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Themed Issue: Non-coding RNA Therapeutics

THEMED ISSUE REVIEW

Discovery platforms for RNA therapeutics

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RNA therapeutics are emerging as a unique opportunity to drug currently "undruggable" molecules and diseases. While their advantages over conventional, small molecule drugs, their therapeutic implications and the tools for their effective *in vivo* delivery have been extensively reviewed, little attention has been so far paid to the technological platforms exploited for the discovery of RNA therapeutics. Here, we provide an overview of the existing platforms and *ex vivo* assays for RNA discovery, their advantages and disadvantages, as well as their main fields of application, with specific focus on RNA therapies that have reached either phase 3 or market approval.

LINKED ARTICLES: This article is part of a themed issue Non-coding RNA Therapeutics. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v182.2/issuetoc

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1 | INTRODUCTION

RNA therapeutics have the ambition to overcome a major limitation of conventional drugs, which is the need to have a protein target with specific clefts and pockets suitable for binding either small molecules or antibodies. For example, RNA therapeutics can target messenger RNAs (mRNAs) or noncoding RNAs via base pairing (Zhu et al., 2022), and *in vitro* transcribed mRNAs can be used for the expression of virtually any therapeutic protein.

RNA therapeutics include the following classes of molecules:

1. Messenger RNAs can be transcribed *in vitro* and delivered into the cell, often encapsulated in lipid nanoparticles, for protein replacement, supplementation or vaccination, as showcased by the COVID-19 pandemic (Damase et al., 2021; Kim, 2022; Zhu et al., 2022; Zogg et al., 2022). mRNA vaccines are also used as personalized medicines for targeting specific tumours (Kim, 2022).
2. Short interfering RNAs (siRNAs) are double-stranded RNAs, 21–25 nucleotides long (Zogg et al., 2022), which use the RNA interference (RNAi) pathway to suppress the expression of their target mRNAs (Zhu et al., 2022).
3. MicroRNAs (miRNAs) are natural, small noncoding RNA molecules that suppress the expression of a multiple mRNAs by either blocking translation or promoting their degradation. miRNA-based therapeutics include both miRNA mimics and miRNA inhibitors. Mimics are double-stranded RNA molecules that have the same sequence as the endogenous miRNA duplexes, resulting in the repression of the target mRNAs and, thereby, of the corresponding proteins, while miRNA inhibitors are designed to interfere with specific miRNAs, thereby restoring protein synthesis (Zhu et al., 2022; Zogg et al., 2022).
4. Antisense oligonucleotides (ASOs) are 15–25 nucleotides-long RNAs, DNAs or RNA–DNA heteroduplexes that can promote alternative splicing, cause nonsense-mediated mRNA decay (NMD), inhibit or activate translation or block the interaction between miRNAs and their target mRNAs (Damase et al., 2021; Zhu et al., 2022; Zogg et al., 2022). ASOs often contain chemical modifications that increase their stability, as in the case of locked

Abbreviations: ALAS1, 5-aminolevulinic acid synthase 1; APO, apolipoprotein; ASO, antisense oligonucleotides; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; COVID-19, Coronavirus disease 2019.

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nucleic acids (LNA), phosphorodiamidate morpholino oligonucleotides (PMOs) and peptide nucleic acids (PNAs).

5. Long noncoding RNAs (lncRNAs) are not translated into proteins but instead function intrinsically as RNA molecules. While their large size makes their delivery challenging and activates an immune response, they can be targeted by either transcriptional or post-transcriptional inhibition, steric hindrance of secondary structure formation or protein interactions (Arun et al., 2018). Some lncRNAs are transcribed in the antisense direction to coding genes and negatively regulate them *in cis*. These are named natural antisense transcripts (NATs) and can be targeted by specific ASOs, named "antagoNATs," which have been used successfully to express **brain-derived neurotrophic factor (BDNF)** and **SCN1A ($Na_v1.1$)** in the central nervous system of mice and primates (Hsiao et al., 2016; Modarresi et al., 2012). These promising pre-clinical results will likely pave the way to the use of lncRNA-based therapeutics in clinical trials.
6. Aptamers are short single-stranded DNA (ssDNA) or RNA molecules chosen for their selective binding to non-nucleic acid targets that include proteins, carbohydrates, small molecules and even entire cells (Diener et al., 2009; Kim, Seo, et al., 2021; Zhou & Rossi, 2017).

Considering the plethora of RNA therapeutics, how to select the best one for a given disease?

Traditionally, RNA drugs have been designed and optimized with an educated guess, based on deep biological understanding of disease mechanisms and identification of the most relevant genes, and pathways to be either inhibited or stimulated by the drug. In genetic diseases, the mutated gene, as well as its direct inhibitors/activators, are often the ones to be targeted by RNA therapies. In the case of aptamers, the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology allows for efficient and automatic selection of the best sequences to bind a defined target (Chang et al., 2023).

For complex diseases, the most relevant pathways are often identified by the so-called "omic" technologies, which include genomics, epigenomics, transcriptomics, proteomics and metabolomics. Highly relevant for RNA therapeutics, transcriptomics wishes to define the complete set of RNA transcripts that are produced by the genome in a specific cell under specific circumstances, for example, in a specific disease.

Both gene expression microarray and high-throughput RNA-sequencing are widely used to discover new drug targets. RNA-sequencing offers the advantage of identifying previously unknown RNAs. Therefore, the comparison of the RNA-sequencing-derived gene expression profiles in diseased and healthy conditions stands as a unique tool in the pursuit of RNA therapeutics (Yang et al., 2020). In addition, genome-wide association studies (GWAS) are genomic studies that involve surveying the genomes of many people, looking for genetic variants that occur more frequently in those with a specific disease or trait compared with those without the disease or trait. These studies wish to identify variants that are statistically associated with a risk or trait, and thus they inform of correlation not causation. However, in some instances, as in the case of PCSK, discussed later, they can identify variants that have a functional consequence and therefore represent potential targets for drug development.

"Omic" approaches are often combined to integrate diverse datasets, thereby identifying coherently matching geno-pheno-envirotype relationships and predicting therapeutic targets. One of the largest efforts for large-scale omic analysis is The Cancer Genome Atlas (TCGA). In this project, over 20,000 cancer samples, spanning 33 cancer types, have been analysed by genomics, epigenomics, transcriptomics and proteomics, together with matched healthy samples. Numerous novel targets, including RNA therapeutics, have been discovered by this approach, as, for example, an ASO to silence the oncogenic lncRNA SAMMSON (survival associated mitochondrial melanoma specific oncogenic non-coding RNA) for the therapy of melanoma (Dewaele et al., 2022, 2023).

While "omic" technologies mainly generate descriptive data, functional screenings are specifically designed to directly identify targets that exert a functional effect. On the other hand, "omic" technologies can be used to understand how the levels of targets, previously identified by functional screening, change in the relevant tissues. In particular, proteomics appears more informative than transcriptomics, as therapeutic targets may be altered at the RNA but not at the protein level (Ragone et al., 2023). Thus, the two approaches provide complementary information and should be used synergistically to maximize the probability of identifying the most relevant targets.

In the following paragraphs, we will review the advantages and limitations of the main screening strategies that holds the highest potential for discovering relevant targets for RNA therapeutics and provide some paradigmatic examples of their successful use in the field of cardiovascular and lung disease.

Figure 1 summarizes the major trajectories followed by RNA therapies from discovery platforms to preclinical validation and, eventually, to human use.

2 | ARRAYED SCREENING

In the traditional drug discovery process, hypotheses are formulated based on existing knowledge, tested experimentally and either confirmed or disproved, according to the results. Iterative cycles are usually needed to get conclusive results. A more modern approach relies on the screening of thousands of molecules (i.e. mRNAs, siRNAs, miRNAs, lnCRNAs or ASOs) to select the ones that exert the highest effect on a given phenotype. This is commonly defined as "functional screening," and it digs into molecular mechanisms of disease in an unbiased manner. As functional screenings, by definition, select molecules that have an impact on a phenotype of interest, the probability that the identified targets eventually exert a functional effect *in vivo* is potentially higher than in "omic" discovery approaches. In addition, the possibility to screen in humanized models, as described below, increases the chance of moving the identified targets from preclinical to clinical experimentation.

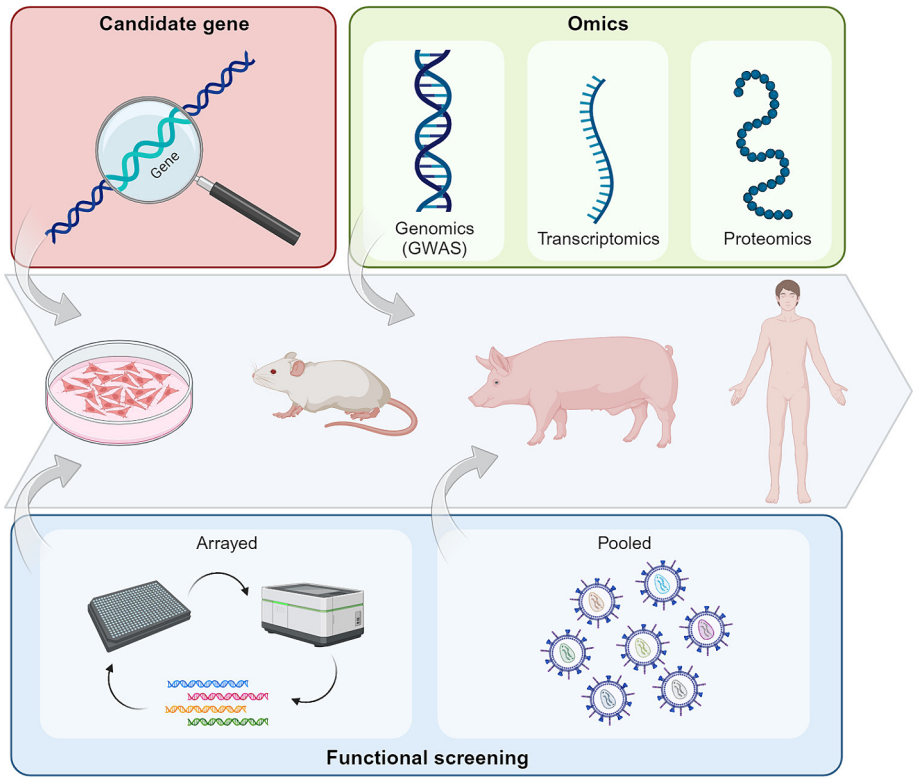

FIGURE 1 Approaches to discover novel RNA therapeutics and their trajectory towards human use. As any drug, RNA therapies must follow a standardized validation of their efficacy from cell culture systems to small and large animals and eventually to patients. While RNA therapies have been traditionally designed based on candidate genes, more recent platforms include both "omic" technologies and functional (arrayed and pooled) screenings.

Functional screenings have been instrumental in some of the greatest discoveries of the last century, including human oncogenes, viral receptors, small molecules with anti-enzymatic activity, and induced Pluripotent Stem (iPS) cell reprogramming factors. One of the earliest and most elegant examples of "screening for function" dates back to 1982 and led to the identification of the first human oncogene Harvey rat sarcoma virus (*HRAS*; Pulciani et al., 1982) by the group of Mariano Barbacid at the National Health Institute (NIH), in the United States. Most recently, a milestone paper by Kazutoshi Takahashi and Shinya Yamanaka identified four factors (octamer-binding transcription factor (Oct) 3/4, SRY-related HMG-box gene 2 [Sox2], cellular myelocytomatosis oncogene [c-Myc] and Kruppel-like factor [4Klf4]) able to reprogram any somatic cell into a pluripotent stem cell. These factors were identified by a simple functional screening, in which all the possible permutations of 24 factors were overexpressed in fibroblasts, screening for their ability to activate the promoter of the F-box protein 15 (*FBX15*) gene, a known marker of pluripotent stem cells (in the assay, the Fbx15 promoter drove the expression of neomycin resistance and cells were selected in the presence of high dose of the neomycin analogue G418).

An indispensable condition to perform functional screening is the possibility to match the desired phenotype with the molecule responsible for it. Arrayed libraries, where each molecule has specific and unique coordinates, allow to maintain precise correlations between treatments and effects. This screening technology was initially implemented by big pharma, due to their wish to screen as many drugs as possible in the shortest amount of time. Thus, much effort has put on increasing screening capacity ("throughput first"), mainly through automation and miniaturization. The first endpoints for high throughput screening (HTS) were biochemical assays, to be measured into multimode plate readers that can scan thousands of wells within a few minutes. In this case, results are monodimensional, as a single parameter (i.e. fluorescence, absorbance and chemiluminescence) is measured and values are averaged per well. Over the past years, the introduction of the technology into academic laboratories has led to a shift from high throughput to high content screening, where cells are visualized by automated imaging systems and classified according to multiple markers. Image-based, phenotypic high-throughput screening (HTS) represents the latest evolution of the "functional" approach. This shift has been made possible by the progresses of automated high content microscopy that allows to scan individual wells by acquiring fluorescent images in multiple wavelengths. This leads to the generation of multiparametric datasets that reflect the cellular phenotype in response to a given treatment. Having the possibility to screen for images has opened the field to single cell analysis, in which each cell can be defined as a unique object, with specific coordinates, classified according to specific markers, followed in time and space for kinetic assays and finally ranked according to the phenotype of interest. The possibility to run single cell analysis in functional screening increases the complexity of the assays that can be implemented, including the analysis of multiple cell types in co-culture and of three-dimensional (3D) organoids that better mimic human tissues during diseases, as discussed later (Figure 2).

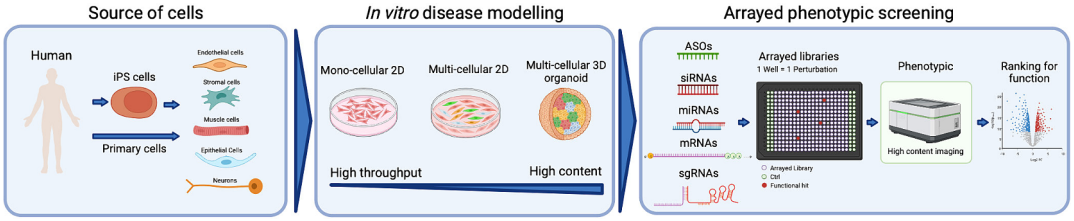

FIGURE 2 Arrayed screening for the discovery of RNA therapies. Discovery platforms for personalized RNA therapies leverage either patient-specific iPS-derived or primary cells, which can be cultured into monocellular/multicellular 2D/3D cultures to create disease-in-a-dish-models. These models can be systematically interrogated with RNA-based arrayed libraries, in which every well corresponds to a specific perturbation (ASO/siRNA/miRNA/mRNA/gRNA for CRISPR knockout). Phenotypic alterations are quantified at both cellular and subcellular levels through automated high-content imaging, followed by automated image analysis. Phenotypes are eventually classified based on the results of image analysis and ranked for potency according to specific biological questions.

3 | SCREENING USING POOLED LIBRARIES

Different from arrayed screens, pooled screens are based on pooled libraries, composed of multiple perturbations, which are administered together to target cells. The screen readout usually detects the effect of each perturbation as an enrichment against a selective pressure and can identify the perturbation itself.

Pooled screens traditionally leverage lentiviral vector libraries of either short hairpin RNA (shRNA) or single guide RNA (gRNA) molecules targeting multiple genes. In this way, each shRNA/gRNA sequence acts as a permanent, genetic barcode in each individual cell. Transduction at low multiplicity of infection (MOI) ensures that target cells do not receive more than one shRNA/gRNA simultaneously. Barcode abundance upon application of the selective pressure allows to identify the most relevant genes regulating the phenotype of interest.

An additional advantage of pooled screens is that they can be conducted both on cell lines, primary cells *ex vivo* and *in vivo*. *Ex vivo* screens involve the harvesting of primary cells, which can be cultured either in 2D or as 3D organoids (Parnas et al., 2015), while in vivo screens entail either vector injection into animals (Jin et al., 2020) or transduction of the cells *in vitro/ex vivo* prior to their implantation (Dubrot et al., 2022; STING Inhibits the Reactivation of Dormant Lung Cancer Metastasis, 2023).

Pooled screens are generally cheaper than arrayed screens, as they do not require high-throughput robotics and are less labour intensive. This is particularly relevant for genome-wide screens, which are significantly cheaper in a pooled than in an arrayed format.

Genome-wide screens are appealing for drug discovery, as they are completely unbiased and do not rely on any a priori knowledge. However, they require many cells to ensure adequate coverage, which makes them unfeasible for rare cell types. Targeted screens, focused on a smaller set of tens to thousands of genes, often serve as a practical alternative to genome-wide screens, albeit with the limitation that their scope is confined to the selected genes, potentially overlooking unexpected biological mechanisms. Combining both strategies is possible by conducting a genome-wide screen with modest coverage (encompassing all genes but with comparatively lower sensitivity for each individual gene), followed by a targeted screen with high coverage (focusing on specific candidate genes or gene sets, thereby achieving higher sensitivity for the detection of these genes).

Multiple readouts can be used to select enriched and depleted cells after the application of the selective pressure. The most common readout is cell viability/proliferation, where the impact of selective pressure is tracked over time. Additional readouts include protein expression by flow cytometry (Tsuchiya et al., 2023), gene reporter activity (Feldman et al., 2019) or physical separation based on specific cell activities, such as cell migration (Prolo et al., 2019).

Pooled screens were initially based on shRNA libraries, which inhibit mRNA post-transcriptionally via endogenous interference through the RNA-induced silencing complex (RISC). More recently, the technology has evolved with the development of CRISPR-Cas9 screens, where sgRNAs are introduced into Cas9-expressing cells. This results in DNA double strand break, followed by repair through error-prone nonhomologous end joining (NHEJ).

Introduced insertions and deletions (indels) can result in either frameshift mutations or the generation of a premature stop codon.

Despite being introduced many years ago, RNAi still offers advantages for specific applications (Schuster et al., 2019). First, shRNA transduction is simpler, as it does not require the activity of endonucleases, which is sometimes inefficient in primary cells. Second, siRNA-based knockdown is not biased by either cell ploidy or chromatin conformation, as the RNAi machinery acts in the cytoplasm. On the other hand, the efficiency of knockdown is difficult to standardize and robust screens require many shRNAs per gene, also considering their propensity to generate off-target effect. In addition, shRNA overexpression in target cells often saturate the endogenous RNAi machinery, resulting in dysregulated processing of endogenous miRNAs, with potential, unexpected consequences on the phenotype of interest.

Clustered regularly interspaced short palindromic repeats (CRISPR) screens exhibit greater sensitivity and specificity in detecting essential genes, especially those with moderate expression levels. Different from shRNA-based screens that only target protein-coding genes, CRISPR screens can also be conducted for noncoding DNA and gene regulatory regions, as Cas9 acts in the nucleus.

Over recent years, several studies capitalized on pooled gRNA screening to discover novel targets across various biological functions.

Because cell viability and proliferation are the most straightforward readouts, cancer has emerged as the most fitting field for the application of this methodology. Indeed, CRISPR knockout screening has unveiled novel targets for cancer therapy related to immune evasion. (Chen, Li, et al., 2022; Dubrot et al., 2022; Frangieh et al., 2021; Griffin et al., 2021; Wang et al., 2020), drug resistance (Gao et al., 2021; Ramaker et al., 2021) and oncogenic pathways (Dai et al., 2021; Gao et al., 2023; Li, Wang, et al., 2023; Sun et al., 2023; Wei et al., 2022).

In addition to gene knockout, nuclease-deficient or dead Cas9 (dCas9) can be fused to either repressor or activation domains, thereby modulating transcription at gene promoters or other regulatory elements near the transcriptional start site (TSS). This strategy is commonly named CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa) (Alerasool et al., 2020; Kanafi & Tavallaei, 2022).

By regulating endogenous transcription, CRISPRi enables the functional assessment of regulatory elements (Ahmed et al., 2021; Leng et al., 2022) and nuclear-retained noncoding RNAs (Cai et al., 2020; Liu et al., 2020). These elements are often challenging to target with shRNAs and may not always be effectively perturbed by CRISPRko, which typically requires substantial modifications to disrupt their function.

Efficient CRISPRa typically targets sequences located upstream TSSs, distinct from those required for CRISPRi, which are located downstream TSSs.

Despite great enthusiasm elicited by CRISPRi and CRISPRa, CRISPRko-based pooled screens remain the most commonly utilized and effective method to discover new therapeutic targets (Bock et al., 2022).

4 | MODELLING HUMAN DISEASE IN CELL CULTURE FOR RNA DRUG SCREENING

In the following paragraphs, we will discuss the main cellular assays able to reproduce human disease, so far considered for both arrayed and pooled screening, with some paradigmatic examples and a few successful drug discovery stories, which hold the promise to lead to future RNA therapies, not only for genetic conditions but also for common, complex diseases. These include primary cells, iPS cells and 3D organoids.

Both healthy and diseased primary cells have been largely used in discovery platforms for RNA-therapeutics in many fields, including miRNAs for cardiac regeneration (Eulalio et al., 2012), cardiac hypertrophy (Jentzsch et al., 2012) and smooth muscle cell proliferation (Fiedler et al., 2014), siRNAs for host restriction factors in HIV-1 infection (Ali et al., 2019) and adeno-associated virus (AAV) transduction (Mano et al., 2015).

As the final goal of modern medicine, including RNA-based therapeutics, is to be molecularly tailored and patient-specific, more sophisticated "disease-in-a-dish-models" based on induced pluripotent stem (iPS) cells have been developed and formulated as 2D co-culture systems, multicellular 3D organoids, engineered tissues and microfabricated devices to mimic tissue dynamics. iPS cells are an inextinguishable source of patient-derived-cells, which can self-assemble in organ-surrogate multicellular 3D structures (Figure 2). They can be cryopreserved and differentiated into virtually any cell type, holding a unique relevance for rare genetic diseases, in which biological samples are scarcely available. In addition, iPS cells can be genetically engineered *in vitro* to generate knock-in/-out lines, as well as endogenous reporter lines for live kinetic assays. For all these reasons, they are more and more used in functional discovery platforms, including arrayed and pooled screenings.

To date, scientific evidence supports iPS cell differentiation into brain cells (neurons, motoneurons, astrocytes and microglia) (Karumbayaram et al., 2009; Penney et al., 2020), various retinal cells including retinal epithelium (Meyer et al., 2009), cardiac myocytes (Burridge et al., 2012; Narazaki et al., 2008), endothelial cells (Narazaki et al., 2008), alveolar cells (Jacob et al., 2017), hepatocytes (Song et al., 2009), pancreatic β cells (Tateishi et al., 2008) and haematopoietic cells including dendritic cells and macrophages (Choi et al., 2009; Senju et al., 2009). All these cell types have been considered for drug discovery for a variety of genetically driven human diseases, but also to screen for miRNAs promoting cardiac regeneration (Diez-Cuñado et al., 2018; Renikunta et al., 2023).

Pooled libraries of gRNAs for CRISPRi and CRISPRa have been used for genome-wide survival screens in iPS cell-derived human neurons to identify neuronal-specific essential genes (Tian et al., 2019, 2021), cytokine-induced inflammatory astrocyte reactivity genes (Leng et al., 2022), genes governing microglia survival, activation, and phagocytosis (Dräger et al., 2022) and functional cardiac lncRNAs (Liu et al., 2017). Additionally, CRISPRko screenings using iPS cells have identified modifiers and therapeutic targets for frontotemporal dementia (Guo et al., 2023), doxorubicin-induced cardiotoxicity (Sapp et al., 2021), telomere stability in ageing (Mannherz & Agarwal, 2023) and Zika virus infection (Li, Muffat, et al., 2019).

Both primary and iPS cells are often cultured as organoids, reproducing human brain, retina, heart, lung, digestive system, liver and kidney (Zhao et al., 2022). As these structures can be generated from both healthy and diseased tissues, they represent unique discovery tools for high-content screening (Hofbauer et al., 2021; Lancaster & Knoblich, 2014; Sharick et al., 2019).

Patient-derived tumour organoids (PDOs) have been largely used as in anticancer drug discovery, as they recapitulate the genetic heterogeneity and the cellular composition of the original tumour, particularly in the case of breast (Sachs et al., 2018; Tebon et al., 2023) and liver cancer (Broutier et al., 2017; Li, Knutsdottir, et al., 2019). Human colorectal cancer organoids have been successfully used in whole genome pooled gRNA screenings for the identification of new tumour suppressors (Michels et al., 2020), genes involved in **TGF-β** resistance (Ringel et al., 2020) and novel druggable targets (Gao et al., 2021). While several RNA-based therapies have been proposed for cancer treatment over the past years, including siRNAs (Golan et al., 2015; Titze-de-Almeida et al., 2017; Zorde Khvalevsky et al., 2013), miRNAs (Zhang et al., 2019) and small activating RNA (saRNA) to reactivate tumour suppressor genes (Sarker et al., 2020), large screening

campaigns for the identification of RNA-based anticancer medications are still missing. Patient-derived organoids (PDO)-based discovery platforms will be particularly useful to develop new patient-tailored RNA-based therapies in cancer.

PDOs are generated from whole tumour biopsies. Thus, they comprise the original tumour microenvironment (TME), including innate and adaptive immune cells (Yuki et al., 2020), which is effectively targeted by both cellular (Rosenberg & Restifo, 2015; Tran et al., 2016) and pharmacological (Larkin et al., 2019; Socinski et al., 2018) immunotherapies (Ou et al., 2023; Shelkey et al., 2022). This offers the possibility to leverage this platform to optimize mRNA-based anticancer vaccines that stand as the next frontier in anticancer therapy (Duan et al., 2022).

Three-dimensional organoids are not limited to cancer and can be generated from iPS cells. In particular, pooled CRISPR functional screenings were successfully performed in brain, kidney and intestinal organoids (Esk et al., 2020; Fleck et al., 2023; Hansen et al., 2023; Li, Fleck, et al., 2023; Ungricht et al., 2022). At present, arrayed screenings in noncancer organoids were performed in the brain (Park et al., 2021), kidney (Czerniecki et al., 2018) and cardiac organoids (Mills et al., 2019) for small molecule drugs, but novel applications, extended to RNA therapies, are expected in the years to come.

5 | RNA THERAPEUTICS AND THEIR ROAD TO THE CLINICS

The first RNA therapeutic approved by the US Food and Drug Administration (FDA) in 2004 for the treatment of age-related macular degeneration was **pegaptanib**, a pegylated RNA-modified aptamer targeting the 165 amino acid isoform of **VEGF-A** (Gragoudas et al., 2004).

After this milestone, other RNA therapeutics have received regulatory approval and are still in use, the majority of which are antisense oligonucleotides (ASO) that either modulate splicing or interfere with gene expression. In the first category, **eteplirsen**, golodirsen, **viltolarsen** and **casimersen** (Charleston et al., 2018) are indicated for the treatment of Duchenne muscular dystrophy, where they modify the splicing of the dystrophin gene, leading to the production of a functional protein, while **nusinersen** restores the expression of survival of motor neuron 2 (SMN2) for the therapy of spinal muscular atrophy (Finkel et al., 2017). In the second category, inotersen has been developed for the treatment of polyneuropathy associated with hereditary transthyretin (TTR)-mediated amyloidosis, in which the mutated TTR gene produces an abnormal protein that aggregates and accumulates in tissues. Inotersen hybridizes with the 3′ UTR of the TTR transcript, preventing its translation and the accumulation of TTR aggregates (Benson et al., 2018). Similarly, **volanesorsen** degrades apolipoprotein C-III (ApoC-III) mRNA for the therapy of familial chylomicronaemia (Witztum et al., 2019).

Globally listed RNA drugs also include four siRNAs targeting TTR for the treatment of familial amyloid polyneuropathy (patisiran) (Adams et al., 2018), 5-aminolevulinic acid synthase 1 (ALAS1) for acute hepatic porphyria (givosiran) (Balwani et al., 2020), hydroxyacid oxidase 1 (HAO1) for primary hyperoxaluria type 1 (lumasiran) (Garrelfs et al., 2021) and **proprotein convertase subtilisin/kexin type 9 (PCSK9)** for hypercholesterolaemia (Inclisiran) (Ray et al., 2020), as well as two mRNAs encoding for the Spike protein of SARS-CoV-2, used as vaccines (tozinameran and elasomeran) (Munro et al., 2022). In addition to these global drugs, a personalized ASO-based drug, developed for a single child affected by Batten disease (milasen), was approved by the FDA in 2018, targeting a specific mutation in the ceroid lipofuscinosis, neuronal 7 (*CLN7*) gene (Kim, 2022).

Additional RNA therapeutics are in phase 3 clinical trials and are expected to reach the market soon. These include three siRNAs targeting the same TTR and ApoC-III mRNAs for the treatment of familial amyloid polyneuropathy (vutisiran) (Adams et al., 2023) and hypertriglyceridaemia (ARO-APOC3) (Hegele, 2022), respectively, but also siRNAs specific for additional targets, such as **TRPV1** for dry eye disease (tivanisiran) (Moreno-Montanes et al., 2018), antithrombin for haemophilia A and B (fitusiran) (Young et al., 2023) and p53 for the prevention of acute kidney injury after surgery (teprasiran) (Thielmann et al., 2021). Seven additional ASO drugs are in phase 3 clinical trials targeting TTR and ApoC-III for amyloidosis (eplontersen) (Coelho et al., 2023) and hypertriglyceridaemia (olezarsen) (Tardif et al., 2022) but also apo(a) to reduce cardiovascular risk (Pelacarsen) (Tsimikas et al., 2021), superoxide dismutase 1 (SOD1) and fused in sarcoma (FUS) to treat amyotrophic lateral sclerosis (tofersen and **ulefnersein [ION363]**) (Korobeynikov et al., 2022; Miller et al., 2022), **huntingtin (HTT)** to treat **Huntington disease** (tominersen) (Tabrizi et al., 2022) and prekallikrein to treat hereditary angioedema (donidalorsen) (Fijen et al., 2022). Finally, new RNA-based vaccines are in phase 3 for SARS-Cov-2 (LUNAR-COV19 and ARCoV) (Chen, Hu, et al., 2022; de Alwis et al., 2021), melanoma (Schuler-Thurner et al., 2015) and advanced renal cell carcinoma (Amin et al., 2015). mRNA replacement therapies have reached the clinical stage for cystic fibrosis, propionic acidaemia and ornithine transcarbamylase deficiency, but they are all in phase 1/2 clinical trials.

If we look at the process that drove to the discovery of the relevant target in these clinically advanced therapeutic RNAs, they have been mainly identified by a candidate gene approach, which is the mutated gene in the case of genetic diseases (spinal muscular atrophy, Duchenne muscular dystrophy, TTR amyloidosis, amyotrophic lateral sclerosis, Huntington's disease, cystic fibrosis, propionic acidaemia and ornithine transcarbamylase deficiency), a key viral gene in the case of antiviral vaccines, a disease-related gene as in the case of ApoC-III and apo(a) for severe hypertriglyceridaemia, 5-aminolevulinic acid synthase 1 for acute hepatic porphyria, hydroxyacid oxidase 1 (HAO1) for primary hyperoxaluria type 1, TRPV1 for dry eye disease, antithrombin for haemophilias, p53 for acute kidney injury and prekallikrein for hereditary angioedema.

PCSK9 is the only example of molecule that has been confirmed as a relevant target by a genome-wide association studies. The whole story started with the evidence that gain-of-function mutations in its sequence were responsible for familial hypercholesterolaemia (Leren, 2004). On the other hand, African individuals who were

double-recessive for non-functional PCSK9 had extraordinarily low levels of LDL cholesterol, and thereby, greatly reduced cardiovascular risk compared with the general population (Cohen et al., 2005). These data have been confirmed by multiple genome-wide association studies (Myocardial Infarction et al., 2016; Saavedra et al., 2014) and led to the idea of inhibiting its activity to control cholesterol levels. PCSK9 is a typical non-druggable target, as it does not contain any small molecule binding site that controls its function. Thus, antibodies and siRNAs stand as the most effective tools to inhibit PCSK9 and improve cardiovascular outcome. Given the size of the target population, these drugs are likely going to open a new era of lipid-lowering therapy (Hajar, 2019).

Table 1 lists the main RNA therapeutics so far approved or in clinical trial. This analysis clearly shows that unfortunately any RNA therapeutic, identified by the novel and functional platforms discussed above, arrayed and pooled libraries, has reached or is close to reach the clinical stage. At the same time, the COVID-19 pandemic has impressively accelerated the pathway to the clinics for numerous RNA therapies, and we can expect that many novel RNA therapeutics will be tested in clinical trials in the upcoming years.

6 | CHALLENGES AND FUTURE OPPORTUNITIES

Because the beauty of RNA therapies is that they can be easily and rationally designed, provided that the target is known, the traditional path in their development stems from the definition of a candidate target, which is either a disease-causing gene, a viral gene or a gene identified as a putative target by "omic" technologies. As a consequence, unbiased screening using RNA-based drugs has been left behind and RNA therapies identified by screening approaches have not yet entered the clinical arena.

Several arrayed screenings have identified candidate miRNA mimics and inhibitors, as well as siRNAs, that could represent powerful therapeutic tools for a whole host of human disorders. However, several challenges have emerged and hampered the transition of miRNA-based therapeutics into clinical use.

First, stability and *in vivo* uptake are often limited using the current delivery methods that mainly include dermal application, intravenous injection and inhalation (Chow et al., 2020; Diener et al., 2022). A local delivery could be considered in situations where surgical access is feasible and entails incorporating miRNA molecules into a biodegradable three-dimensional matrix, to be implanted into the tissue. This method effectively circumvents challenges associated with poor miRNA stability over time and potential side effects stemming from systemic administration, thereby allowing continuous and tissue-specific delivery of miRNA-based therapies (Zhang et al., 2016).

To overcome the challenge of poor cellular membrane permeability to nucleic acids, including miRNAs, various approaches have been explored, including nanosized carriers (liposomes), bacterial minicells and extracellular vesicles (EVs) (Diener et al., 2022). Chemical modifications also enhance both stability and cellular uptake of miRNA-based therapeutics. These modifications usually focus on the phosphate backbone, the nucleobases, or the ribose sugar and aim at reducing the negative charge of the miRNAs, to ultimately enhance their adhesion to the cell surface and facilitate their uptake (Diener et al., 2022). Common modifications include the use of LNA bases (Grunweller & Hartmann, 2007) and biomolecule conjugates, such as N-acetylgalactosamine (GalNAc; Huang, 2017). Nonpathogenic viral vectors, as adeno-associated virus vectors, represent an additional tool for the intracellular delivery of miRNA-based therapeutics, although they are fraught by two major drawbacks, immunogenicity and chronic expression of the therapeutic miRNA.

Second, cell- and tissue-specific delivery is difficult, if not impossible, to achieve *in vivo*, primarily because of the accumulation of nanoparticles in the liver upon intravenous administration. Consequently, liver-targeted LNPs, which capitalize on endogenous lipid transport proteins for hepatocyte targeting, dominate the clinical pipeline of RNA therapeutics (Dilliard & Siegwart, 2023). Extrahepatic delivery, that is, to the lungs and lymphoid tissues, necessitates detargeting the liver by disrupting ApoE binding (Dilliard et al., 2021) or by altering the lipid charge in the LNP (Kim, Jeong, et al., 2021; Kranz et al., 2016). Alternatively, RNAs can be transported to target cells by antibody fragments, peptides, aptamers or other ligands that specifically bind receptors on surface of the target cell. In this approach, a ligand that binds to a specific biomolecule is incorporated into the delivery system, as in the case of N-acetylgalactosamine (GalNAc)–siRNA conjugates givosiran, lumasiran and inclisiran.

Third, off-target effects remain a major concern for both siRNAs and gRNAs, despite progresses in designing sequences with strengthened on-target specificity. As a general strategy to reduce off-target effects is to combine multiple siRNAs/gRNAs targeting the same sequence, so that each molecule can be delivered at a lower dose (Hannus et al., 2014). Chemical modifications that enhance RNA stability, reduce immunogenicity, and improve cellular uptake may also allow a further reduction of the dose, thereby also being effective on off-targets. In the case of CRISPR-Cas9, nuclease engineering can produce Cas9 variants with reduced off-target activity (Casini et al., 2018). Finally, computational tools to predict potential off-target sites are exponentially improving their capacity to ensure guide specificity (Wessels et al., 2023).

Additional limitations stem from the assay used for the screening, which is often too simple, not able to reproduce the complexity of the human condition. The discrepancy between *in vitro* and *in vivo* stands as an important factor accounting for the high failure rate in drug development. Thus, screening platforms are progressively shifting from high throughout to high content, becoming able to image and analyse multiple features in multicellular, 3D cell culture systems, which better reflect the *in vivo* behaviour of most cell types, as discussed above.

An additional wave of novelty is expected to come from artificial intelligence (AI) discovery platforms for RNA therapies. Several companies are investing in AI algorithms able to predict which RNAs can be targeted by small molecules. Others are combing phenotypic,

TABLE 1 RNA therapies that are either approved by regulatory agencies or in clinical trial.

RNA therapeutics that have received regulatory approval and are still in use				
RNA molecule	**Drug**	**Target gene**	**Disease**	**Ref.**
ASO	**Eteplirsen**	Dystrophin	Duchenne muscular dystrophy	(Charleston et al., 2018)
	Golodirsen	Dystrophin	Duchenne muscular dystrophy	(Charleston et al., 2018)
	Viltolarsen	Dystrophin	Duchenne muscular dystrophy	(Charleston et al., 2018)
	Casimersen	Dystrophin	Duchenne muscular dystrophy	(Charleston et al., 2018)
	Nusinersen	SMN2	Spinal muscular atrophy	(Finkel et al., 2017)
	Inotersen	**Transthyretin**	Transthyretin-mediated amyloidosis	(Benson et al., 2018)
	Volanesorsen	ApoC-III	Familial chylomicronaemia	(Witztum et al., 2019)
	Milasen	CLN7	Batten disease	(Kim et al., 2019)
siRNA	Patisiran	Transthyretin	Familial amyloid polyneuropathy	(Adams et al., 2018)
	Givosiran	5-aminolevulinic acid synthase 1	Acute hepatic porphyria	(Balwani et al., 2020)
	Lumasiran	Hydroxyacid oxidase 1	Hyperoxaluria type 1	(Garrelfs et al., 2021)
	Inclisiran	**PCSK9**	Hypercholesterolaemia	(Ray et al., 2020)
mRNA	Tozinameran	**Spike (SARS-CoV-2)**	COVID-19	(Munro et al., 2022)
	Elasomeran	Spike (SARS-CoV-2)	COVID-19	(Munro et al., 2022)
RNA therapeutics that are in clinical trial				
RNA molecule	**Drug**	**Target gene**	**Disease**	**NCT/Eudra CTN**
ASO	Eplontersen	Transthyretin	Amyloidosis	NCT04136171
	Olezarsen	ApoC-III	Hypertriglyceridaemia	NCT05355402
	Pelacarsen	Apo(a)	Cardiovascular risk	NCT04023552
	Tofersen	Superoxide dismutase 1	Amyotrophic lateral sclerosis	NCT02623699
	ION363	Superoxide dismutase 1	Amyotrophic lateral sclerosis	NCT04768972
	Tominersen	**Huntingtin**	Huntington's disease	NCT03342053
	Donidalorsen	**Prekallikrein**	Hereditary angioedema	NCT05139810
	Temavirsen	miR-122	Chronic hepatitis C	2015-001535-21
	AZD4046	miR-103/107	Steatohepatitis	NCT02826525
siRNA	Vutisiran	Transthyretin	Familial amyloid polyneuropathy	NCT03759379
	ARO-APOC3	ApoC-III	Hypertriglyceridaemia	NCT04720534
	Tivanisiran	Transient receptor potential vanilloid 1	Dry eye disease	NCT04819269
	Fitusiran	**Antithrombin**	Haemophilia A and B	NCT03549871
	Teprasiran	p53	Acute kidney injury after surgery	NCT03510897
miRNA	MRX34	miR-34a	Selected cancers	NCT01829971
	Mesomir 1	miR-16	Pleural mesothelioma and non-small cell lung cancer	NCT02369198
	miHTT	Huntingtin	Huntington's disease	NCT04120493
mRNA	LUNAR-COV19	Spike (SARS-CoV-2)	COVID-19	NCT05012943
	ARCoV	Spike (SARS-CoV-2)	COVID-19	NCT04847102
	mRNA-4,157	Tumour RNA	Melanoma	NCT03897881
	MRT5005	**CFTR**	Cystic fibrosis	NCT03375047
	mRNA-3,927	**PCCA** and **PCCB** subunits of the PCC enzyme	Propionic acidaemia	NCT04159103
	ARCT-810	Ornithine transcarbamylase	Ornithine transcarbamylase deficiency	NCT05526066

arrayed screens with AI to elucidate the mechanisms of action of small molecule mRNA drugs. As incredible developments in RNA-based discovery are expected over the next 5 years, it seems to be an excellent time to combine and synergize RNA with AI, which will further accelerate the progress of RNA therapies and their entrance into the clinical arena.

6.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Christopoulos, et al., 2023; Alexander, Fabbro, et al., 2023).

AUTHOR CONTRIBUTIONS

All authors revised the literature and wrote the manuscript, with specific focus on arrayed screens (LB), pooled screens (GC) and clinical application (SZ).

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CONFLICT OF INTEREST STATEMENT

Authors do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

N/A-Review.

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