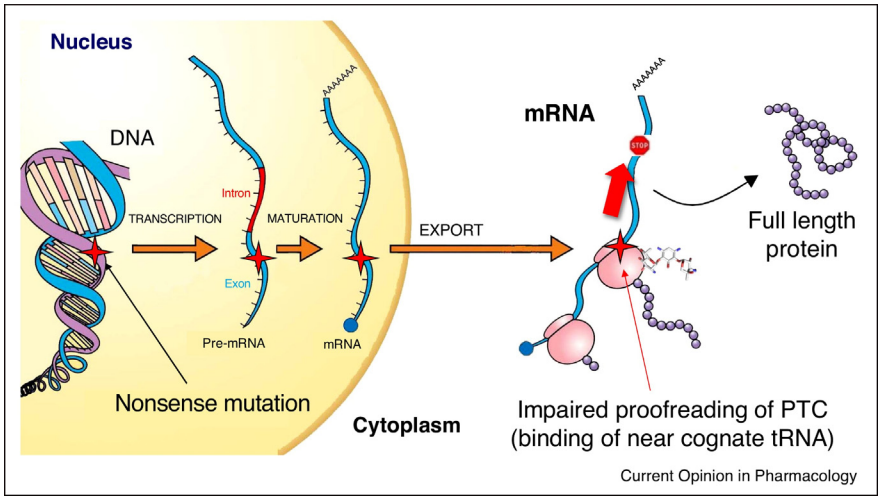
Splicing mutations

The splicing machinery

Precursor messenger RNA (pre-mRNA) is processed to mature mRNA by removal of introns in a process named RNA splicing. This process is carried out by the spliceosomes, ribonucleoprotein complexes that recognize the exon–intron junctions and catalyze the precise removal of introns and subsequent joining of exons. Alternative splicing is the process by which a single primary transcript yields different mature RNAs leading to the production of different protein isoforms with diverse functions. Most intron-containing transcripts are alternatively spliced [37].

Splicing involves the precise removal of introns from pre-mRNA, such that exons are spliced together to form mature RNAs with intact translational reading frames. Splicing requires exon recognition, accurate cleavage and rejoining of exons. RNA splicing depends on the proper recognition of exons guided in part by conserved sequence elements at the exon–intron junctions: GT and AG intronic dinucleotides at the donor and acceptor intron/exon junctions, respectively. The recognition of exon–intron boundaries also depends on less-conserved consensus motifs, adjacent to both donor and acceptor sites including the polypyrimidine tract and the branch site [38–41]. In addition to the splice sites, exons are defined by *cis*-acting regulatory elements, which have been divided into four functional categories: exonic

Figure 1



Antisense PTC read-through therapy. Drugs should allow re-expression of a full length protein by binding of near-cognate tRNA.

splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers ((ISEs) also known as intronic activators of splicing (IASs)) and intronic splicing silencers (ISSs). These *cis*-acting elements interact with *trans*-acting activators or repressors of splicing. Exons are activated by ESEs that promote their inclusion and are repressed by ESSs.

Splicing is carried out by the interaction of the splice-site sequences with the spliceosome, which consists of five small nuclear ribonucleoprotein (snRNP) complexes and a large number (over 100) of non-snRNP proteins which include splicing factors from the large family of serine–arginine (SR) proteins and the heterogeneous nuclear ribonucleoprotein (hnRNP) family. These splicing factors interact with the spliceosome (through protein–protein interactions) as well as with RNA (through RNA binding domains), and are essential for constitutive splicing as well as for alternative splicing (reviewed in [42]). Differences in the levels of functional splicing factors were found among different tissues, suggested to regulate the tissue specific level of alternatively spliced transcripts.

Alternative splicing and CF

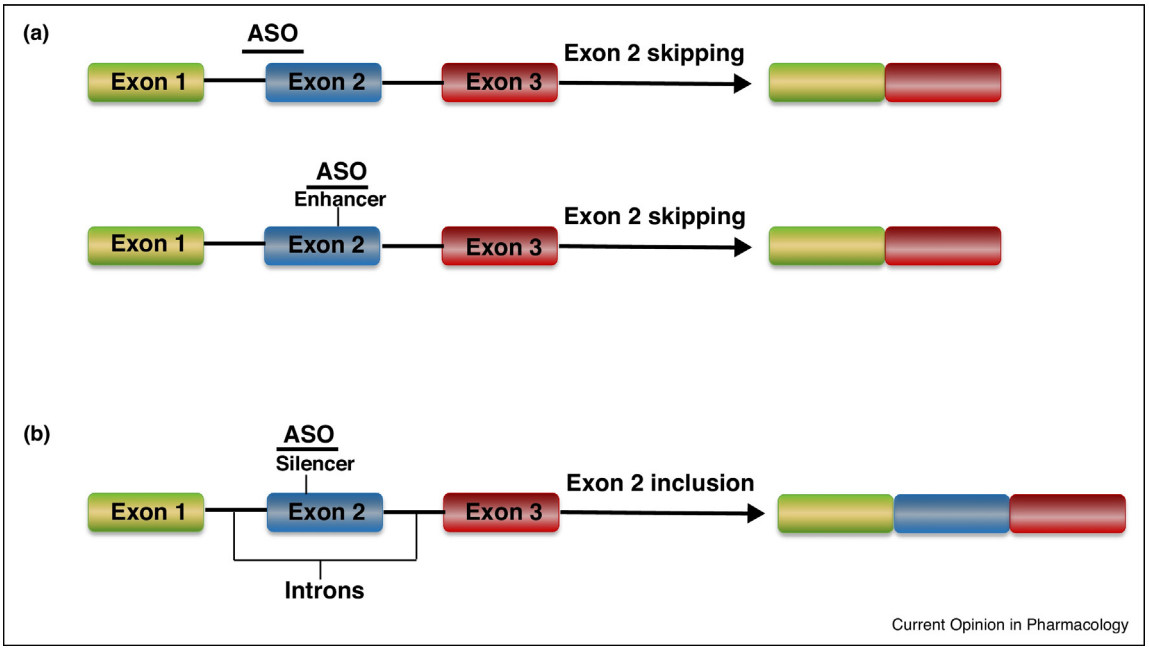
A significant proportion (20–30%) of disease-causing mutations in humans affect pre-mRNA splicing (reviewed in [43]). These mutations disrupt intronic or exonic splicing motifs [44]. Splicing mutations can lead to both aberrantly and correctly spliced transcripts, by partial skipping of exons or inclusion of intronic sequences, or can change the ratio of the programmed alternatively spliced isoforms.

Mutations that alter splicing in cis can either enhance or create splicing elements (creation of a cryptic splice site), or weaken/destroy a splice site. Both types of mutations can lead to abnormal exon definition, resulting in inappropriate inclusion or skipping of an exon. Most mutations have a loss-of-function phenotype, as they usually result in severe disruption of the open reading frame and often also destabilize the mRNA through NMD [36].

The CFTR gene comprises 27 coding exons, all required for a functional CFTR protein. Nevertheless, several exons were shown to undergo partial aberrant skipping, generating non-functional CFTR proteins [45]. As of today, more than 2000 mutations are now reported in the CF mutation database (http://www.genet.sickkids.on.ca/app). A significant fraction (10–15%) of CFTR mutations affect pre-mRNA splicing of the gene by either creating or destroying canonical splice sites or altering ISE, ISS, ESE and ESS regulatory elements throughout the gene [36]. Additionally, PTCs like any exonic mutation can also induce exon skipping by altering ESE and ESS motifs. In this regard, PTCs have even been shown to be statistically more inclined to induce exon skipping as compared to other exonic mutations [29••].

Splicing mutations can be divided into two subclasses. Mutations in subclass I completely abolish exon recognition while those in subclass II lead to both aberrantly and correctly spliced CFTR transcripts, by either weakening or strengthening exon-recognition motifs. Subclass II also includes intronic mutations, which generate cryptic donor or acceptor sites and can lead to partial inclusion of intronic sequences. In addition, there are mutations and polymorphisms that disrupt exonic splicing motifs, which also affect the CFTR splicing pattern. Subclass II mutations are associated with relatively milder forms of

Figure 2



Antisense oligonucleotide-mediated therapy. **(a)** Masking splice junctions or splicing enhancers by ASO is expected to reduce exon recognition resulting in exon skipping. **(b)** Masking splicing silencers by ASO is expected to promote exon inclusion. Abbreviation: ASO, Antisense Oligonucleotide.

CF, due to the partial generation of normal transcripts, translated into normal proteins [46].

Nevertheless, there is a marked variability in disease severity both between different patients in the same organ and in the same patient among different organs [47,48] and reviewed in [46]. An inverse correlation was found between the level of correctly spliced transcripts and disease severity [48–52] suggesting a role for splicing regulation as a genetic modifier of disease severity, in CF patients carrying splicing mutations [46].

Splicing modulation

Overexpression of splicing factors was shown to modulate the level of correctly spliced CFTR RNA [53]. Several of the factors promoted higher levels of correctly spliced CFTR transcripts leading to activation of the CFTR channel and restoration of its function [53]. These results explain the correlation between the level of correctly spliced CFTR transcripts and disease severity. They also support the development of therapies that modulate splicing for CF and other genetic diseases.

Antisense oligonucleotide-mediated therapy

One novel therapeutic approach for genetic disorders is based on the administration of Antisense Oligonucleotides (ASOs) [54]. ASOs are single stranded short synthetic RNA-like molecules that are chemically modified to improve their ability to recognize their target RNAs, confer resistance to nucleases and provide favourable pharmacokinetic properties [55]. Gene-specificity is accomplished by targeting the ASOs by base pairing to the desired transcripts and to specific *cis*-acting elements within the transcript. Oligonucleotide-based therapies have been used to inhibit or activate specific splicing events, by binding to an element and sterically blocking its activity or recruiting effectors to this site (illustrated in Figure 2). ASOs were shown to modulate splicing in cells with the CFTR splicing mutation 3849+10 kb C-to-T and c.2657+5G>A [56,57••]. For c.2657+5G>A, ASO treatment increased the amount of correctly localized CFTR protein at the plasma membrane and hence, CFTR function [57••].

The potential of ASOs as a therapeutic approach is demonstrated in several human genetic diseases resulting from splicing mutations, including Spinomuscular atrophy (SMA). Recently, an ASO-based drug (Nusinersen) completed successfully phase-3 clinical trials in patients with infantile-onset SMA, leading to the drug's approval by the FDA (FDA approves first drug for spinal muscular atrophy. 2017; https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm534611.htm). This example demonstrates the great potential of ASO-based splicing modulation for the treatment of genetic diseases and emphasizes the importance of local delivery for efficient treatment with minimal toxicity. An additional extensively studied genetic disease is Duchenne muscular dystrophy (DMD), characterized by progressive

muscle degeneration. Recently, the FDA granted a conditional approval to Exondys 51 (eteplirsen), an ASO-based drug treatment for DMD (FDA grants accelerated approval to first drug for Duchenne muscular dystrophy; https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm521263.htm).

In light of the exciting supporting data from other genetic diseases modulating the level of correctly spliced CFTR transcripts using ASO-based approach has great therapeutic potential for CF patients. The challenge in CF is the delivery of the ASO in sufficient amounts to achieve clinical benefit in the lungs. Many drugs are being delivered to CF patients by inhalation. Importantly, previous studies have shown that inhaled ASOs have excellent distribution throughout the lung, achieving cellular penetration and activity without toxicity [58].

Conclusion

Among rare medical disorders, CF is considered a model disease as it has pioneered studies in genetics, molecular and cellular pathogenesis and drug discovery, paving the way for other rare and common disorders. Excitingly, forthcoming novel drugs targeting the molecular basis of nonsense or splicing mutations might represent 'curative' treatments for CF patients carrying these mutations. These will serve as a model for future highly specific protein-function modulating therapies for other rare and common diseases such as Haemophilia, severe epilepsy and several genetically induced cancer types.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Zhou-Suckow Z, Duerr J, Hagner M, Agrawal R, Mall MA: **Airway mucus, inflammation and remodeling: emerging links in the pathogenesis of chronic lung diseases**. *Cell Tissue Res* 2017, **367**:537-550.

2. Pranke IM, Sermet-Gaudelus I: **Biosynthesis of cystic fibrosis transmembrane conductance regulator**. *Int J Biochem Cell Biol* 2014, **52**:26-38.

3. Kerem E: **Pharmacologic therapy for stop mutations: how much CFTR activity is enough?** *Curr Opin Pulm Med* 2004, **10**:547-552.

4. •• Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Dřevínek P, Griese M, McKone EF, Wainwright CE, Konstan MW *et al.*: **A CFTR potentiator in patients with cystic fibrosis and the G551D mutation**. *N Engl J Med* 2011, **365**:1663-1672.

This paper provides evidence that treatment of G551D compound heterozygote patients with ivacaftor associated with a clinically significant improvement in respiratory and nutritional status and a normalization of sweat chloride.

5. Kellermayer R: **Translational readthrough induction of pathogenic nonsense mutations**. *Eur J Med Genet* 2006, **49**:445-450.

6. Keeling KM, Wang D, Conard SE, Bedwell DM: **Suppression of premature termination codons as a therapeutic approach**. *Crit Rev Biochem Mol Biol* 2012, **47**:444-463.

7. Shoshani T, Augarten A, Gazit E, Bashan N, Yahav Y, Rivlin Y, Tal A, Seret H, Yaar L, Kerem E: **Association of a nonsense mutation (W1282X), the most common mutation in the Ashkenazi Jewish cystic fibrosis patients in Israel, with presentation of severe disease**. *Am J Hum Genet* 1992, **50**:222-228.

8. Frischmeyer PA, Dietz HC: **Nonsense-mediated mRNA decay in health and disease**. *Hum Mol Genet* 1999, **8**:1893-1900.

9. Maquat LE: **When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells**. *RNA* 1995, **1**:453-465.

10. Maquat LE: **Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics**. *Nat Rev Mol Cell Biol* 2004, **5**:89-99.

11. Chang Y-F, Imam JS, Wilkinson MF: **The nonsense-mediated decay RNA surveillance pathway**. *Annu Rev Biochem* 2007, **76**:51-74.

12. Mendell JT, Sharifi NA, Meyers JL, Martinez-Murillo F, Dietz HC: **Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise**. *Nat Genet* 2004, **36**:1073-1078.

13. Linde L, Boelz S, Nissim-Rafinia M, Oren YS, Wilschanski M, Yaacov Y, Virgilis D, Neu-Yilik G, Kulozik AE, Kerem E *et al.*: **Nonsense-mediated mRNA decay affects nonsense transcript levels and governs response of cystic fibrosis patients to gentamicin**. *J Clin Invest* 2007, **117**:683-692.

14. Linde L, Boelz S, Neu-Yilik G, Kulozik AE, Kerem B: **The efficiency of nonsense-mediated mRNA decay is an inherent character and varies among different cells**. *Eur J Hum Genet* 2007, **15**:1156-1162.

15. Tarpey PS, Raymond FL, Nguyen LS, Rodriguez J, Hackett A, Vandeleur L, Smith R, Shoubridge C, Edkins S, Stevens C *et al.*: **Mutations in UPF3B, a member of the nonsense-mediated mRNA decay complex, cause syndromic and nonsyndromic mental retardation**. *Nat Genet* 2007, **39**:1127-1133.

16. Wang D, Wengrod J, Gardner LB: **Overexpression of the c-myc oncogene inhibits nonsense-mediated RNA decay in B lymphocytes**. *J Biol Chem* 2011, **286**:40038-40043.

17. Wang D, Zavadil J, Martin L, Parisi F, Friedman E, Levy D, Harding H, Ron D, Gardner LB: **Inhibition of nonsense-mediated RNA decay by the tumor microenvironment promotes tumorigenesis**. *Mol Cell Biol* 2011, **31**:3670-3680.

18. Walter P, Ron D: **The unfolded protein response: from stress pathway to homeostatic regulation**. *Science* 2011, **334**:1081-1086.

19. •• Oren YS, McClure ML, Rowe SM, Sorscher EJ, Bester AC, Manor M, Kerem E, Rivlin J, Zahdeh F, Mann M *et al.*: **The unfolded protein response affects readthrough of premature termination codons**. *EMBO Mol Med* 2014, **6**:685-701.

A feedback loop between the NMD and UPR homeostatic mechanisms was uncovered and characterized in this study. This feedback loop enhances the response to readthrough treatment, highlighting its clinical importance, demonstrating the importance of the UPR and its regulatory network for genetic diseases caused by PTCs and for cell homeostasis under normal conditions.

20. Sermet-Gaudelus I, Namy O: **New pharmacological approaches to treat patients with cystic fibrosis with nonsense mutations**. *Am J Respir Crit Care Med* 2016, **194**:1042-1044.

21. Wilschanski M, Yahav Y, Yaacov Y, Blau H, Bentur L, Rivlin J, Aviram M, Bdolah-Abram T, Bebok Z, Shushi L *et al.*: **Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations**. *N Engl J Med* 2003, **349**:1433-1441.

22. Sermet-Gaudelus I, Renouil M, Fajac A, Bidou L, Parbaille B, Pierrot S, Davy N, Bismuth E, Reinert P, Lenoir G *et al.*: **In vitro prediction of stop-codon suppression by intravenous gentamicin in patients with cystic fibrosis: a pilot study**. *BMC Med* 2007, **5**:5.

23. Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, Paushkin S, Patel M, Trotta CR, Hwang S *et al.*: **PTC124 targets genetic disorders caused by nonsense mutations**. *Nature* 2007, **447**:87-91.

24. Kerem E, Hirawat S, Armoni S, Yaakov Y, Shoseyov D, Cohen M, Nissim-Rafinia M, Blau H, Rivlin J, Aviram M *et al.*: **Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial**. *Lancet (London, England)* 2008, **372**:719-727.

25. Clancy JP, Rowe SM, Bebok Z, Aitken ML, Gibson R, Zeitlin P, Berclaz P, Moss R, Knowles MR, Oster RA *et al.*: **No detectable improvements in cystic fibrosis transmembrane conductance regulator by nasal aminoglycosides in patients with cystic fibrosis with stop mutations**. *Am J Respir Cell Mol Biol* 2007, **37**:57-66.

26. Sermet-Gaudelus I, Boeck KDe, Casimir GJ, Vermeulen F, Leal T, Mogenet A, Roussel D, Fritsch J, Hanssens L, Hirawat S *et al.*: **Ataluren (PTC124) induces cystic fibrosis transmembrane conductance regulator protein expression and activity in children with nonsense mutation cystic fibrosis**. *Am J Respir Crit Care Med* 2010, **182**:1262-1272.

27. •• Roy B, Friesen WJ, Tomizawa Y, Leszyk JD, Zhuo J, Johnson B, Dakka J, Trotta CR, Xue X, Mutyam V *et al.*: **Ataluren stimulates ribosomal selection of near-cognate tRNAs to promote nonsense suppression**. *Proc Natl Acad Sci U S A* 2016, **113**:12508-12513.

PTC124 targets the ribosome promotes the insertion of near-cognate tRNAs at the site of the nonsense codon without apparent effects on transcription, mRNA processing, mRNA stability, or protein stability. The proteins contain amino acid replacements similar to those derived from endogenous readthrough, namely Gln, Lys, or Tyr at UAA or UAG PTCs and Trp, Arg, or Cys at UGA PTCs.

28. Bidou L, Hatin I, Perez N, Allamand V, Panthier J-J, Rousset J-P: **Premature stop codons involved in muscular dystrophies show a broad spectrum of readthrough efficiencies in response to gentamicin treatment**. *Gene Ther* 2004, **11**:619-627.

29. •• Hinzpeter A, Aissat A, de Becdelièvre A, Bieth E, Sondo E, Martin N, Costes B, Costa C, Goossens M, Galietta LJV *et al.*: **Alternative splicing of in-frame exon associated with premature termination codons: implications for readthrough therapies**. *Hum Mutat* 2013, **34**:287-291.

A screen by *in vitro* minigene assay of all six PTCs described in exon 15 of the CFTR gene demonstrated alternative splicing to differing degrees for five of them. Of the five, PTC mutations c.2537G>A (p.Trp846*(UAG)) and c.2551C>T (p.Arg851*) cause the greatest proportion of transcripts lacking exon 15; The total amount of transcripts together with the splicing profile should be assessed to anticipate and improve efficacy of read-through therapy.

30. Xue X, Mutyam V, Tang L, Biswas S, Du M, Jackson LA, Dai Y, Belakhov V, Shalev M, Chen F *et al.*: **Synthetic aminoglycosides efficiently suppress cystic fibrosis transmembrane conductance regulator nonsense mutations and are enhanced by ivacaftor**. *Am J Respir Cell Mol Biol* 2014, **50**:805-816.

31. Rowe SM, Sloane P, Tang LP, Backer K, Mazur M, Buckley-Lanier J, Nudelman I, Belakhov V, Bebok Z, Schwiebert E *et al.*: **Suppression of CFTR premature termination codons and rescue of CFTR protein and function by the synthetic aminoglycoside NB54**. *J Mol Med (Berl)* 2011, **89**:1149-1161.

32. •• Mutyam V, Du M, Xue X, Keeling KM, White EL, Bostwick JR, Rasmussen L, Liu B, Mazur M, Hong JS *et al.*: **Discovery of clinically approved agents that promote suppression of cystic fibrosis transmembrane conductance regulator nonsense mutations**. *Am J Respir Crit Care Med* 2016, **194**:1092-1103.

Two independent screens, firefly luciferase and CFTR-mediated transepithelial chloride conductance assay, were performed on a library of 1600 clinically approved compounds. Eight agents were advanced for secondary screening in primary cells. Ivacaftor significantly increased CFTR activity in cells treated with pyranoradine tetraphosphate, potassium p-aminobenzoate, and escin, an herbal agent as compared with vehicle control.

33. •• Haggie PM, Phuan P-W, Tan J-A, Xu H, Avramescu RG, Perdomo D, Zlock L, Nielson DW, Finkbeiner WE, Lukacs GL *et al.*: **Correctors and potentiators rescue function of the truncated W1282X-cystic fibrosis transmembrane regulator (CFTR) translation product**. *J Biol Chem* 2017, **292**:771-785.

Corrector and potentiator combinations were tested in primary cultures and conditionally reprogrammed cells generated from nasal brushings from one W1282X homozygous subject. There was no CFTR function in W1282X cells despite the presence of adequate transcript levels. The authors speculate that corrector and potentiator combinations may have therapeutic efficacy in cystic fibrosis caused by the W1282X mutation.

34. •• Xue X, Mutyam V, Thakerar A, Mobley J, Bridges RJ, Rowe SM, Keeling KM, Bedwell DM: **Identification of the amino acids inserted during suppression of CFTR nonsense mutations and determination of their functional consequences**. *Hum Mol Genet* 2017, **26**:3116-3129.

The authors determined the amino acids inserted at the W1282X PTC (a UGA codon) in the context of its three upstream and downstream CFTR codons during G418-mediated suppression (respectively leucine, cysteine and tryptophan). These amino acids (and their proportions) were significantly different from those obtained for the CFTR G542X UGA mutation (cysteine, tryptophan and arginine) which demonstrates the importance of the mRNA context in the readthrough therapy. The study also confirms that some variant CFTR proteins generated by PTC suppression can exhibit reduced maturation and activity.

35. Nomakuchi TT, Rigo F, Aznarez I, Krainer AR: **Antisense oligonucleotide-directed inhibition of nonsense-mediated mRNA decay**. *Nat Biotechnol* 2016, **34**:164-166.

36. Gonzalez-Hilarion S, Beghyn T, Jia J, Debreuck N, Berte G, Mamchaoui K, Mouly V, Gruenert DC, Déprez B, Lejeune F: **Rescue of nonsense mutations by amlexanox in human cells**. *Orphanet J Rare Dis* 2012, **7**:58.

37. Garcia-Blanco MA, Baraniak AP, Lasda EL: **Alternative splicing in disease and therapy**. *Nat Biotechnol* 2004, **22**:535-546.

38. Fairbrother WG, Yeh R-F, Sharp PA, Burge CB: **Predictive identification of exonic splicing enhancers in human genes**. *Science* 2002, **297**:1007-1013.

39. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR: **ESEfinder: a web resource to identify exonic splicing enhancers**. *Nucleic Acids Res* 2003, **31**:3568-3571.

40. Fairbrother WG, Chasin LA: **Human genomic sequences that inhibit splicing**. *Mol Cell Biol* 2000, **20**:6816-6825.

41. Zhang XH-F, Heller KA, Hefter I, Leslie CS, Chasin LA: **Sequence information for the splicing of human pre-mRNA identified by support vector machine classification**. *Genome Res* 2003, **13**:2637-2650.

42. Black DL: **Mechanisms of alternative pre-messenger RNA splicing**. *Annu Rev Biochem* 2003, **72**:291-336.

43. Faustino NA, Cooper TA: **Pre-mRNA splicing and human disease**. *Genes Dev* 2003, **17**:419-437.

44. Cartegni L, Chew SL, Krainer AR: **Listening to silence and understanding nonsense: exonic mutations that affect splicing**. *Nat Rev Genet* 2002, **3**:285-298.

45. Bienvenu T, Beldjord C, Chelly J, Fonknechten N, Hubert D, Dusser D, Kaplan JC: **Analysis of alternative splicing patterns in the cystic fibrosis transmembrane conductance regulator gene using mRNA derived from lymphoblastoid cells of cystic fibrosis patients**. *Eur J Hum Genet* 1996, **4**:127-134.

46. Nissim-Rafinia M, Kerem B: **Splicing regulation as a potential genetic modifier**. *Trends Genet* 2002, **18**:123-127.

47. Pagani F, Stuani C, Tzetis M, Kanavakis E, Efthymiadou A, Doudounakis S, Casals T, Baralle FE: **New type of disease causing mutations: the example of the composite exonic regulatory elements of splicing in CFTR exon 12**. *Hum Mol Genet* 2003, **12**:1111-1120.

48. Ramalho AS, Beck S, Meyer M, Penque D, Cutting GR, Amaral MD: **Five percent of normal cystic fibrosis transmembrane conductance regulator mRNA ameliorates the severity of pulmonary disease in cystic fibrosis**. *Am J Respir Cell Mol Biol* 2002, **27**:619-627.

49. Chiba-Falek O, Kerem E, Shoshani T, Aviram M, Augarten A, Bentur L, Tal A, Tullis E, Rahat A, Kerem B: **The molecular basis of disease variability among cystic fibrosis patients carrying the 3849+10 kb C–> T mutation**. *Genomics* 1998, **53**:276-283.

50. Chillón M, Casals T, Mercier B, Bassas L, Lissens W, Silber S, Romey MC, Ruiz-Romero J, Verlingue C, Claustres M: **Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens**. *N Engl J Med* 1995, **332**:1475-1480.

51. Rave-Harel N, Kerem E, Nissim-Rafinia M, Madjar I, Goshen R, Augarten A, Rahat A, Hurwitz A, Darvasi A, Kerem B: **The molecular basis of partial penetrance of splicing mutations in cystic fibrosis**. *Am J Hum Genet* 1997, **60**:87-94.

52. Highsmith WE, Burch LH, Zhou Z, Olsen JC, Strong TV, Smith T, Friedman KJ, Silverman LM, Boucher RC, Collins FS *et al.*: **Identification of a splice site mutation (2789 +5 G>A) associated with small amounts of normal CFTR mRNA and mild cystic fibrosis**. *Hum Mutat* 1997, **9**:332-338.

53. Nissim-Rafinia M, Aviram M, Randell SH, Shushi L, Ozeri E, Chiba-Falek O, Eidelman O, Pollard HB, Yankaskas JR, Kerem B: **Restoration of the cystic fibrosis transmembrane conductance regulator function by splicing modulation**. *EMBO Rep* 2004, **5**:1071-1077.

54. Kole R, Krainer AR, Altman S: **RNA therapeutics: beyond RNA interference and antisense oligonucleotides**. *Nat Rev Drug Discov* 2012, **11**:125-140.

55. Bennett CF, Swayze EE: **RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform**. *Annu Rev Pharmacol Toxicol* 2010, **50**:259-293.

56. Friedman KJ, Kole J, Cohn JA, Knowles MR, Silverman LM, Kole R: **Correction of aberrant splicing of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by antisense oligonucleotides**. *J Biol Chem* 1999, **274**:36193-36199.

57. •• Igreja S, Clarke LA, Botelho HM, Marques L, Amaral MD: **Correction of a cystic fibrosis splicing mutation by antisense oligonucleotides**. *Hum Mutat* 2016, **37**:209-215.

ASO strategy to correct the aberrant splicing caused by c.2657+5G>A CFTR mutation showed that exon 16 inclusion was almost completely restored resulting in increased levels of correctly localized CFTR protein at the plasma membrane with increased function, highlighting the therapeutic potential of ASOs for the specific correction of splicing mutations.

58. Crosby JR, Guha M, Tung D, Miller DA, Bender B, Condon TP, York-DeFalco C, Geary RS, Monia BP, Karras JG, Gregory SA: **Inhaled CD86 antisense oligonucleotide suppresses pulmonary inflammation and airway hyper-responsiveness in allergic mice**. *J Pharmacol Exp Ther* 2007, **321**:938-946.