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Review article

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tRNA therapeutics for genetic diseases

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Abstract

Transfer RNAs (tRNAs) have a crucial role in protein synthesis, and in recent years, their therapeutic potential for the treatment of genetic diseases — primarily those associated with a mutation altering mRNA translation — has gained significant attention. Engineering tRNAs to readthrough nonsense mutation-associated premature termination of mRNA translation can restore protein synthesis and function. In addition, supplementation of natural tRNAs can counteract effects of missense mutations in proteins crucial for tRNA biogenesis and function in translation. This Review will present advances in the development of tRNA therapeutics with high activity and safety in vivo and discuss different formulation approaches for single or chronic treatment modalities. The field of tRNA therapeutics is still in its early stages, and a series of challenges related to tRNA efficacy and stability in vivo, delivery systems with tissue-specific tropism, and safe and efficient manufacturing need to be addressed.

Sections

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Introduction

Modern medicine is undergoing a remarkable transformation owing to the rapid progress in gene therapy methods. Through the application of advanced molecular biology technology and enhanced delivery systems, an era of unprecedented opportunities has emerged. This new era is characterized by the development of precision medicines tailored to individual genetic requirements. A notable illustration of this breakthrough is the extraordinary success achieved by employing two messenger RNA (mRNA) vaccines in the global fight against the SARS-CoV-2 pandemic. The utilization of mRNA as a therapeutic approach has unlocked countless possibilities[1,2], inspiring the exploration of innovative RNA-based treatments for a wide range of previously untreatable human conditions.

mRNA facilitates the transient transmission of genetic information from DNA to the ribosomes — the biosynthetic macromolecular machines of the cell. To accurately decipher the genetic information embedded in mRNA, the ribosomes are assisted by transfer RNAs (tRNAs). tRNAs are classically thought of as interpreters of the genetic code, translating nucleic acid identity into amino acids. The four nucleotides in mRNA form 64 unique triplets (codons). Of these, 61 are sense codons and collectively encode 20 amino acids via base pairing between the mRNA codon and the complementary part of the anticodon of cognate tRNA (Box 1). The remaining three codons, UGA, UAA and UAG, referred to as stop codons, also termed natural termination codons (NTCs), do not have a cognate tRNA but are recognized by a specific protein, termed release factor (or eRF1 in eukaryotes)[3]. Stop codons are crucial to faithfully signal termination of protein synthesis.

Owing to the degeneracy of the genetic code, most amino acids except for methionine and tryptophan are encoded by two, three, four or six codons, known as synonymous codons. Certain tRNAs undergo modifications at the first nucleotide of the anticodon loop (nucleotide 34, Box 1), enabling them to simultaneously decode two codons. One codon is recognized through Watson–Crick interactions with all three nucleotides, whereas the other codon establishes a wobble interaction with the last nucleotide within the triplet[4]. Thus, in total, less tRNAs are needed to decode the 61 sense codons. Human cells express 41 different tRNA isoacceptors, whose concentration varies among different tissues and at different stages of cell differentiation[5–8]. The composition of tRNA pools is closely regulated to shape the proteomes according to the cellular state[9–11].

A nonsense mutation can convert 18 out of the 61 sense codons into one of the three stop codons. This premature termination codon (PTC) leads to a premature termination of protein translation and development of loss-of-function disease phenotypes. Nonsense mutations account for approximately 11% of genetic diseases, making them a predominant category of disease-causing mutations in the human population as a whole[12]. Under native conditions, a small fraction of the PTCs undergoes spontaneous suppression[13]. More than 30 low molecular weight pharmacological compounds (for example, aminoglycosides, non-aminoglycoside antibiotics and oxadiazoles) that suppress translational termination at PTCs have been identified and characterized in preclinical studies[14,15]. However, the multiple clinical trials have yielded conflicting outcomes, probably because of the low efficacy, unspecific insertion of amino acids at the PTCs and off-target effects at natural stop codons[14,15]. So far, only ataluren has received a conditional approval for a subset of patients with a single disease, Duchenne muscular dystrophy[16].

Spurred by natural suppressor tRNAs (sup-tRNAs), which have the ability to recognize stop codons as sense codons[17,18], Kan and coworkers pioneered the concept of using anticodon-altered sense tRNAs to decode PTCs and restore protein synthesis and function[19]. Genetic mutations in tRNA genes or in genes encoding proteins participating in tRNA biogenesis that alter tRNA and/or aminoacyl-tRNA levels — a potent modulator or mRNA translation and protein production (reviewed in ref. 20) — expand the scope of tRNA therapeutics. Supplementation with the affected tRNA could counteract the mutational effect and restore mRNA translation. However, there are various challenges that may face the use of tRNAs as therapeutics such as low efficacy, instability, immunogenicity and safety concerns, hampering their clinical translation. Critically, however, advancements in mRNA and small RNA therapeutics made over the past three decades have begun to address many of these challenges, including stability enhancement and reduction of immunogenicity through using modified nucleotides[21], and the development of various materials for efficient encapsulation and in vivo delivery of nucleic acids[1,2,22]. These technological breakthroughs are applicable to tRNA therapeutics, renewing the interest of the scientific community and industry[23,24] in utilizing tRNAs as innovative gene therapy approaches for monogenic disorders with diverse underlying molecular mechanisms.

This Review will discuss key strategies for leveraging tRNAs as therapeutic agents for monogenic disorders. Recent developments in delivery platforms for systemic and tissue-specific delivery will be highlighted, evaluating their suitability for administering tRNA payloads. Furthermore, challenges encountered in establishing tRNAs as effective and safe therapeutics will be addressed.

History of tRNA therapeutics

Nearly half a century ago, three crucial studies laid the foundation for tRNA-based therapy (Fig. 1, Table 1). Sveda and colleagues fused erythrocytes preloaded with heterologous native sup-tRNAs (*Escherichia coli* or yeast) with mouse cells expressing truncated hypoxantine–guanine phosphoribosyl transferase (HGPRT) and restored full-length protein expression[25]. Adding a native yeast sup-tRNA^{Ser} to erythrocyte lysates derived from a β-thalassaemia patient with a nonsense mutation at a lysine AAG codon in the β-globin gene, Kan and colleagues achieved a correction of up to 10% of the full-length β-globin protein[26] (Table 1). Motivated by this success, Kan and colleagues mutated the anticodon of the cognate tRNA^{Lys} to pair to the UAG PTC and microinjected the anticodon-modified sup-tRNA^{Lys} gene into the *Xenopus* oocyte nuclei[19], thus showing that episomally expressed sup-tRNA genes are transcribed and functional in cells. Efforts to expand the applications of sup-tRNAs in various tissues and disease contexts continued, using two routes for sup-tRNA supplementation: (1) as a plasmid-encoded sup-tRNA gene for episomal expression in the nucleus[27–33] and (2) transfection of in vitro-transcribed (IVT) sup-tRNA into the cytoplasm of the recipient cell[31,33–36] (Table 1). For the sup-tRNA designs, the efforts revolved around engineering the anticodon of the native tRNA that decodes the wild-type codon to pair with the newly emerged PTC, thereby reintroducing the same amino acid at the affected codon[19,27–32]. However, the inefficient activity of those anticodon-engineered sup-tRNAs in clinically relevant model systems, even when supplemented at higher doses as multiple gene copies[28], and the early stages of delivery platforms for clinical application have impeded clinical developments.

Parallel developments in the synthetic biology to refactor tRNAs for genetic code expansion and incorporation of noncanonical amino acids at natural stop codons[37–42] set examples to improve sup-tRNA efficiency by engineering the tRNA bodies. However, because of the noncanonical amino acids these tRNAs bear, the most promising

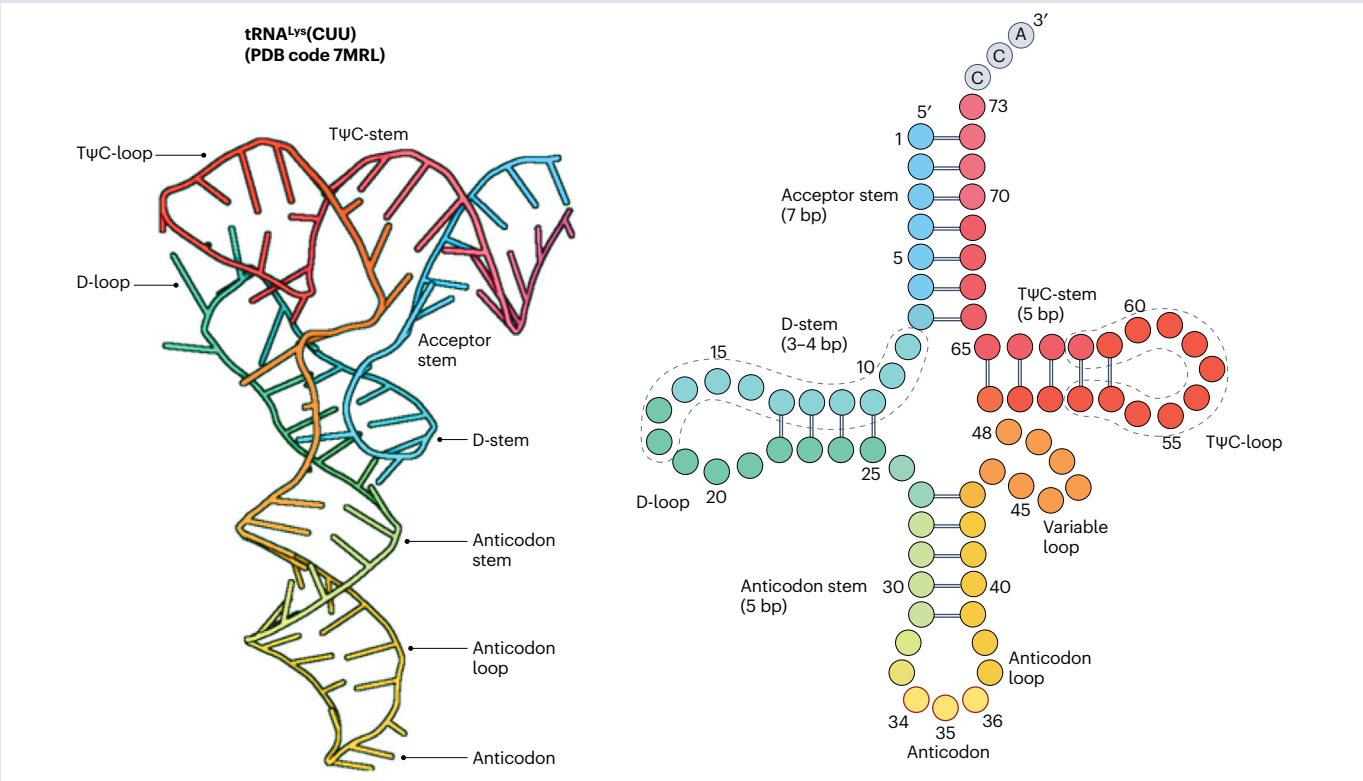
Box 1

Structure and modifications of natural tRNAs

tRNAs are the most abundant group of cellular RNAs when measured in moles. To fit the same ribosomal site and enable similar decoding efficacy of all tRNAs, their 3D L-shaped structure (see left image of figure; PDB, protein data bank) is restricted by a narrow set of structural parameters; therefore, sequence variations compensate for the chemical diversity of the amino acid moiety. tRNAs establish a partially double-stranded structure arranged in four stems with a conserved length in base pairs (bp; see right image of figure); the stems are connected with loops resulting in an overall very narrow tRNA length range of 73–90 nucleotides. Despite differences in the length, the anticodon is always numerated 34–36. In eukaryotes, cytoplasmic tRNAs are nuclear-encoded and transcribed as precursor tRNA by RNA polymerase III, supported by the concerted action of two transcription factors (TFIIIC binding to intragenic tRNA regions (dashed line selected regions) and TFIIIB binding to sequences 5′ upstream of tRNA transcription start) (reviewed in refs. 6,20,46).

Precursor tRNAs are processed in a sequence of maturation events, including removal of 5′ leader, 3′ trailer, splicing of introns (in some tRNAs), 3′ addition of the CCA extension by a CCA-adding enzyme, posttranscriptional modifications and attachment of the amino acid by the cognate aminoacyl-tRNA synthetase (AARS)[43].

tRNAs undergo extensive modifications (that is, more than 80 chemically distinct modifications), with an average of 13 modifications per tRNA in mammals[236]. Modifications in the anticodon and anticodon loop are indispensable and affect the fidelity of decoding. Modifications in the tRNA body affect stability and modulate interactions with tRNA interacting proteins; however, the degree of modification at each residue largely varies[203,237,238]. Modifications at position 34 enable wobbling base pairing with the third nucleotide of the mRNA codon[4], so that on average, less tRNA species (that is, 35–55 isoacceptors in different organisms) are necessary to decode all 61 sense codons[6,203].



candidates often scored with changes in the acceptor stem[39], which is densely enriched of recognition elements for the AARSs[43] and changes in it may alter the amino acid identity. Convergent de novo computational designs that harness evolutionarily selected signatures of sense codon decoding tRNAs for function in translation and interactions with tRNA-binding proteins (Box 1) provide a rationale for engineered sup-tRNAs with enhanced suppression activity[34].

The past decade has witnessed new mechanistic discoveries that link aberrancies in tRNA(s) with pathology and suggest tRNA as a novel therapeutic modality[44–48]. Two recent studies have exploited the therapeutic potential of tRNAs in preclinical settings, administering them as adeno-associated virus (AAV) formulations for episomal expression[33] or as lipid nanoparticles (LNPs) directly into the cytosol[35] and solidified the feasibility of tRNAs as

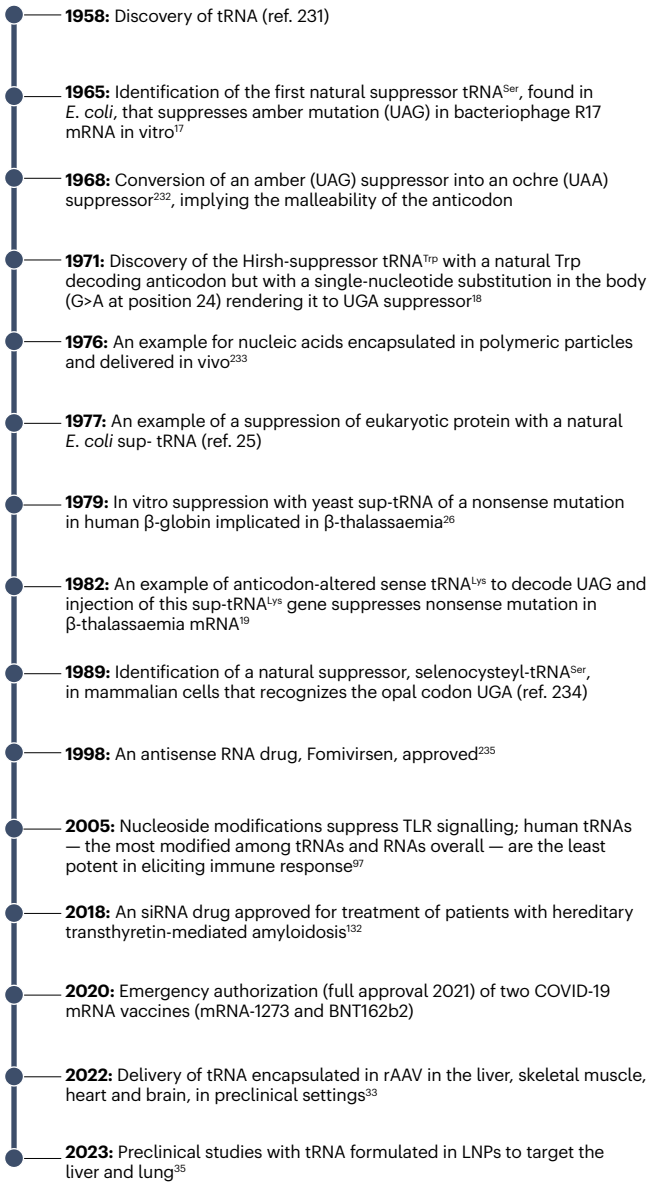


Fig. 1 | Timeline of some key milestones fostering the development of tRNA therapeutics. sup-tRNA, suppressor tRNA; TLR, Toll-like receptor; siRNA, short interfering RNA; COVID-19, coronavirus disease 2019.

a therapeutic with high molecular safety and suppression efficacy (Table 1, Fig. 1). Innovations in viral and non-viral delivery systems for other RNA-based gene therapies will promote the clinical translation of tRNA-based therapies for nonsense mutation-associated diseases and other pathologies linked to alterations of endogenous tRNA(s).

Applications of tRNA therapeutics

There are two groups of monogenic diseases that could potentially be addressed with tRNA-based therapeutics: (1) pathologies associated with nonsense mutations and (2) pathologies linked to AARS mutations associated with tRNA depletion (Fig. 2).

Correction therapies with sup-tRNAs

Nonsense mutations — a common type of disease-causing mutations in the human population[12] — introduce a PTC, which shares the functional characteristics of the natural stop codons, that is, lack of tRNA to decode the PTCs (Fig. 2a). A total of 19 sup-tRNAs would be required to suppress all possible nonsense mutation-associated PTCs and, thereby, introduce the correct amino acid (Fig. 3). The utilization of tRNAs as therapeutics requires their adequate activity in translation to support sufficient production of the affected protein(s) that is defined by the therapeutic threshold, or the amount of a protein to ameliorate a disease phenotype. The therapeutic threshold is usually individually defined for each disease and can be as low as 10% for the recessive cystic fibrosis[49,50], or be asymptotic to full correction of various oncogene suppressors[51]. To reach those measures, operationally potent tRNAs with high translation efficacy are needed.

A notable advantage of sup-tRNAs as a therapeutic modality is that a single therapeutic formulation has the potential to be utilized for a wide range of clinically distinct indications. For example, the mutation of the arginine CGA codon to UGA PTC is the most frequent nonsense mutation (that is, 23.7% of all known nonsense mutations associated with human pathologies[52]). Thus, one potent sup-tRNA^{Arg} could be utilized to treat the most common nonsense mutation at arginine codons in various diseases. Critically, however, as discussed in the following sections, sequence context-driven variations in the readthrough efficiency and differences in tissue-specific delivery are hurdles that need to be addressed.

sup-tRNAs only target the natural pool of transcripts of the affected gene, which eliminates the risk of overexpression beyond the normal range. Thus, sup-tRNAs are well-suited for treating conditions associated with nonsense mutation in genes (for example, goldilocks genes) which require precise regulation, as both overexpression and underexpression can be harmful[53]. MeCP2 is a well-known example of a goldilocks gene, in which X-linked haploinsufficiency results in Rett syndrome, whereas trisomy of *MECP2* causes MECP2 duplication syndrome, which primarily affects males and leads to intellectual disability of varying severity[54].

Many tRNAs, dependent on their evolutionary age and selection for high accuracy in translation[55,56], may resist engineering to decode PTCs or the efficacy of suppression may be insufficient for therapeutic benefit. In such cases, an alternative approach could be adopted: a potent sup-tRNA bearing an unrelated amino acid (that is, different than the wild-type amino acid at the affected codon) could be used to restore translation at the PTC. This strategy, mimicking a missense mutation, may be applicable to specific proteins. For example, the dystrophin gene associated with Duchenne muscular dystrophy has been shown to tolerate missense mutations[57], whereas the disease-associated proteins in Dravet syndrome or cystic fibrosis (SCN1A or CFTR, respectively) are sensitive to missense mutations; the missense mutation is often disease-causing[58,59]. At some PTCs in CFTR, miscoding with amino acids with physicochemical properties similar to those of the original amino acid might be tolerated[60]. There is potential for augmenting sup-tRNA-driven amino acid misincorporation with approved therapies. For instance, combining sup-tRNAs with Trikafta — an FDA-approved CFTR modulator therapeutic for many cystic fibrosis missense mutations[61] — could alleviate the misfolding effect of the misincorporated amino acid by the sup-tRNA.

tRNA supplementation therapies

A shared characteristic among some clinically distinct pathologies is the transient or permanent depletion of a tRNA isoacceptor family that

Table 1 | Overview of potential sup-tRNA applications

Target disease indication	Strategy	Key findings	Ref.
Mouse HGPRT	Fusion of HGPRT-defective cells with erythrocytes preloaded with *E. coli* or yeast sup-tRNA	Specific suppression of HGPRT UAA PTC with sup-tRNA (UAA) and not with sup-tRNA (UGA)	25
β-Globin (β-thalassaemia)	Suppression with yeast sup-tRNA^{Ser} in vitro, inpatient-derived reticulocyte lysate with UAG PTC at Lys	PTC suppression of approximately 15%, with a missense incorporation of Ser at the Lys codon	26
β-Globin (β-thalassaemia)	Co-injection into *Xenopus* oocyte nuclei of human sup-tRNA^{Lys} gene and β-globin mRNA with UAG PTC at Lys codon	Evidence for functional expression of human anticodon-altered sense tRNA^{Lys} as plasmid-encoded sup-tRNA^{Lys} gene	19
Model protein	SV40-vector delivery of anticodon modified *Xenopus laevis* tRNA^{Tyr} to sup-tRNA^{Tyr} (UAG suppressor) and its functional expression in monkey kidney cells (CV-1)	sup-tRNA^{Tyr} gene is not deleterious to CV-1 cell metabolism	30
Xeroderma pigmentosum (XP) (XP group A)	Transfection of sup-tRNA^{Arg} gene (UAG suppressor) into XP cells with R207X mutation in the *XP* gene	A twofold increase in ultraviolet radiation survival dose, but no detectable increase in XP protein levels	32
Model protein — chloramphenicol acetyltransferase	Injection of sup-tRNA^{Ser} gene (UAA suppressor) into skeletal muscle and hearts of transgenic mice; plasmids expressing multiple copies (up to 16) of sup-tRNA^{Ser}	A demonstration of suppression in mice with efficacy of 1–2%; dose-dependent effect of suppression	28
Dystrophin (Duchenne muscular dystrophy)	Co-transfection of sup-tRNA and LacZ-PTC reporter genes; direct injection of sup-tRNA gene (UAA suppressor) into skeletal muscle of mdx mouse	Inverse correlation of the suppression effect and sup-tRNA gene dose in HeLa cells; 2.5% efficiency and full-length dystrophin production in mdx mouse muscles	29
Model proteins — luciferase reporters	Transfection of IVT sup-tRNA^{Ser} into A549 cells co-expressing plasmid-encoded PTC–luciferase variants	Direct transfection of IVT sup-tRNA; equal suppression activity at all three PTCs (UAA, UGA and UAG)	36
E-cadhedrin (hereditary diffuse gastric cancer)	E-cadherin-deficient cells co-transfected with sup-tRNA^{Arg} gene and E-cadherin mini-gene with PTC	sup-tRNA^{Arg} expressed from a plasmid under the control of the native tRNA^{Arg} promoter	27
CFTR (cystic fibrosis)	Screening of anticodon-engineered sup-tRNAs among isodecoders of 10 human tRNA families in HEK cells; sup-tRNA^{Arg} gene electroporated into mouse skeletal muscle with luciferase reporter; co-transfection of sup-tRNA with PTC–CFTR genes in *Xenopus* oocytes	Similar suppression efficiency when co-transfected as sup-tRNA gene or as IVT sup-tRNA; minimal suppression activity at natural stop codons; difference in suppression efficacy among isodecoders	31
Alpha-L-iduronidase (IDUA) (mucopolysaccharidosis type I)	Intravenous rAAV delivery of single or multiple copies of sup-tRNA^{Tyr} gene in *IDUA*(W402X) knock-in mouse; lentiviral transfection of sup-tRNA gene in fibroblasts from patient with *IDUA*(W402X)	Targeting multiple tissues: IDUA activity restored to 9.5% in the liver, 27% in hearts and 1.3% in the brain; stable IDUA levels at 6 months after administration; limited effect on natural stop codons and no alterations of the endogenous tRNA homoeostasis	33
CFTR (cystic fibrosis)s	Readthrough at three PTCs with IVT sup-tRNAs, or with plasmid-encoded sup-tRNAs transfected in immortalized cells (16HBEge) gene-edited to express three full-length PTC–CFTR	5–7% restoration of R1162X–CFTR activity with 16 copies of plasmid-encoded sup-tRNA^{Arg}; miscoding and some rescue of channel activity with four copies of sup-tRNA^{Leu} at W1282X, but not with the cognate sup-tRNA^{Trp}	230
CFTR (cystic fibrosis)	Intravenous and intratracheal LNP delivery of IVT sup-tRNA^{Ser} in mouse; transfection of sup-tRNA^{Ser} and sup-tRNA^{Arg} in cystic fibrosis cell models and epithelial cells of patient with R1162X-*CFTR*	Optimized sup-tRNAs for high-efficacy CFTR activity restored to 14% beyond the clinical threshold for cystic fibrosis; in tissue stability of IVT sup-tRNAs >72 h; no effect on natural stop codons	35

HGPRT, hypoxantine–guanine phosphoribosyl transferase; IVT, in vitro-transcribed; LNP, lipid nanoparticle; PTC, premature termination codon; rAAV, recombinant adeno-associated virus; sup-tRNA, suppressor tRNA.

often leads to abnormalities in translation at the affected codons. An illustrative example of such diseases is the subgroup of the Charcot–Marie–Tooth (CMT) pathologies associated with heterozygous mutations in several AARS genes and characterized by length-dependent axonal atrophy and degeneration[62]. In the majority of incidences, mutations do not alter the aminoacylation activity of the AARS[63,64], indicating that loss of enzymatic activity is not a prerequisite for disease causality. A recent study with CMT-associated mutations in glycyl-tRNA synthetase proposes an altered kinetics of release of tRNAs^{Gly} (ref. 48). This transient tRNAs^{Gly} sequestration markedly decreased the translation velocity at all four Gly codons, altered expression of Gly-containing transcripts and activated the integrated stress response[47,48] (Fig. 2b). Drosophila CMT models for other AARSs show a reduced global protein synthesis in motor and sensory neurons[63]; thus, the tRNA sequestration might be a common underlying mechanism for the majority, if not all, of CMT pathologies associated with AARS mutations. Increasing the copy number of the most abundant tRNA^{Gly}(GCC) in CMT mice alleviated the atrophy and degeneration, implying that supplementation of the cognate tRNAs might be a suitable therapeutic strategy[48]. Elevating the level of the major tRNA isoacceptor alone was enough to overcome tRNA sequestration caused by the mutated glycyl-tRNA synthetase[48] (Fig. 2b). Nonetheless, it would be more advantageous to administer cocktails that include all cognate tRNA isoacceptors charged by the mutated AARS in a ratio that mimics their natural concentrations. This would ensure a better overall balance and efficacy in restoring translation functions at all Gly codons.

Design of tRNA cargo

tRNA administration

There are two primary approaches for administering tRNA therapeutics: (1) episomal delivery of a tRNA gene to the nucleus (Fig. 4a) and

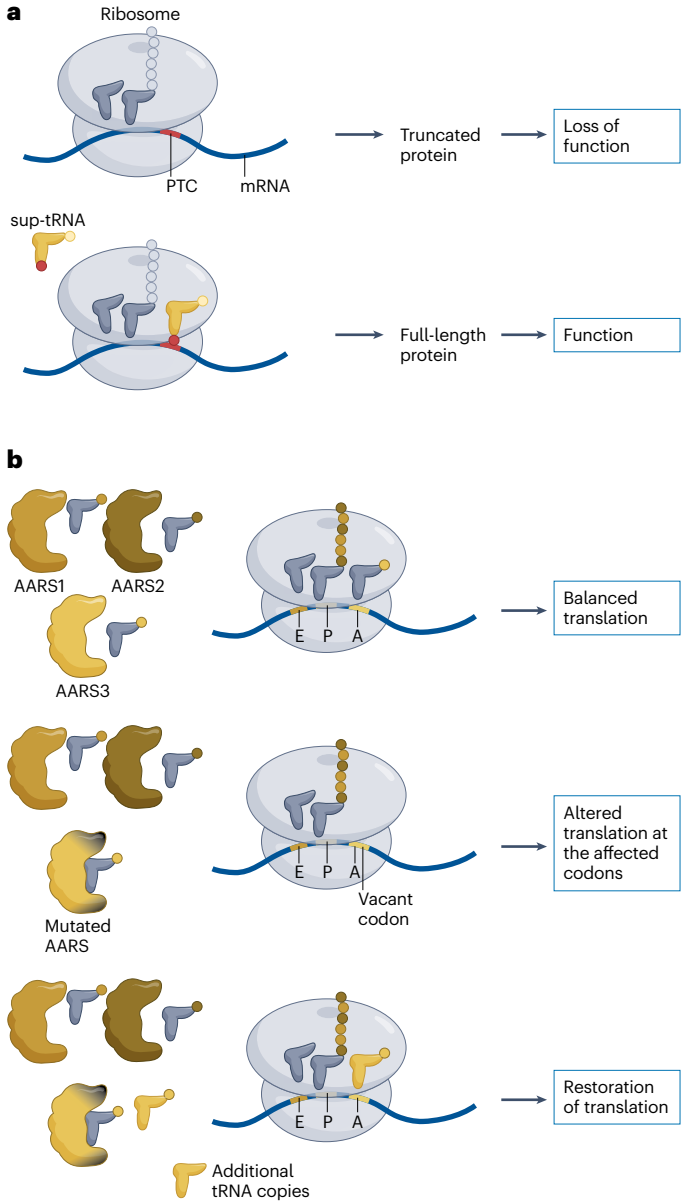


Fig. 2 | tRNA-based therapeutic modalities. a, Suppression of nonsense mutations. A nonsense mutation introduces a PTC (red) which signals termination of translation and, consequently, production of truncated non-functional protein (upper schematic). sup-tRNA tailored to decode PTC enables smooth translation through the PTC and production of full-length functional protein (lower schematic). **b**, Supplementation with native tRNAs to counteract mutation-driven tRNA sequestration by the cognate AARS. Native tRNAs aminoacylated with the corresponding amino acid by the cognate AARS (codon, amino acid and AARS depicted in matching colours) participate in the translation of the cellular proteome (upper schematic). A pathogenic mutation in one AARS changes the kinetics of aminoacyl-tRNA release, causing a transient ribosome stalling at the cognate codons (yellow) and consequently alters the synthesis of all proteins containing these codons (middle schematic). tRNA supplementation (blue tRNA) compensates for the mutation-induced aminoacyl-tRNA sequestration (yellow) and restores translation at the affected codons (lower schematic). The three tRNA-binding sites at the ribosome (A, P and E) depicted from right to left in three different codon colours. AARS, aminoacyl-tRNA synthetase; mRNA, messenger RNA; PTC, premature termination codon; sup-tRNA, suppressor tRNA.

(2) cytosolic delivery of a mature tRNA (Fig. 4b). Each have their own design considerations.

Episomal delivery. With episomal delivery technologies (Fig. 4a), the tRNA gene-expressing cassette is transported to the nucleus, in which it utilizes the endogenous transcription machinery centred around RNA polymerase (Pol) III (Box 1). In clinical studies of full transcript replacement therapies, the most commonly used promoters are strong heterologous promoters (CMV and CAG) or promoters of highly expressed cellular transcripts (albumin and synapsin) to achieve a high tissue-specific expression[65]. For tRNA therapeutics, this would translate into using a strong Pol III promoter (U6 or H1)[28,33] or a highly expressed cellular tRNA ($tRNA^{Tyr}$ or $tRNA^{Gln}$). The episomal delivery as a tRNA gene enables continuous and independent expression of the tRNA in the target tissue and should be ideally achieved with a single administration.

The two strong Pol III promoters (U6 and H1), commonly used for small RNAs and guide RNA production in vector-based CRISPR-Cas9 systems[66], have been recently exploited in in vivo administration of sup-tRNAs resulting in a stable restoration of the alpha-L-iduronidase activity over 6 months[33]. The human H1 Pol III promoter is also active as a Pol II promoter[67], which may decrease the sup-tRNA expression levels with time. Alternatively, native promoters of highly expressed cellular tRNAs ($tRNA^{Tyr}$ and $tRNA^{Gln}$) could be used. Highly expressed endogenous tRNAs usually have strong internal and 5′ upstream promoter sequences (Box 1). The genomic context, which relates to the regulation of the chromosomal accessibility, also determines the expression potential of tRNA genes[8]. Thus, considering the upstream promoter sequence and the flanking genomic context of highly expressed cellular tRNAs could represent a suitable strategy for sustainable and high expression of sup-tRNA that is comparable to the expression levels of the cellular tRNAs. In addition, targeted nucleotide substitutions in the internal tRNA promoters (Box 1) can be explored to further modulate the expression level[68].

Among human tRNAs, approximately 6% (for example, $tRNA^{Tyr}$(GTA), $tRNA^{Ile}$(UAU), $tRNA^{Arg}$(UCU) and $tRNA^{Leu}$(CAA)) possess an intragenic intron between nucleotide 37 and 38, which has a crucial role in tRNA maturation[69]. A recent study investigating administration of various anticodon-engineered sup-tRNAs demonstrates that in the case of sup-$tRNA^{Tyr}$, the intron does not enhance the production and charging of sup-$tRNA^{Tyr}$ [33]. However, certain introns serve as recognition signals for modifying enzymes[70–72]; thus, the inclusion of an intron in the tRNA gene-expressing cassette might be beneficial for some sup-tRNAs and should be evaluated on a case-by-case basis.

Cytosolic tRNA delivery. An alternative strategy for tRNA administration is to supply operational tRNA directly in the cytosol, that is, translationally active tRNAs competent for selection by the ribosome during protein synthesis (Fig. 4b). In this case, therapeutic tRNA is readily available for translation; therefore, the therapeutic effect is observed nearly at the onset of administration[35]. The therapeutic effect depends on the tRNA stability and turnover; thus, cytosolic tRNA delivery requires repetitive administrations.

For cytosolic administration, the therapeutic tRNA is synthesized in a cell-free in vitro transcription reaction using a linear DNA template

encoding tRNA preceded by the T7 promotor[73]. Following purification, through organic solvent-extracting methods (for example, phenol–chloroform followed by precipitation in ethanol), the in vitro synthesized tRNA is renatured to adopt its natural 2D and 3D structure (Box 1). The administered IVT tRNA utilizes the natural machinery of the cell to be repeatedly loaded with the cognate amino acid by the corresponding AARS[34]. Aminoacyl-tRNA administrations (that is, as a pre-charged tRNA with the cognate amino acid) are not feasible; the ester bond between the 3′ terminal adenosine of the tRNA and the cognate amino acid is highly pH sensitive[74] and will be rapidly hydrolysed during packaging and/or administration in the cell.

Anticodon-engineering for sup-tRNAs

The most common approach to design sup-tRNAs is to modify the anticodon sequence of natural tRNAs to decode PTCs[19,27–32]. Systematic alterations of the anticodon of 10 human tRNA isoacceptor families have yielded some important conclusions[31]. Firstly, not all tRNA isoacceptors can accommodate changes in the anticodon[31]. Secondly, certain tRNA isoacceptors exhibit a clear preference for a particular PTC identity; for example, sup-tRNA^{Trp}CUA has higher suppression efficiency at UAG than sup-tRNA^{Trp}UCA at UGA and UAG, whereas tRNA^{Gln} shows a clear preference for UAA over UAG PTCs[31]. Thirdly, within the same tRNA family, some tRNA isodecoders or isoacceptors tolerate alterations of the anticodon, whereas others do not. An anticodon-engineered sup-tRNA may not establish the ideal Watson–Crick geometry for decoding in the ribosomal A site[56] to efficiently antagonize nonsense-mediated mRNA decay (NMD) and the mRNA degradation process[33,35]. NMD recognizes only PTCs and not the normal termination codons[75].

The anticodon is a (strong) recognition signal for many AARS[43]. Consequently, the anticodon-engineered sup-tRNAs might be mischarged and insert an amino acid that is different from the original, non-mutated one at the PTC, resulting in a missense mutation. For instance, the anticodon of tRNA^{Ser}, tRNA^{Leu} and tRNA^{Tyr} does not serve as a recognition signal for the cognate AARS[43,76–78] and engineering the anticodon of these tRNAs to decode UAG introduces the desired amino acid at the PTC[33]. By contrast, the anticodon of tRNA^{Trp} is recognized by the tryptophanyl-tRNA synthetase[79] and anticodon-engineered sup-tRNA^{Trp}CUA inserts preferably lysine (79%) rather than the cognate tryptophan (20%)[33].

To enable decoding of UGA and UAA, a uridine is introduced at position 34 of the sup-tRNA designs, which is the most naturally modified nucleotide in tRNAs (Box 1). If this U34 would be modified, it may involve the sup-tRNA in wobbling interactions, potentially leading to amino acid misincorporations at unrelated codons and activation of the integrated stress response[80]. Moreover, changes in the anticodon sequence of sup-tRNAs may disrupt other conserved modifications in the anticodon loop that modulate the decoding accuracy[81,82].

Overall, the suppression efficiency of anticodon-engineered sup-tRNAs is relatively modest and may fall short of reaching the therapeutic threshold, particularly for dominantly inherited pathologies with a relatively high disease threshold. However, these simple sup-tRNA designs might still be adequate for addressing diseases with a low therapeutic threshold.

Enhancing sup-tRNA efficacy

Idiosyncratic evolutionary constraints acting on the entire sequence have shaped the similar decoding efficiency of different tRNA sequences[83,84]. A recent approach has emerged that capitalizes on the functionally conserved features of tRNA and enhances suppression efficacy by modulating various regions of tRNAs outside the anticodon[34,35]. Different segments of tRNAs have vital roles for their function in translation (Box 1); for example, the anticodon-stem and loop regulate decoding accuracy, the TΨC-stem sequence determines the binding affinity to elongation factor, the D-loop coordinates long-range interactions to establish the functional 3D architecture of tRNAs, and the acceptor stem carries identity elements for AARS[43,85–88].

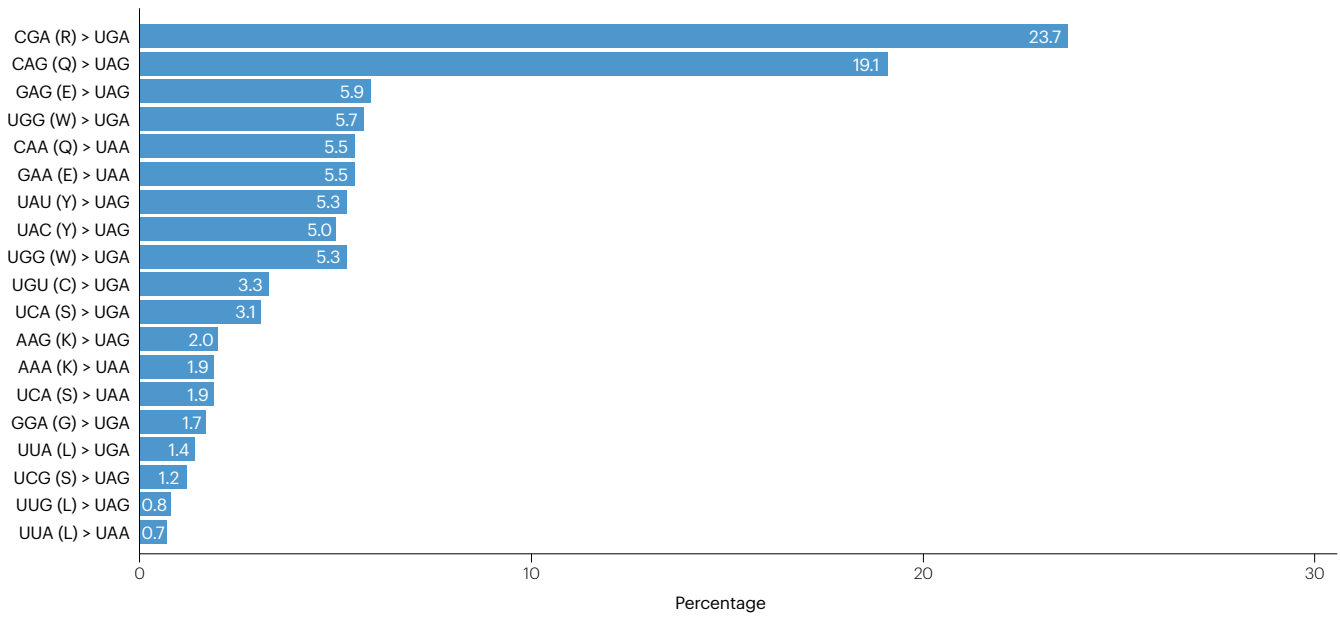


Fig. 3 | Frequency of nonsense mutations in the human population associated with various genetic diseases. Of the sense codons, 18 can be mutated into premature termination codons, with four of them potentially having dual premature termination codon identities. For some theoretical possibilities, no pathogenic mutation has been described. Amino acid identity is included as a single-letter code (in parentheses).

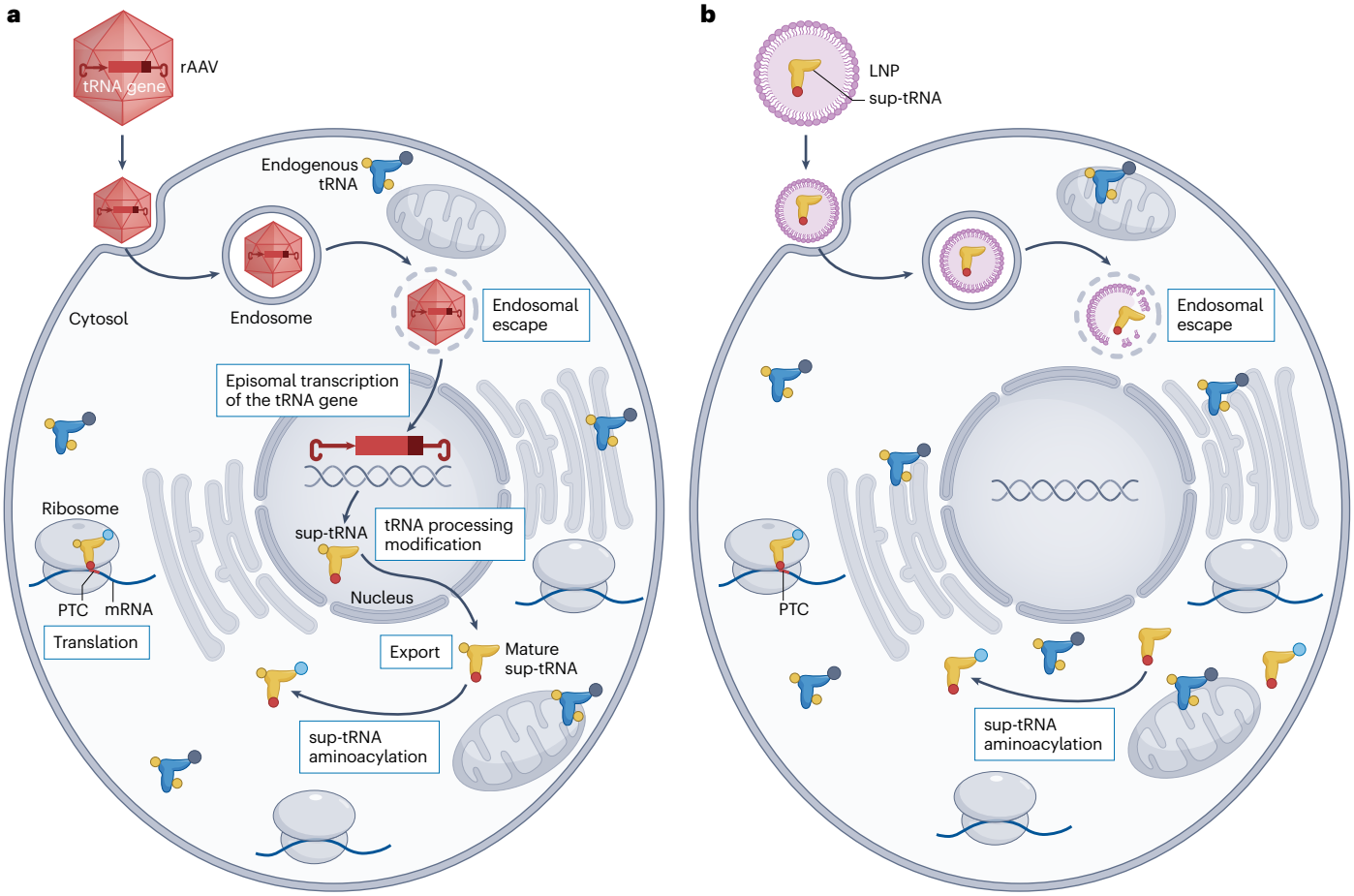


Fig. 4 | Therapeutic tRNA delivery approaches. a, Delivery as tDNA formulations exemplified using rAAV vehicles. The gene encoding the tRNA of interest is flanked by the rAAV inverted terminal repeats into a 'transgene expression cassette' which is transcribed episomally in the nucleus using the natural transcription machinery of the cell. Processed tRNA is probably modified and exported into the cytosol for translation. **b**, Delivery as tRNA formulations exemplified with LNP vehicles. In vitro transcribed tRNA is released in the cytosol, most probably remains unmodified, but is aminoacylated and participates in multiple rounds of translation. Administered tRNAs are depicted in yellow with a red anticodon to pair to the PTC (red); natural tRNAs are depicted in blue, posttranscriptional tRNA modifications by yellow circles and cognate amino acid by a larger blue circles. LNP, lipid nanoparticle; PTC, premature termination codon; rAAV, recombinant adeno-associated virus.

However, the physicochemical properties of the amino acid carried by each tRNA (that is, stabilizing or destabilizing the aminoacyl-tRNA[89]) require unique design principles for each tRNA family[35]. For example, in the case of arginine, a neutral amino acid, enhancing the PTC suppression efficacy of sup-tRNA^{Arg} involves altering the TΨC-stem sequence to stabilize interactions with the elongation factor. Conversely, for sup-tRNA^{Ser} and sup-tRNA^{Ala} carrying a stabilizing and destabilizing amino acid, respectively, fine-tuning of both the anticodon stem and TΨC-stem proves beneficial[34,35]. By leveraging this approach, the readthrough efficacy of sup-tRNA^{Ser} and sup-tRNA^{Arg} has been enhanced by 4-fold and 2.5-fold, respectively, compared with the readthrough efficacy of the anticodon-engineered tRNAs[35]. A significant readthrough (approximately 10%) at UAA PTC, the most challenging to suppress[90], has been achieved with IVT sup-tRNA^{Ser} transfected into a human cystic fibrosis bronchial epithelial cell line expressing the pathogenic S455X PTC of *CFTR*[35].

Despite the unprecedented achievements in PTC suppression in preclinical settings[35], the suppression efficacy is still too low to provide a clinical benefit for many dominantly inherited disorders with a high therapeutic threshold. Anticodon-engineered isodecoders with high basal suppression activity[31] could be selected as a starting point for further sup-tRNA sequence optimization. For isoacceptors with low suppression activity or resistant to anticodon engineering, an alternative strategy could be applied. The nucleotides serving as identity elements for the AARS of a highly efficient sup-tRNA can be exchanged with those of another AARS to preferably charge the sup-tRNA with a desired amino acid[77]. Alterations in the tRNA body sequence to enhance efficiency may abolish recognition signals for natural modifications and affect the cellular stability of the sup-tRNA (Box 1). Thus, sup-tRNA designs should balance between optimal efficiency and stability for a maximal clinical benefit for each disease.

The design of sup-tRNAs can also draw inspiration from natural sup-tRNAs. A recently discovered tRNA variant with a shorter anticodon stem efficiently decodes the UGA stop codon as tryptophan in the trypanosomatid *Blastocrithidia nonstop*[91]. In natural instances of stop-codon reassignment, such as the incorporation of the essential

trace element selenium as L-selenocysteine (Sec) at specific in-frame UGA stop codons, $tRNA^{Sec}$ has a crucial role[92,93]. However, in these cases, the natural sup-tRNA is 'assisted' either by a concurrent mutation in the release factor 1 for tripanosomatids[91] or by a dedicated translation factor and secondary structural element in the mRNA for selenocysteine[93]. Therefore, mimicking such natural sup-tRNAs may not be sufficient to convert other native tRNAs into efficient suppressors[34].

Along with these tailored designs to selectively target specific regions to enhance sup-tRNA efficacy, approaches based on random mutagenesis and combinatorial libraries[39,94] or using machine-learning (AlltRNA, www.alltrna.com) could also generate tRNA candidates with enhanced suppression activity. The identity elements of the cognate AARS should be strictly preserved to ensure the incorporation of the desired amino acid at the PTC.

A strong context-dependent readthrough efficiency for nonsense mutations with the same amino acid and PTC identity signature (for example, R553X and R1162X in CFTR) has been observed[35]. This issue still remains enigmatic and may depend on the utilization of the particular mRNA and/or the degree in which each mutated mRNA is subject to NMD. Notably, sup-tRNAs efficiently antagonize the NMD at some PTCs[33,35]. This antagonism may manifest only when readthrough levels are substantially elevated[95], suggesting that further development of the sup-tRNA designs to enhance their suppression activity would be beneficial.

Evading the immune response

Regarding cytosolic tRNA deliveries (Fig. 4b) for suppression (sup-tRNA) or supplementation (natural tRNAs) therapies, the IVT tRNA may stimulate innate immunity by activating the endosomal Toll-like receptors (TLRs), a main family of pattern recognition receptors responding to viral RNA and expressed primarily but not exclusively in immune cells[96]. Many of the naturally occurring modified nucleotides, such as pseudouridine (ψ), N^1-methyl-pseudouridine ($m^1\psi$), N^6-methyladenosine (m^6A), 5-methylcyitidine (m^5C) and 2-thiouridine (s^2U), render RNAs invisible for the human TLR7 and TLR8 and reduce cytokine production[97–99]. The reverse transcriptase used for in vitro tRNA transcription is naturally promiscuous for many modified nucleosides and incorporates them into the IVT RNA — a production strategy used in the COVID-19 mRNA vaccines to fully replace unmodified uridine with $m^1\psi$ and avoid undesired innate immunity responses. However, when incorporated during an in vitro transcription reaction, modified nucleotides alter secondary interactions[100–102], which would be deleterious for the function of tRNAs in translation. Specific natural tRNA modifications are potent silencers of the innate immune response. For example, the 2′-*O*-methylation of guanine at position 18 of a bacterial tRNA is sufficient to suppress the activation of the TLR signalling cascade in human cells[103,104]. In principle, double-stranded RNA is nearly non-immunogenic[97]; thus, site-specific incorporation of modified nucleosides into the single-stranded tRNA regions should be favoured. Naturally occurring nucleotides in the loops, for example, deoxyuridine (D) in the D-loop and ψ in the TψC, could be the preferred choices as they also tend to facilitate the native-like interactions that maintain the translationally functional L-shaped structure (Box 1). A Lego-like synthesis strategy of sup-tRNAs, including IVT synthesis of unmodified tRNA parts and chemical synthesis with site-specific incorporation of modified nucleosides, would be more cost-intensive as the ligation of those tRNA fragments unavoidably decreases production yields of full-length sup-tRNA. A full-length chemical sup-tRNA synthesis could be an option. Currently, however, chemical synthesis has not reached sufficient yields for full-length tRNAs. Independent of the production approach, the purity of the final preparation must be very high and free of short linear contaminants that could stimulate an undesired immune response and the production of cytokines, and decrease the efficiency of the tRNA cargo.

Delivery of tRNA therapeutics

To fully harness the therapeutic potential of tRNAs for precision treatments of monogenic diseases, it is essential to develop and customize formulation strategies that address the specific limitations associated with tRNA payloads. This requires simultaneous advances in various formulation approaches, tailored to the unique requirements of tRNA-based therapies. The negative charge of tRNAs, along with their vulnerability to degradation by RNases in tissues or body fluids, makes entering cells extremely challenging for them, like other RNA therapeutics. Therefore, a crucial aspect of ensuring the clinical effectiveness of tRNA-based gene therapy is achieving efficient delivery to specific tissues or cell types. Notable progress in the field of viral and non-viral delivery systems has provided viable options for delivery of different RNA cargos and for targeting different tissues (reviewed in refs. 1,2,65,105–118). The potential of tRNA as a therapeutic has only recently been recognized and no clinical study to date has been registered. However, two recent studies on sup-tRNA administrations in mice use delivery platforms developed for other RNAs[33,35], thus supporting their potential suitability for tRNA therapeutics. Here, we summarize the advances in delivery platforms that would potentially be also suitable for administration of tRNA payloads.

Delivery vehicles

To design clinically relevant drug delivery vehicles for tRNA cargo, it is important to consider the mechanism of action and the tissue of onset for the corresponding pathology. The natural tropism of viral-derived vehicles or exosomes to cross the blood–brain barrier (BBB) is an attractive solution for episomal tRNA delivery (Fig. 4a), to treat pathologies with the central or peripheral nervous system as a primary tissue of onset[112]. Synthetic carriers (that is, LNPs and biocompatible polymers) might be more suitable for repeated systemic administrations and provide a complementary alternative for tissues that are inaccessible to AAV vehicles (such as the kidney and lung).

AAV-based delivery. Viral vector-based deliveries harness the natural potential of viruses to deliver nucleic acids (DNA or RNA genomes) into both proliferating and postmitotic cells, including the central nervous system (CNS). The first approval of recombinant AAV (rAAV) delivery for gene-replacement therapy of lipoprotein lipase deficiency in Europe[119] has deemed rAAV vehicles safe and efficacious for other gene therapeutic applications. With more than 60 active clinical trials which utilize AAV capsids as a delivery vehicle to target different tissues, for example, the brain[120,121], eye[122], lung[123], liver[124] and muscle[125], the AAV vectors have achieved a remarkable standing from a therapeutic standpoint and dominate the realm of gene therapies[65]. A recent study has pioneered a rAAV delivery platform for tRNA therapy, and in preclinical settings, it showed efficient suppression of PTCs in different mouse tissues (for example, the liver, heart, skeletal muscle and brain)[33].

Improved AAV capsids with refined characteristics for higher safety and efficacy have broadened the range of tissues and conditions that can be targeted, ultimately enhancing the number of disease targets[126]. Currently, there are 13 human and non-human primate AAV serotypes, all of which are considered non-pathogenic. AAV serotype 2

(AAV2) is the first AAV isolate with natural tropism towards muscles and hepatocytes. Engineered natural and synthetic AAV (or rAAV) vectors with unique capsid structures have been developed, allowing targeting of different tissues[105,111,112,115,127]. Luxturna, the first FDA-approved AAV-mediated gene therapy, utilized the AAV2 serotype[111]. A systematic review of 149 unique clinical trials reveals that the AAV2 serotype is still the most used[65]. AAV9 and AAV2 display similar widely disseminated transduction efficiency and several tissues can be effectively targeted with these two serotypes. AAV9 is more efficient than AAV2 and its ability to bypass the BBB makes AAV9 a promising candidate for widespread delivery to the central and peripheral nervous system[127]. In seven clinical trials, AAV8 and AAV9 capsids are used for delivery to the CNS.

Capsid evolution techniques are becoming increasingly sophisticated, enabling highly specific transduction of specific cell types. Novel capsids (for example, rAAV-LK03, SPK-100 and AAV-HSC15) have been developed to expand the transduction efficiency. For example, rAAV-LK03 originates from a capsid-shuffled library which had been selected in a xenograft humanized liver mouse model and is 10 to 20 times more efficient at transducing human hepatocytes in vivo than AAV8[128]. It performs robustly in primates and humans, but not in mouse models[129]. Epigenetic regulation is the reason for selectivity of AAV capsids in different organisms[130], emphasizing on the poor predictability of animal studies for efficacy in humans. These new developments hold great promise for tRNA delivery, although preclinical studies remain to be performed in order to understand their tissue specificity and safety.

LNP-based delivery. With the mRNA-based vaccines to protect against COVID-19[131] and the FDA-approved short interfering RNA (siRNA) therapeutics (patisiran, which is sold under the brand name Onpattro) to target hereditary transthyretin amyloidosis in the liver[132], LNP administration secured a prominent place among safe and efficient classes of vehicles for gene therapies. The FDA-approved LNPs are four-component systems: an ionizable (cationic) lipid, cholesterol, a helper lipid and a polyethylene glycol (PEG)-conjugated (reviewed in refs. 1,110,114).

Historically, cationic lipids, which possess a permanent positive charge, have been used for encapsulation of negatively charged mRNA[1,133]; however, their short blood circulation time, endosomal trapping and degradation[134] have impeded their wide clinical use. Major efforts are being dedicated to the development of new ionizable lipids, for example, DLin-MC3-DMA (in the siRNA-based drug patisiran of Alnylam) or ALC-0315 (COVID-19 vaccine of Pfizer–BioNTech–Acuitas). These lipids are ionizable at acidic pH 4–5 to encapsulate the negatively charged RNA entities, but uncharged at the neutral cytosolic pH, and this property facilities endosomal escape of the LNPs[110,135]. The ionizable lipid DLin-MC3-DMA[132,136] used in the first FDA-approved LNP product exhibits poor degradability, leading to toxicity concerns associated with repeated local and systemic administrations. To address this issue, Moderna has developed a biodegradable lipid 5 (ref. 137) which demonstrated no apparent toxicity following systemic administration in animal models of acute intermittent porphyria[138], or following local intratumoral administration of IL-23, IL-36γ and OX40L mRNAs[139]. The utilization of biodegradable lipids for RNA delivery, including tRNAs, holds great promise for the future of LNP technology owing to their improved safety profiles and reduced safety risks.

The other three components of the LNPs, for example, PEG lipid, cholesterol and helper lipid, also affect delivery and pharmacokinetics[140,141]. PEG lipid improves the blood circulation time of the LNPs by interacting with water in the blood and creating an aqueous barrier[142]. Helper lipids also facilitate endosomal escape by adjusting the fluidity of the LNP[108]. Unmodified cholesterol is a primary stabilizing component of the LNPs, although some oxidized or esterified cholesterols have shown some improvements[143]. However, reported changes in LNP structure by incorporating modified cholesterols[144] warrant caution when replacing cholesterol.

The optimized lipid formulations[145–147] and local delivery to the retina, eye, lung and myocardium[113,148–150] expand the portfolio of tissues transfected by LNPs, ultimately expanding the number of disease targets. Adding a fifth component to the classic four-component LNP system that binds receptors selectively expressed on specific cells can also increase tissue selectivity. For example, a fifth lipid that binds to vibronectin or to β2-gylcoprotein facilitates delivery to the lung or spleen, respectively[151,152].

By changing the molar ratio of the LNP components and lipid-to-RNA, the LNPs can be retargeted to other tissues[107,114]. Arcturus Therapeutics has achieved robust and persistent suppression of PTCs in mouse liver using ionizable LUNAR® lipid[35]. By changing the ionizable lipid and total lipid-to-tRNA weight ratio from 25:1 to 15:1, the LUNAR® LNPs were retargeted to deliver sup-tRNA to the lungs[35]. Organ-targeted delivery can also be improved by using biomimetic lipids that are enriched in the membranes of the target tissue. For example, as neurotransmitters cross the BBB, neurotransmitter-derived lipids can redirect LNPs to the brain following intravenous injection[110,114,153,154]. By functionalizing the LNPs with receptor-specific antibodies, LNPs can also reach the extracellular space of the brain[145]. The antibodies bind to the exofacial epitopes of certain endothelial receptors, such as insulin or transferrin receptors, and through receptor-mediated transcytosis cross the BBB and subsequently localize in the nucleus for transcription of the therapeutic gene[145]. Using such functionalized LNPs, plasmid DNA has been delivered into the brain of mice, rats and monkeys[155,156]. Chronic treatment of rats with weekly intravenous injections shows no evidence for toxicity or brain inflammation[157]. Thus, the functionalized LNPs could provide an alternative to the AAVs for re-administration of tRNAs to brain tissues.

Extracellular vesicle-based delivery. Exosomes are endosome-derived lipid bilayered intraluminal vesicles of 40–150 nm in diameter, which through fusion to the plasma membrane and endocytosis are secreted into the extracellular space[109,158,159]. They contain various constituents of the cell of origin, including DNA, RNA, lipids, metabolites, and cytosolic and cell-surface proteins[160]. With the latter, they interact with the recipient cell and insert through different mechanisms, including lipid fusion, endocytosis, micropinocytosis and receptor-mediated uptake[159]. Although the physiological purpose of exosome generation remains elusive, they have gained attention as drug delivery systems owing to their inherent ability to mediate near-distance and long-distance intercellular communication between cells[159,161,162]. The minimal immune clearance and enhanced bioavailability, the lack of malignant transformation[163,164] and the potential to circumvent the BBB[165] make exosomes ideal carriers of diverse therapeutic payloads, including DNA, RNA and oligonucleotides. The recent FDA approval of exoASO-STAT6, an antisense oligonucleotide-loaded exosome for intravenous systemic administration[166], developed by Codiak Biosciences and currently in a phase I study for colorectal cancer (NCT 05375604), opens up new possibilities to explore the therapeutic utilization of exosomes for tRNA-based treatments.

Exosomes can be derived from different cells and tissues and bear the composition and the homing effect of the parental cell type[162,164].

The stable lipid bilayer guards them against the action of native immune cells and digestive enzymes, and determines their pharmacokinetic and pharmacodynamic properties[158]. Exosomes have been used to deliver functionalized DNA to treat cancer[167]; however, their small size limits the DNA cargo to small synthetic DNA nucleotides or small plasmid DNA. The relatively small size of the tRNA gene makes tRNA gene-expressing plasmids suitable for exosomal delivery.

Exosomes have also been engineered to deliver siRNAs and antisense oligonucleotides to a desired target. Co-incubation, transfection and electroporation are the frequently used approaches of loading of the therapeutic RNA molecules into exosomes, although the efficiency differs and needs to be optimized for each exosome and cell type[118]. A common way to load them with an RNA cargo is to transfect the parental cells with the corresponding RNA[168,169]. Although exosomes have not yet been exploited in tRNA delivery, production pathways similar to those of siRNA or oligonucleotide delivery are possible for tRNA payloads. The ability of the exosomes to bypass BBB[165], their higher stability in body fluids than that of LNPs, and their suitability for repeated administrations[170,171] unlock innovative options for cytosolic tRNA administrations into the CNS and peripheral neuronal tissues.

Currently, the inefficient packaging with RNA cargo limits the clinical applications of exosomes. Synthetic biology-inspired control devices have been used to enhance mRNA packaging, and engineered producer cells implanted in living mice could consistently deliver cargo mRNA to the brain[172]. Whether this approach could be used for tRNA packing remains to be proven, as it was deemed unsuitable for other structured RNAs[173]. Along with this, the intricacies of loading, standardization of manufacturing and functioning uncertainties[161] warrant further exploration and development to unleash the full potential of exosomes in tRNA therapeutics.

Other types of delivery systems. The biocompatible poloxamine-based copolymers could be a promising alternative to LNPs or AAVs for delivery of tRNA cargo. Leveraging the low immunogenicity and favourable safety profiles of poloxamine, a self-assembling particle for plasmid DNA payload delivery to the lungs, has been developed[174]. The system contains three synthetic peptides, an anchor with hydrophobic blocks from poloxamine, a cationic moiety with basic amino acids to encapsulate nucleic acids and facilitate endosomal escape, and a targeting block to target the particles to a specific tissue[174]. The particles have been tested in vitro, in cellular models and in vivo in a mouse model for mRNA replacement therapy of cystic fibrosis. Considering the favourable safety profile of poloxamines and their ability to specifically target different tissues, for example, lung epithelia and cardiac and skeletal muscles[174,175], the potential of poloxamine-based copolymers to encapsulate tRNA and their suitability as delivery platforms for tRNA therapeutics warrant investigation.

Considerations and challenges of tRNA therapeutics

The field of tRNA therapeutics holds immense promise for the development of innovative treatments; however, it also faces several challenges that require careful consideration. A fundamental obstacle lies in the efficiency of delivery of therapeutic tRNAs to target cells. As discussed in the previous section, strategies for enhancing delivery efficiency while maintaining tRNA stability and integrity during transit need to be addressed. Achieving the balance between desired therapeutic properties and potential off-target effects or unintended interactions with cellular processes remains a complex task. Establishing robust manufacturing processes to ensure consistent quality, purity and scalability is vital for clinical translation. Importantly, the regulatory landscape surrounding tRNA therapeutics is still evolving. Developing standardized guidelines and frameworks for evaluating the safety, efficacy and quality of these novel therapeutic modalities is essential to facilitate their regulatory approval and widespread adoption.

Safety

As with any gene therapy, toxicity of tRNA therapeutics is a perennial concern. The primary concern with sup-tRNA-based therapeutics is the creation of neoantigens by reading through natural stop codons[51]. Inherent mechanisms operate to detect and activate signalling cascades which clear C-terminally extended proteins resulting from the spontaneous readthrough of the natural stop codons[176–178]. The spontaneous readthrough for the majority of the cellular transcripts is below 1%, but for a few transcripts, it can reach up to 10%[179]. Using ribosome profiling (that is, a transcriptome-wide analysis of translating ribosomes[180]), recent studies show that anticodon-engineered sup-tRNA formulations induce a marginal readthrough at the cognate natural stop codons[31,33] (Table 1), which, however, is at least onefold lower than the unspecific readthrough induced by the aminoglycoside G418 at all three stop codons[33,90]. Improved sup-tRNA designs with higher suppression efficiency do not cause any discernible readthrough at any of the native stop codons[35] (Table 1), suggesting that higher readthrough efficacy parallels higher molecular safety. The sequence context of the natural termination codons that has been evolutionarily selected for high termination efficiency[90,181], along with the ability of a cell to eliminate proteins C-terminally extended by a spontaneous readthrough[177], is probably the reason for the lack of off-target readthrough at natural stop codons. Despite the extremely low frequency of the sup-tRNA-induced readthrough at natural stop codons, some C-terminally extended proteins might be very stable and accumulate with time. For this, mass spectrometry should also be used to assess the molecular safety profile of the sup-tRNAs.

The concentration of natural tRNAs must be kept in a defined narrow margin, as dysregulation of a single tRNA isoacceptor[182] or broader changes of tRNA repertoires[9] may unlock cell reprogramming into a more proliferative, potentially cancerous state. A recent study has presented evidence that an imbalance in tRNA levels can be compensated by other isodecoders and this compensation comes at the cost of compromised translation accuracy[183], potentially leading to erroneous protein synthesis. Furthermore, the effect may differ depending on whether naturally low-abundance or high-abundance isoacceptors are targeted in a potential tRNA-supplementation therapy. This would require a precise dosing, probably in a very small concentration window, which should be determined in each case to minimally alter the levels of the remaining tRNAs. Even sup-tRNAs, which do not target a natural sense codon, bear some risk in misbalancing the tRNA households. The expression of a small yet sizeable number of isodecoders (38 out of 243) was altered following systemic administration of sup-tRNA^{Tyr}–rAAV in mice[33]. Thus, in the preclinical phase, any dysregulation of native tRNA pools should be carefully assessed for each tRNA preparation. The dosing in episomal tRNA delivery is less precisely controllable; thus, IVT tRNA formulations suitable for re-dosing could be an option.

A safety issue related to IVT tRNA formulations is the inefficiency of the purification procedure and contamination of the final preparations with shorter truncated IVT products, which may cause some undesired responses in their clinical use[184]. Optimized purification

Glossary

Adeno-associated virus
(AAV). Non-pathogenic, small single-stranded DNA virus, whose genome (4.7 kb) encodes four non-structural rep proteins, three capsid (cap) proteins and assembly-activating protein, flanked by two AAV-specific palindromic ITRs (145 bp).

Aminoacyl-tRNA synthetase
(AARS). A universal enzyme family that aminoacylates tRNAs with their cognate amino acid.

Basket trials
Also known as bucket trials; a type of clinical trial for patients with different diseases with the same mutation or biomarker.

Episomal expression
A non-integrated extrachromosomal circular DNA from a viral genome that replicates and is transcribed independently in the eukaryotic nucleus.

Missense mutation
A genetic alteration within the protein-coding sequence leading to a change of the encoded amino acid which may alter the function of a protein.

Natural suppressor tRNAs
Native suppressors of nonsense mutations (mostly in bacteria and yeast) arise from mutation in sense codon decoding tRNA genes enabling the mutant tRNA to translate a stop codon.

Nonsense-mediated mRNA decay
(NMD). A pathway that rapidly degrades mRNA in response to nonsense mutations, which can arise owing to errors in transcription or failure to remove intronic regions, altering the natural reading frame.

Nonsense mutation
A genetic alteration within the protein-coding sequence exchanging a sense codon (that is, encoding an amino acid) to a termination or stop codon, resulting in a loss of protein function.

Recombinant AAV
(rAAV). Engineered AAV, in which the DNA of interest replaces the viral sequences encoding for rep and cap genes, whereas both *cis*-packaging ITR signals are retained.

Termination codons
Also known as stop codons. Three codons, named amber (UAG), ochre (UAA) and opal (UGA), terminate mRNA translation and, through pairing with the release factor (eRF1 in eukaryotes), release the newly synthesized protein.

Therapeutic threshold
The therapeutic threshold, which is established for each disease based on calculations, descriptive methods and clinical practice, describes the probability of disease at which the condition between treatment and no treatment is the same.

Toll-like receptors
Mediators of inflammatory pathways mediating the immune response towards a variety of pathogen-derived ligands, including DNA, double-stranded RNA, single-stranded RNA and oligonucleotides.

tRNA isoacceptor family
A group of all tRNA isoacceptors carrying the same amino acid.

tRNA isoacceptors
Different tRNA species, which are aminoacylated with the same amino acid, but differ in their anticodon sequences.

tRNA isodecoders
tRNA species that are aminoacylated with the same amino acids and bear the same anticodon, but differ elsewhere in their sequences.

procedures should be adopted to fully eliminate impurities. The IVT tRNA contains exclusively unmodified nucleotides. Although the administration of IVT sup-tRNAs in cell model systems with integrated TLRs has been shown not to stimulate the innate immune response[35], high-dose re-administrations in tissue may cause some mild immune responses. Current developments in small RNA-based therapies may provide solutions to abolish immune stimulation and enhance operational stability of tRNAs (for example, incorporating modified natural and unnatural nucleotides)[21,22]. Although the RNA stability could be immensely improved, some unnatural nucleotides might be harmful and cause cytotoxicity[185]. To enhance the safety profile of the tRNA payloads, naturally occurring nucleotides, which should be site-specifically incorporated to preserve the operational activity of tRNA (that is, the 3D architecture), might be a better choice.

Irrespective of the tRNA payloads, the delivery systems can present safety concerns. AAV vectors have dominated the realm of in vivo transgene delivery in gene therapies; however, the recent deployment of several trials by the FDA with the pushback for presenting longitude efficacy and safety assessments over extended periods of follow-up[186] have exposed several limitations of the approach. AAV vectors may cause toxicity problems related to the immune system, especially when higher doses are required to treat a disease[187]. In clinical studies, the expression of an AAV-mediated gene has often been lower in patients than in preclinical animal models, even after accounting for differences in weight between mice and humans. It is believed that immune responses against the AAV vector contribute significantly to this reduced expression[111,112,115]. The pre-existing neutralizing antibodies, which are prevalent in humans[188], also reduce the effectiveness of AAV-mediated therapy[189,190]. Removing pre-existing anti-AAV antibodies by partial immunosuppression with rapamycin has been proposed, to allow for AAV re-administration[191]. The endopeptidase imlifidase tested in transplant patients[192] efficiently degrades circulating IgG and eliminates anti-AAV antibodies, both in in vitro tests with human plasma and in vivo in non-human primates[193], providing a solution for overcoming humoral and treatment-induced immunity to AAVs.

In addition, viral genomes may randomly integrate into the host DNA and genotoxicity is an issue of concern. Delivered DNA typically persist episomally in the nucleus of transduced cells and only rarely integrate (that is, 0.1–1%) into the host genome[194]. Several studies in animal models, including longitudinal monitoring in dogs and non-human primates, show that viral genomes remain mainly extra-chromosomal (reviewed in ref. 106), although the genotoxicity risk may differ depending on the AAV serotype. Clonal integration of AAV2 genome sequences in pro-oncogenes was detected in biopsies of hepatocellular carcinoma patients[195]. The inserted sequences represent mostly fragments of the AAV-specific palindromic inverted terminal repeats (ITRs). The sequences within ITRs that are essential for viral propagation are known (reviewed in ref. 196), suggesting that AAV serotypes with altered ITRs could improve clinical safety.

The safety of LNPs depends on the lipid components utilized. Cationic and ionizable lipids can trigger the host immune response and the secretion of pro-inflammatory cytokines[197]. Dependent on the dose and tissue type, the cytotoxicity of some lipid components

might be a safety concern[198]. A way to decrease possible cytotoxic effects and improve the biocompatibility of the lipid nanoparticle is to utilize biodegradable lipids[110]. Endosomal trapping, which decreases the active concentration of RNA therapeutics, is a significant problem in LNP formulations. New types of ionizable lipids outcompete, at least in part, the endosomal deactivation of the RNA entities[110,135]. It is worth mentioning that PEGylated lipids induce allergic reactions owing to the pre-existing antibodies in a portion of the human population, and this is suggested to be one of the reasons for the rare anaphylactic reactions following administration of the COVID-19 vaccines[199]. The underlying factors associated with these rare allergic reactions are worthy of investigation, as for gene therapies, much higher doses and/or re-dosing are required.

Dosing and biodistribution

Determining the optimal dosage regimen for therapeutic tRNA molecules is crucial for achieving the desired therapeutic effect while minimizing potential side effects. However, establishing appropriate dosing schemes is complex, as factors such as tRNA stability, cellular uptake and intracellular processing dynamics must be considered. The therapeutic window for tRNA supplementation therapies might be much narrower than that for sup-tRNAs because multiple codons of the same kind across many transcripts are affected.

Classical rAAV delivery introduces an episomal genome (for example, tRNA gene expression cassette) into the nucleus of transduced cells (Fig. 4a). tRNA gene expression cassettes represent a genetically independent unit and potentially provide sustained tRNA expression throughout the whole life cycle of the cell with a single administration. In quiescent tissues and postmitotic cells, such as neuronal tissues, it is theoretically possible that a single administration of a tRNA gene could lead to sustained longitudinal expression (Fig. 4a). However, the exact duration of persistence in each specific tissue is currently unknown and may vary[126]. The durability of the effect remains fundamentally unanswered and the classical view of 'one-and-done' concept of AAV-based treatments would need robust evidence and lifelong follow-up of paediatric patients, to demonstrate a sustainable effect.

In regenerative and mitotic cells, long-term expression from a single administration is not probable. However, repeated tRNA administration using the AAV delivery platform may prove difficult owing to AAV capsid-induced immunogenicity[200] (see 'Safety' subsection). Alternatively, because of their minimal immune clearance, exosomes could be an ideal carrier for repeated administration of episomal tRNA payloads, following the scheme of a phase I clinical trial for colorectal cancer (NCT 05375604).

Compared with the single-dose concept of episomal deliveries, cytosolic IVT tRNA deliveries require re-administration. This should not be seen as a disadvantage as it may enable therapeutic termination in the case of any unforeseen side effects. The small size of tRNAs is a significant advantage compared with the bulky mRNA cargos used in whole-gene replacement therapies. Improved systems with higher endosomal escape and cytosolic delivery efficiency[201], and designed to carry higher tRNA payloads, would enhance the concentration and, consequently, the efficacy of the tRNA therapeutics. The persistence of the therapeutic effect by cytosolic administrations (Fig. 4a) depends on the operational stability of the individual tRNA (that is, the tissue-specific or cell-specific stability of the tRNA) and on the stability of the translated therapeutic protein, and cumulatively they determine the frequency of re-administration. Because of their highly folded structure (Box 1), tRNAs are among the most stable cellular RNAs with a half-life of approximately 100 h (refs. 202,203), which exceeds by several times the doubling time of rapidly dividing mitotic cells. In both mouse liver or human cells, when administered in an LNP formulation directly into the cytosol, the levels of sup-tRNA were unchanged over 3–5 days[35], suggesting that depending on the stability of the target protein, the frequency of re-administration could range from weekly to even monthly in certain cases.

Understanding the biodistribution of tRNA therapeutics is essential to ensure their effective delivery to target tissues or organs. The systemic circulation and various physiological barriers within the body can influence the distribution and accumulation of tRNA molecules, potentially impacting their therapeutic efficacy. The tropism of LNPs can change with the biomolecules they interact with in the tissues[204]. In AAV-based therapies, transduction efficiency is a major limitation. High doses of AAV, frequently used in preclinical stages to overcome limited biodistribution, can lead to increased liver toxicity[115]. Local administrations seem more feasible in achieving desired therapeutic readthrough levels: intrahippocampal injection of sup-tRNA^{Tyr}–rAAV9 resulted in 10% readthrough efficiency compared with 1.3% by systemic administration[33]. However, two important lessons from the current clinical trials with other RNA cargos have emerged that should guide the development of AAV capsids for tRNA payloads: (1) intravenous and intrathecal AAV administrations are safer than other administration modalities and (2) the high-dose treatment (for example, 2×10^{14} vg/kg) ends up mostly in the liver[65]. A feasible avenue for the development of AAV vehicles would be to use engineered capsids and tissue-specific tRNA promoters to escape liver accumulation[122]. Although much remains to be discovered about tissue-specific tRNA promoters, the flanking genomic context[8] of tRNA isodecoders with strong tissue-specific expression bias[45,183], combined with mutations in the internal tRNA promoters to boost expression[68], should be investigated. In parallel, engineered AAV capsids with enhanced tropism have emerged;[65] the higher tissue-specific transduction efficiency should be tested for tRNA administrations.

Achieving precise control over dosing and biodistribution of tRNA therapeutics requires a comprehensive understanding of their pharmacokinetics, as well as improvement of delivery systems with tissue-specific tropism. Addressing these challenges will be crucial for advancing the clinical translation of tRNA-based treatments.

Preclinical validation

Preclinical validation is a crucial step in evaluating the efficacy and safety parameters of a potential therapeutic. For diseases with large patient groups, the FDA has recommended various cell and animal models for preclinical studies. However, for rare disorders, there are no such extensive regulations for preclinical evaluations in place. The disease indications suitable for treatment with tRNA therapeutics are categorized as rare to ultra-rare, with some mutations unique to a single individual. Currently, there are no appropriate animal models for each individual mutation and their creation is not feasible. Thus, a key advance in the development of tRNA therapeutics would be the identification of systems that are maximally predictive of efficacy in humans.

Cell culture models with reporter constructs (for example, luciferase and fluorescent proteins) are instrumental in initial screening procedures; however, the efficacy of selected lead tRNA designs may differ under physiological settings. NMD represents a substantial obstacle to suppression therapies[205]; therefore, it is imperative to

assess the effectiveness of sup-tRNA on native NMD-sensitive transcripts. Ideally, patient-derived primary cells should be used to assess tRNA efficacy and molecular safety issues such as readthrough at natural stop codons by ribosome profiling or mass spectrometry, as well as activation of the integrated stress response and dysregulation of the tRNA households by quantitative deep sequencing-based approaches (for example, mim-tRNAseq[206], QuantM-seq[207], YAMAT-seq[208], Hydro-tRNAseq[209], DM-tRNA-seq[210], PANDORA-seq[211], four-leaf clover qRT-PCR[212], LOTTE-seq[213], OTTER-seq[214], i-tRAP[215] and direct tRNA sequencing with nanopore[216,217]). A limited availability of primary material with individual mutation signatures might be an issue. Pluripotent stem cells individualized by introducing the mutation (for example, by CRISPR-Cas technologies) and differentiated to the major cell type targeted for administration could be a suitable alternative[218,219].

A proper readout, which captures specific disease protein features, is also essential in the validation process. Ideally, several molecular parameters should be assessed: (1) full-length protein production, (2) correct protein localization and/or function and (3) restoration of tissue function[15]. Measurements of protein levels using conventional approaches (for example, western blot) are the most convenient. The physiological measurements are more challenging and might be available for a few intensively studied diseases, such as cystic fibrosis (for example, electrical current measurements in Fisher rat thyroid cells or patient-derived nasal or bronchial airway cells, or swelling assays in patient-derived organoids[220–222]), and Duchene muscular dystrophy (muscle cell contractility of patient-derived induced pluripotent stem cells differentiated into muscle cells[223,224]). Parallel development of functional assays whose outcome correlates with clinical manifestation is pivotal.

The delivery systems, which are developed for use in vivo, do not efficiently transduce in cell culture or primary cell material, probably because of gene expression changes that occur when cells are isolated and removed from their natural tissue environment that, in turn, changes the uptake mechanisms and transduction efficiency[117,127]. Commonly used transfection reagents (for example, lipofectamine[35]) or transduction approaches (for example, lentivirus[33]) are suitable for cell models.

The ex vivo analysis needs to be complemented with animal studies which provide additional systemic validation in terms of toxicity, biodistribution and immunogenicity. Given the lack of appropriate animal models for many rare diseases, generic testing on wild-type animals could be an option. The rAAVs and LNPs, which are developed for therapeutic use in humans, show poor performance as delivery vehicles in rodents that could potentially limit the feasibility and predictability of such animal studies. Intravenous administrations of sup-tRNA^{Tyr} delivered in rAAV9 — the most used in clinical studies for administration in the CNS — reached poor suppression in mouse brain[33]. By contrast, the same construct delivered in AAV-PHPeB, a capsid engineered to cross the BBB of some mouse strains[105], enhanced the suppression efficacy by 100-fold[33]. The effects of different organism-specific delivery systems may not be fully comparable and, thus, compromise the predictability of such studies. The growing evidence that animal studies are not always predictive of human outcome has facilitated the new FDA Modernization Act 2.0 that does not require animal models and enables the clearing of drugs for clinical trials using alternative approaches, such as organ on chip or human organoids[225]. It is expected that this would promote drug development, including that of tRNA-based therapeutics, for rare and ultra-rare diseases.

Conceptualization of clinical studies

At present, all tRNA therapeutic platforms are still in the discovery phase or preclinical development (Table 1). Conducting clinical trials using tRNA therapeutics necessitates careful considerations to ensure their safety, efficacy and regulatory compliance. First and foremost, thorough preclinical studies are essential to establish the pharmacological and toxicological profiles of tRNA-based treatments, such as biodistribution, metabolism and potential adverse effects. A challenge facing clinical trials for rare diseases is the small patient population with the same disease indication, which is even smaller when considering the specific mutation signature[226,227]. Given this heterogeneity, the diseases to be potentially suitable to treatment with tRNA-based therapeutics demand a unique approach to clinical trials. In this regard, basket trials, already applied for precision treatment in cancer, which refer to clinical trials in which a targeted therapy is evaluated on multiple diseases that have common molecular alternations[228,229], might be applicable to tRNA therapies. In this context, disorders of the CNS that result in common symptoms such as encephalopathies, such as Dravet Syndrome, Rett Syndrome, CDKL5 deficiency and Angelman syndrome, might lend themselves to basket trials using a common formulation (that is, a single sup-tRNA targeting a common nonsense mutation). In this manner, much larger groups of patients could be reached. Determining the control group can be difficult because of the heterogeneity of the symptoms of the multiple diseases being studied. Thus, it might be more appropriate to pursue a single-arm basket trial with no placebo group[227,229].

Clinical trials are of pivotal importance in determining dosing regimens and long-term safety and efficacy through monitoring over long periods of time (>15 years) and ideally establishing a lifelong monitoring. Furthermore, the potential immunogenicity of exogenous tRNA molecules and the risk of immune reactions must be carefully evaluated. Quantifiable disease biomarkers but also robust methodologies for quantifying and characterizing tRNA molecules in biological samples should be established to ensure accurate analysis and comparisons during clinical trials. Importantly, close collaboration with regulatory agencies and adherence to ethical guidelines are crucial throughout the trial process. By addressing these considerations, clinical trials involving tRNA therapeutics can provide valuable insights into their safety and efficacy, paving the way for their eventual approval and integration into clinical practice.

Outlook

tRNA therapies have the potential to treat a variety of disease conditions. Although the majority of current efforts (Table 1) remain in preclinical development, they are edging closer to the clinic. There are now several companies exploring the therapeutic potential of tRNAs[23,24]. Owing to the small number of patients for each potential indication, the progress of tRNA therapeutics will closely depend on translational developments of other nucleic acid-based gene therapies.

Although it will probably be several years before the first tRNA therapeutic is approved for clinical trial, with the ongoing breakthroughs in tRNA biology and design, nucleotide chemistry, delivery systems and bioinformatics, tRNA-based gene therapy is taking shape. The more than half a century-old concept of tRNA therapeutics may soon enter a new realm of efficacious and safe personalized nanomedicine to treat incurable monogenic diseases.

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Competing interests
Z.I. is co-inventor on several licensed and non-licensed patents related to the use of tRNAs as therapeutics and sup-tRNA design and is a member of the scientific advisory board of Tevard Biosciences. J.C. is scientific co-founder of Tevard Biosciences and a member of their scientific advisory board.

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