Discovery platforms for RNA therapeutics

Giulio Ciucci[1], Luca Braga[1], and Serena Zacchigna[1]

[1]ICGEB

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Abstract

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.

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Giulio Ciucci[1], Luca Braga[2]* and Serena Zacchigna[1, 3]*

[1] Cardiovascular Biology Laboratory, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy
[2] Functional Cell Biology Laboratory, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy
[3] Department of Medicine, Surgery and Health Sciences, University of Trieste, Trieste, Italy

* contributed equally

Address for correspondence

Serena Zacchigna, MD, PhD
Group Leader, Cardiovascular Biology
International Centre for Genetic Engineering and Biotechnology (ICGEB)
Padriciano, 99
34149 Trieste, Italy
Tel. +39 040 3757 354/214/357
Fax +39 040 226555
e-mail: zacchign@icgeb.org
https://www.icgeb.org/cardiovascular-biology/

Abstract

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.

Introduction

RNA therapeutics have the ambition to overcome a major limitation of conventional drugs, that 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, Zhu, Wang & Jin, 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) mRNAs 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, Sukhovershin, Boada, Taraballi, Pettigrew & Cooke, 2021; Kim, 2022; Zhu, Zhu, Wang & Jin, 2022; Zogg, Singh & Ro, 2022). mRNA vaccines are also used as personalized medicines for targeting specific tumors (Kim, 2022).
2) short interfering RNAs (siRNAs) are double-stranded RNAs, 21-25 nucleotides long (Zogg, Singh & Ro, 2022), which use the RNA interference (RNAi) pathway to suppress the expression of their target mRNAs (Zhu, Zhu, Wang & Jin, 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, Zhu, Wang & Jin, 2022; Zogg, Singh & Ro, 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, Sukhovershin, Boada, Taraballi, Pettigrew & Cooke, 2021; Zhu, Zhu, Wang & Jin, 2022; Zogg, Singh & Ro, 2022). ASOs often contain chemical modifications that increase their stability, as in the case of locked 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, Diermeier & Spector, 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 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.

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. 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 to 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 analyzed 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, a ASO to silence the oncogenic lncRNA SAMMSON for the therapy of melanoma (Dewaele et al., 2023; Dewaele et al., 2022).

Different from 'omic' technologies, that mainly generate descriptive data and only in rare, fortunate cases, identify relevant therapeutic targets, functional screenings are specifically designed to directly identify targets that exert a functional effect. 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 pre-clinical validation and, eventually, to human use.

Arrayed screening

In the traditional drug discovery process, hypotheses are formulated based on existing knowledge, tested experimentally and either confirmed or disproved, according on 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 therapeutic effect *in vivo* is 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 pre-clinical to clinical experimentation.

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, 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 *hras* (Pulciani, Santos, Lauver, Long, Aaronson & Barbacid, 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 (Oct3/4, Sox2, c-Myc, and Klf4) 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 over-expressed in fibroblasts, screening for their ability to activate the promoter of the 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 analog 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 mono-dimensional, as a single parameter (i.e. fluorescence, absorbance, 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 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 multi-parametric 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**).

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 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 (2023; Dubrot et al., 2022).

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, Tachibana, Nagao, Tamura & Hamachi, 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 standardise 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.

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 et al., 2022a; Dubrot et al., 2022; Frangieh et al., 2021; Griffin et al., 2021; Wang et al., 2020), drug resistance (Gao et al., 2021; Ramaker, Hardigan, Gordon, Wright, Myers & Cooper, 2021) and oncogenic pathways (Dai et al., 2021; Gao et al., 2023; Li et al., 2023b; 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, Segal, Lee & Taipale, 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).

Modeling 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 AAV transduction (Mano, Ippodrino, Zentilin, Zacchigna & Giacca, 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 iPS cells have been developed and formulated as 2D co-culture systems, multi-cellular 3D organoids, engineered tissues, and micro-fabricated 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, Ralvenius & Tsai, 2020), various retinal cells including retinal epithelium (Meyer et al., 2009), cardiac myocytes (Burridge, Keller, Gold & Wu, 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, He, Taranova, Liang, D'Alessio & Zhang, 2008), hematopoietic 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-Cunado 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., 2021; Tian et al., 2019), cytokine-induced inflammatory astrocyte reactivity genes (Leng et al., 2022), genes governing microglia survival, activation, and phagocytosis (Drager 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 aging (Mannherz & Agarwal, 2023), and Zika virus infection (Li et al., 2019b).

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 tumor organoids (PDOs) have been largely used as in anti-cancer drug discovery, as they recapitulate the genetic heterogeneity and the cellular composition of the original tumor, particularly in the case of breast (Sachs et al., 2018; Tebon et al., 2023) and liver cancer (Broutier et al., 2017; Li et al., 2019a). Human colorectal cancer organoids have been successfully in whole genome pooled gRNA screenings for the identification of new tumor 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, David & Titze-de-Almeida, 2017; Zorde Khvalevsky et al., 2013), miRNAs (Zhang, Liao & Tang, 2019) and small activating RNA (saRNA) to reactivate tumor suppressor genes (Sarker et al., 2020), large screening campaigns for the identification of RNA-based anticancer medications are still missing. PDO-based discovery platforms will be particularly useful to develop new patient-tailored RNA-based therapies in cancer.

PDOs are generated from whole tumor biopsies, thus they comprise the original tumor microenvironment (TME), including innate and adaptive immune cells (Yuki, Cheng, Nakano & Kuo, 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 anti-cancer therapy (Duan, Wang, Zhang, Yang & Zhang, 2022).

3D 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 et al., 2023a;

Ungricht et al., 2022). At present, arrayed screenings in non-cancer organoids, were performed in 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.

RNA therapeutics and their road to the clinics

Among the few RNA therapeutics that have already received regulatory approval and are still in use, the majority are 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 modifiy the splicing of the dystrophin gene, leading to the production of a functional protein, while Nusinersen restores the expression of 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 ApoC-III mRNA for the therapy of familial chylomicronemia (Volanesorsen) (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 PCSK9 for hypercholesterolemia (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 CLN7 gene (Kim, 2022).

Additional RNAs 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 hypertriglyceridemia (ARO-APOC3) (Hegele, 2022), respectively, but also siRNAs specific for additional targets, such as Transient Receptor Potential Vanilloid 1 (TRPV1) for dry eye disease (Tivanisiran) (Moreno-Montanes, Bleau & Jimenez, 2018), antithrombin for hemophilia 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 hypertriglyceridemia (Olezarsen) (Tardif et al., 2022), but also apo(a) to reduce cardiovascular risk (Pelacarsen) (Tsimikas, Moriarty & Stroes, 2021), superoxide dismutase 1 (SOD1) and fused in sarcoma (fus) to treat amyotrophic lateral sclerosis (Tofersen and ION363) (Korobeynikov, Lyashchenko, Blanco-Redondo, Jafar-Nejad & Shneider, 2022; Miller et al.,

2022), huntingtin (HTT) to treat Huntington's 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 et al., 2022b; de Alwis et al., 2021), uveal 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 acidemia 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 acidemia and ornithine transcarbamylase deficiency), a key viral gene in the case of anti-viral vaccines, a disease-related gene as in the case of ApoC-III and apo(a) for severe hypertriglyceridaemia, ALAS1 for acute hepatic porphyria, HAO1 for primary hyperoxaluria type 1, TRPV1 for dry eye disease, antithrombin for hemophilias, 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 GWAS. The whole story started with the evidence that gain-of-function mutations in its sequence were responsible for familial hypercholesterolemia (Leren, 2004). On the other hand, African individuals who were double-recessive for nonfunctional PCSK9 had extraordinary low levels of LDL cholesterol, and thereby, greatly reduced cardiovascular risk compared to the general population (Cohen, Pertsemlidis, Kotowski, Graham, Garcia & Hobbs, 2005). These data have been confirmed by multiple GWAS (Myocardial Infarction et al., 2016; Saavedra, Dufour, Davignon & Baass, 2014). Hence, the idea of inhibiting its activity to control cholesterol level. 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).

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 pandemics 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.

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 beyond 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, relying on lipidic carriers that are often highly inflammatory. Second, cell- and tissue-specific delivery are difficult, if not impossible, to achieve *in vivo*. Third, off-target effects remain a major concern, despite progresses in designing sequences with strengthened on-target specificity.

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 analyze multiple features in multi-cellular, 3-D cell culture systems, which better reflect the *in vivo* behavior of most cell types.

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, 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 five 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.

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Figures with legends

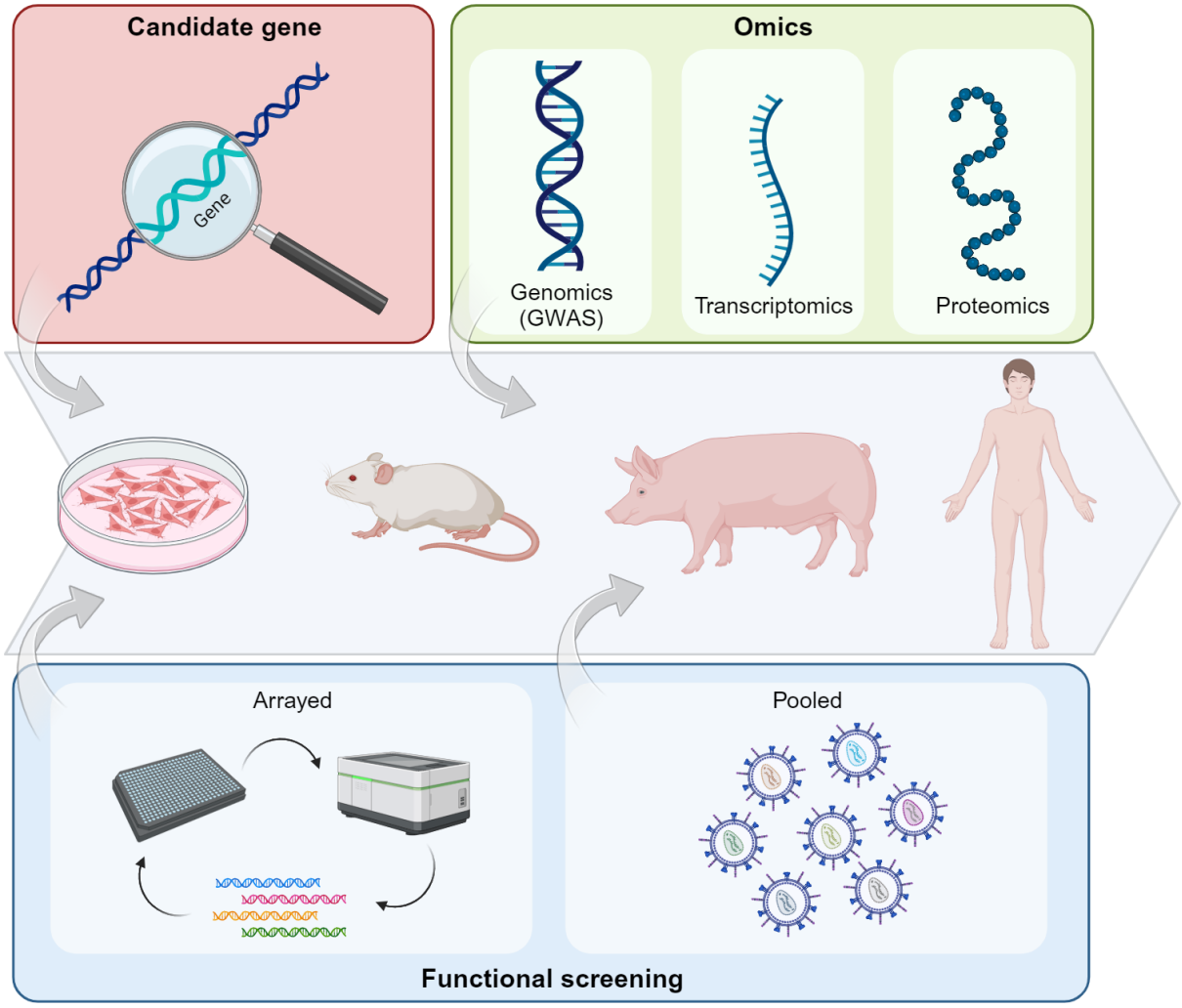

Figure 1. Approaches to discover novel RNA therapeutics and their trajectory toward human use. As any drug, RNA therapies must follow a standardized validation of their efficacy from cell culture systems to small and large animals, and eventually to patients. While RNA therapies have been traditionally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

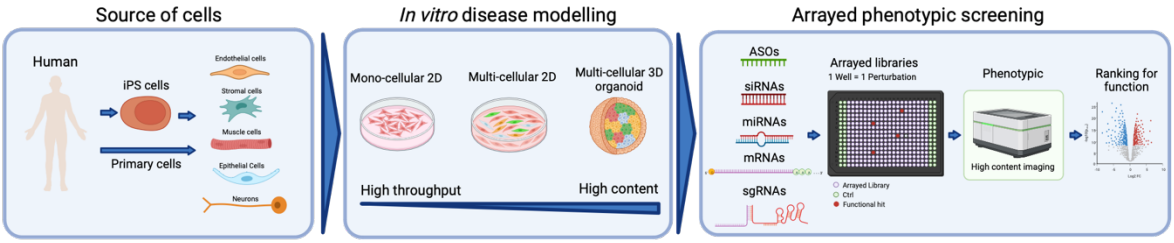

Figure 2. Arrayed screening for the discovery of RNA therapies. Discovery platforms for personalized RNA therapies leverage either patient-specific iPS-derived or primary cells, which can be cultured into mono/multicellular 2D/3D cultures to create disease-in-a-dish-models. These models can be systematically interrogated with RNA-based arrayed libraries, in which every well corresponds to a specific perturbation (ASO/siRNA/miRNA/mRNA/gRNA for CRISPR knock-out). Phenotypic alterations are quantified at both cellular and sub-cellular 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.